Human Endogenous Retrovirus K Gene Expression in Cutaneous Melanoma

Dr Sarita Singh

Imperial College London
Department of Medicine

Thesis submitted in fulfilment of the requirements for the Degree of Doctor of Medicine Research of Imperial College London
2012

Declaration

Application for ethical approval for this work and patient recruitment and consent were undertaken by Dr Sarita Singh. All experimental work, including cell culture, RNA isolation from cells, PCR and cloning, was performed by Dr Sarita Singh in the Retrovirology Laboratory at the Wright-Fleming Institute, Imperial College London. The PCR primers were designed by Dr Sarita Singh and Dr Steve Kaye, Senior Virology Research Officer. Sectioning of archival tissue blocks was carried out by Mr David Peston, Biomedical Scientist, and extraction of RNA from tissue sections by Dr Sarita Singh at the Department of Histopathology, Charing Cross Hospital. Histology slides were reviewed by Dr Nick Francis, Consultant Histopathologist. Sequencing data were analysed by Dr Sarita Singh under the guidance of Dr Steve Kaye and Dr Mike Tristem, Reader in Evolutionary Virology. Statistical tests were performed by Dr Sarita Singh under the guidance of Professor Elena Kulinskaya, Director of the Statistical Advisory Service at Imperial College.

Dr Sarita Singh

Abstract

Introduction:

In recent years, the key environmental and genomic changes that "drive" melanoma have become more clearly elucidated but much remains to be understood. Data published in 2003 demonstrated expression of Human Endogenous Retrovirus K (HERV-K) in melanomas but not in normal tissues, indicating a potential oncogenic role. Furthermore, HERV-K proviruses encode the putative oncogenic proteins Rec and Np9. In this work the feasibility of reliable detection of HERV-K expression from formalin-fixed paraffin-embedded (FFPE) tissue has been determined. Expression has been correlated with melanoma subtype and histological markers of poor outcome.

Methods:

HERV-K mRNA expression in melanoma cell lines (A375 and WM2664), FFPE primary melanoma (n=39), normal skin (n=31) and benign naevus (n=16) tissues was investigated by a novel real-time quantitative RT-PCR (qRT-PCR). Expressed HERV-K *env* sequences were cloned and sequenced.

Results:

A375 cells expressed all HERV-K genes and produced putative viral particles. Full-length mRNA was expressed in all primary melanoma and control tissues investigated with no differences in expression levels between the groups (*pol*: n=47, p=0.267; unspliced *env*: n=40, p=0.823). No correlation was observed with melanoma subtype or stage (Breslow). A proportion of all tissue types expressed *np9* with no differences in expression levels between them (p=0.964). Spliced *env* was expressed in 8/39 melanoma, compared with 1/16 benign naevus and 0/31 normal skin samples (p=0.003). Expression of *rec* was found in 9/39 melanomas but not in control samples (p=0.009) nor extra-lesional skin in five *rec*-positive cases (p=0.06). The *rec*-positive melanomas were histologically in vertical growth phase and thicker than *rec*-negative tumours (median Breslow 2.1mm versus 1.05mm, p=0.009). Sequencing of expressed *env* cDNA clones derived from melanoma and control samples (n=8) revealed expression of multiple HERV-K loci.

Conclusions:

Gene expression can be studied in FFPE tissue. The expression of potentially oncogenic *rec* in approximately 25% primary melanomas merits further evaluation.

Acknowledgements

I owe my deepest gratitude to my supervisor, Professor Chris Bunker, for his unwavering support throughout this work and my career. Notwithstanding his heavy academic and professional commitments, he has been a constant source of encouragement, considered advice and brilliant ideas. His belief in achieving the highest standards has always inspired and motivated me. It has been a privilege and an honour to be his student.

I am indebted to Dr Steve Kaye. With his wisdom, thoughtfulness, enthusiasm and great ability to explain things clearly and simply, he has consistently made molecular virology fun for me. I am grateful to Dr Nick Francis, Dr Mike Tristem, Professor Elena Kulinskaya, Professor Gavin Screaton, Professor Martin Gore and Professor Richard Marais for their invaluable advice. I would also like to thank Mr David Peston for his help with sectioning tissue blocks.

This work would not have been possible without the generous support of Spyros Retsas, Skin Treatment and Research Trust (START), Chelsea and Westminster Healthcare Charities and Westminster Medical School Research Trust. I would like to express my profound gratitude to them.

Finally, I wish to thank my family for their love and encouragement. This work has been one of the most significant academic challenges I have faced and would not have been completed without their guidance and support. To them I dedicate this thesis.

For Diya

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Abbreviations

AA	alopecia areata
Ab	antibody
Ag	antigen
AIDS	acquired immunodeficiency syndrome
AKT3	v-akt murine thymoma viral oncogene homologue 1
ALMM	acral lentiginous melanoma
AMS	atypical mole syndrome
ASIP	agouti signalling protein
ATLL	adult T-cell leukaemia/lymphoma
BAD	Bcl-2 associated agonist of cell death
BAK	Bcl-2 antagonist/killer
BAX	Bcl-2 associated X protein
BCG	Bacillus Calmette-Guerin
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra large
BID	BH3 interacting domain death agonist
BLAST	Basic Local Alignment Search Tool
BLV	bovine leukemia virus
BN	benign naevus
bp	base pairs
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CA	capsid protein
CDK	cyclin dependent kinase
CDKN2A	cyclin dependent kinase inhibitor 2A
cDNA	complementary DNA
c-KIT	cellular homologue of v-kit feline sarcoma viral oncogene
c-*myc*	v-myc myelocytomatosis viral oncogene homologue
corf	central open reading frame
c-onc	cellular oncogene
Ct	cycle threshold
CTL	cytolytic T-lymphocyte

CWH	Chelsea and Westminster Hospital
CXH	Charing Cross Hospital
DASL	cDNA-mediated annealing, selection, extension and ligation assay
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dok 5	docking protein 5
dsDNA	double-stranded DNA
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra acetic acid
EIAV	equine infectious anaemia virus
EM	electron microscopy
env	*env*elope
ERK	extracellular signal-regulated kinase
ERV	endogenous retrovirus
F	forward primer
FCS	foetal calf serum
FFPE	formalin-fixed, paraffin-embedded
gag	*g*roup-specific *a*nti*g*en
gDNA	genomic DNA
H&E	haematoxylin and eosin
HBRC	Human Biomaterials Resource Centre
HBV	hepatitis B virus
HCV	hepatitis C virus
HDM2	human double minute 2
HERV	human endogenous retrovirus
HHV-8	human herpesvirus 8
HIV	human immunodeficiency virus
HKPF	HERV-K *pol* forward
HKPR	HERV-K *pol* reverse
HLA	human leucocyte antigen
HML	human endogenous MMTV-like
HPRT	hypoxanthine-guanine phosphoribosyltransferase

HPV	human papillomavirus
HTLV	human T-lymphotropic virus
IkBa	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IN	integrase
itga2	integrin α2
JSRV	Jaagsiekte sheep retrovirus
KS	Kaposi's sarcoma
lacZα	β-galactosidase alpha peptide
LB	Luria-Bertani medium/broth/agar
LINE	long interspersed element
LMM	lentigo maligna melanoma
LNX	Ligand of Numb protein X
LPD	lymphoproliferative disorders
LTR	long terminal repeat
MA	matrix
MAPK	mitogen-activated protein kinase
MART-1	melanoma antigen recognized by T cells-1
MC1R	melanocortin-1 receptor
MCF	mink cell focus forming virus
Mcl-1	induced myeloid leukaemia cell differentiation protein Mcl-1
MCPyV	merkel cell polyomavirus
MEK	MAPK ERK Kinase
MelARV	melanoma-associated retrovirus
MITF	microphthalmia-associated transcription factor
MLV	murine leukaemia virus
MM	melanoma
MMIS	melanoma *in situ*
MMTV	mouse mammary tumour virus
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MW	molecular weight
MYR	millions of years
NC	nucleocapsid protein

NEHM	neonatal human melanocyte
NMM	nodular melanoma
NOXA	NADPH oxidase activator
Np9	HERV-K nuclear protein 9 kilodaltons
NRAS	neuroblastoma RAS viral (v-ras) oncogene homologue
NS	normal skin
nt	nucleotide
OD	optical density
ORF	open reading frame
P	primer
p53	protein 53
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
P_{lac}	promoter for the *lacZα* gene
PLZF	promyelocytic leukaemia zinc finger
pol	*pol*ymerase
PR	protease
Pr	probe
PTEN	phosphate and tensin homologue
PUMA	p53 upregulated modulator of apoptosis
pUC ori	origin of replication in *E.coli*
qRT-PCR	quantitative reverse transcription PCR
R	reverse primer
RAF	rapidly accelerated fibrosarcoma
RAS	rat sarcoma
RB	retinoblastoma protein
RCF	relative centrifugal force
Rec	regulator of expression encoded by *corf*
RGP	radial growth phase
Rev	regulator of virion expression
Rex	regulator x
RNA	ribonucleic acid

RNAi	RNA interference
RNAse	ribonuclease
RPM	revolutions per minute
RSV	Rous sarcoma virus
RT	reverse transcriptase
RT-PCR	reverse transcription PCR
RVLP	retrovirus-like particle
SA	splice acceptor
SD	splice donor
SLE	systemic lupus erythematosus
SOC	super optimal broth with catabolite repression
SPSS	Statistical Package for Social Sciences
SSM	superficial spreading melanoma
SU	surface
TAE	tris acetate EDTA
Tax	trans-activator x
TE	transposable element
TF	transcription factor
TGCT	testicular germ cell tumour
Tm	melting temperature
TM	transmembrane
Treg	regulatory T cell
tRNA	transfer RNA
TYR	tyrosinase
U	untranslated
UTR	untranslated region
UVR	ultraviolet radiation
VGP	vertical growth phase
VLP	virus-like particle
v-onc	viral oncogene
v-src	viral-sarcoma

Gene abbreviations included in the list above are the official gene names as designated by the HUGO Gene Nomenclature Committee (see http://www.genenames.org/).

Deoxyribonucleic acids (DNA):

Adenine (A)

Cytosine (C)

Guanine (G)

Thymine (T)

Amino acids:

Alanine (A)

Arginine (R)

Asparagine (N)

Aspartic acid (D)

Cysteine (C)

Glutamic acid (E)

Glutamine (Q)

Glycine (G)

Histidine (H)

Isoleucine (I)

Leucine (L)

Lysine (K)

Methionine (M)

Phenylalanine (F)

Proline (P)

Serine (S)

Threonine (T)

Tryptophan (W)

Tyrosine (Y)

Valine (V)

SI units:

g	gram
kb	kilobase
M	molar
mM	millimolar
mm	millimetre

ml	millilitre
ng	nanogram
nm	nanometre
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
nm	nanometer
U	units
V	volts
mA	milliampere

References to buffers:

The letters set out after certain buffers (for example buffer RPE) have been assigned to the buffer by the manufacturer and the meaning of the letters has not been made public.

Chapter 1:

Introduction

1.0 Abstract

Melanoma is an increasing public health problem due to a rise in incidence and mortality rates in fair-skinned populations worldwide and the lack of successful treatments for advanced disease. Although significant insights into the importance of inordinate sun exposure have emerged and knowledge is accumulating about genetic influences, the pathogenesis of melanoma remains unresolved. It is thought that some genetically susceptible individuals are at higher risk when they have greater intermittent ultraviolet radiation (UVR) exposure but, to date, there is no convincing evidence for interactions between genes and environment in the aetiology of melanoma. An unanswered question is why some people are more likely to develop melanoma than others in ostensibly phenotypically homogeneous populations such as Queensland, Australia. It is likely that, in addition to the pigmentation phenotype, there are other as yet undetermined background genetic elements that contribute to the UVR-induced transformation of melanocytes.

Retroviruses are RNA viruses which, by reverse transcription, can form double-stranded DNA (dsDNA) copies of their genomes that can integrate into the chromosomes of an infected cell. Retroviruses are known to cause certain cancers in both animals and humans. Human endogenous retroviruses (HERVs) are remnants of ancestral exogenous retroviral infections that are fixed in the germ-line DNA and are thus transmitted vertically in a Mendelian fashion. Although no longer infectious, they may be transcriptionally active and have been implicated in the pathogenesis of certain diseases. Members of one family, the HERV-K proviruses, have open reading frames (ORFs) for all viral genes and are, therefore, the most likely to be biologically active and potentially pathogenic. Genes for two hypothetically oncogenic accessory proteins, Rec and Np9, have been described in the genome of HERV-K. Additionally insertionally polymorphic HERVs, defined as proviruses that are present only in a proportion of the human population, might represent novel genetic risk factors for disease. HERVs have been implicated in the pathogenesis of melanoma. Retrovirus-like particles (RVLPs) and the expression of HERV-K messenger RNA (mRNA) and proteins have been demonstrated in melanoma tissue and antibodies to HERV proteins have been observed in melanoma patients.

A significant challenge in melanoma research is the lack of availability of fresh or cryopreserved primary melanoma tissue. In the last decade, however, considerable advances have been made in techniques to extract nucleic acids of sufficient quality and quantity from archival FFPE tissue for use in downstream applications such as real-time qRT-PCR.

When this plan of work was conceived there was only a handful of studies in the literature regarding HERV expression in human melanoma, largely based on melanoma cell lines and metastasis tissue. Therefore, it was envisaged to carry out a detailed investigation of HERV-K gene expression in archival primary melanoma with the hope that the findings might illuminate the pathogenesis of melanoma.

1.1 Cutaneous melanoma

1.1.0 Summary

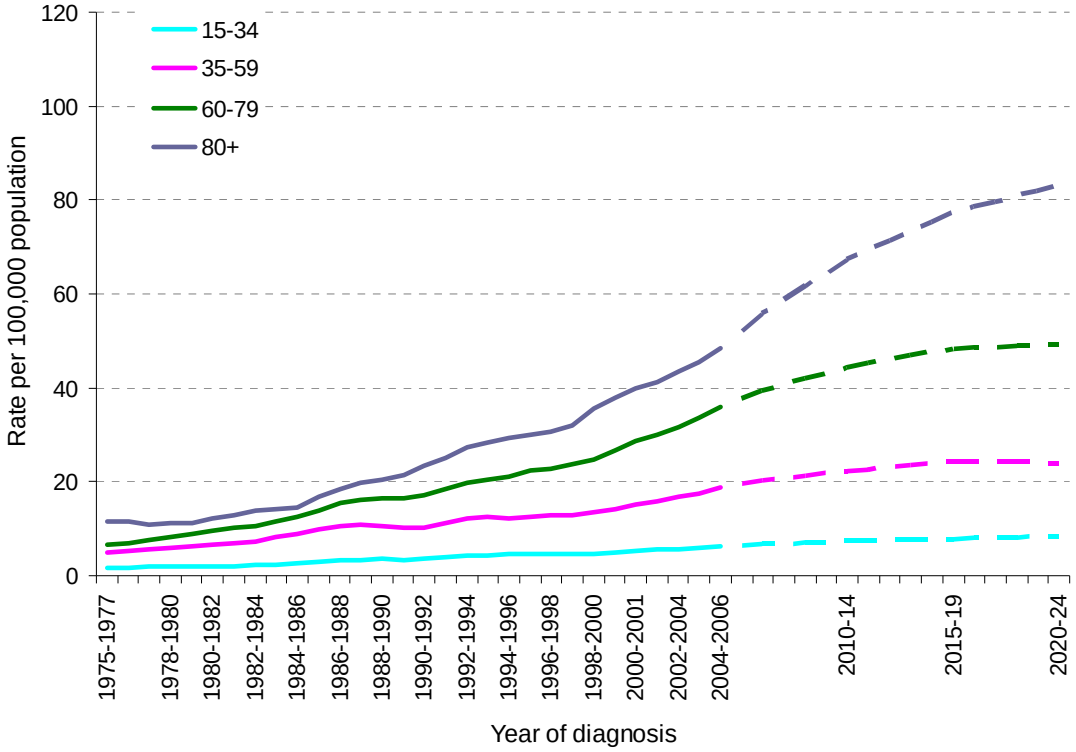
Over the past several decades, there has been an increase in the incidence of melanoma and a concomitant increase in mortality in most fair-skinned Caucasian populations worldwide. Much of the increase in incidence has occurred in young adults, thus the number of life years lost per melanoma death is higher than that for most other solid tumours. Increased risk for melanoma is associated with pigmentation phenotypes, the number of melanocytic naevi, immunosuppression, family history of the disease, severe sunburn during childhood or adolescence and sunbed use. Four major clinicopathological subtypes are recognised and the depth of tumour invasion (Breslow thickness) is the most important determinant of prognosis. Surgical excision is the only curative treatment for melanoma. Regarding pathogenesis, significant insights into genetic influences and the importance of inordinate sun exposure have emerged but the molecular pathogenesis remains elusive. Recent work has shed new light on the molecular events associated with melanoma subtypes, leading to novel approaches to therapeutic development in the setting of advanced disease.

1.1.1 Epidemiology

World melanoma incidence rates reflect the high risk for white populations in sunny climates with the highest age-standardised rates seen in Australia and New Zealand. The highest recorded incidence of melanoma worldwide is in Queensland, Australia, at a figure of 55.8 per 100,000 population for males and 41.1 per 100,000 population for females for the time period 1998–2002 (MacKie et al., 2009). These rates are more than three times that of any European country. In Europe, the highest rates are seen in Switzerland and the fairer-skinned Northern Europeans from Norway, Sweden and Denmark (MacKie et al., 2009). In the UK, rates for both sexes are above the European Union average, at figure of 14.6 per 100,000 population for males and 15.4 per 100,000 population for females in 2007 (Cancer Research UK, 2010a). In 2007, 4,975 cases of melanoma were diagnosed in men and 5,697 cases in women and it was the sixth most common cancer for both sexes combined. Incidence rates rise steadily with age but, although the highest rates are seen in the over 70s, there is a substantial number of cases affecting young adults with almost one third of all cases occurring in people aged less than 50.

Worldwide, the incidence of melanoma is increasing faster than any other common cancer with an approximate doubling of rates every 10-20 years in countries with white populations (Cancer Research UK, 2010a). In Great Britain, male rates have increased more than five times over the last 30 years, while female rates have more than tripled. There have been increases in all age groups with the largest increase in people aged over 60 and, based on trends in incidence from 1975 to 2004, rates are projected to increase up until 2024 (Figure 1.1). In most populations, the increase has been mainly for thin melanomas (<1 mm Breslow thickness). Although partly attributable to increased surveillance and early detection, the current trend of increased melanoma incidence is almost certainly due to lifestyle factors in terms of excessive recreational exposure to high-intensity sunlight and sunburns, which are major risk factors for melanoma (Chang et al., 2009a; Gandini et al., 2005b). Cheap flights from high-latitude countries, such as Scandinavia, to sunny holiday resorts are available all year round and it has been postulated that some of the increase could be attributed to greater opportunities for burning of fair, non-acclimatised Caucasian skin (MacKie et al., 2009).

Figure 1.1: Age specific incidence rates of melanoma in adults in Great Britain, 1975-2006 and projected to 2024



Legend:

Age-specific melanoma incidence rates have risen significantly over the last 30 years. Projections based on trends in melanoma incidence from 1975-2004 suggest that incidence rates in many age groups will continue to increase up until 2024. Incidence rates in people aged 60-79 are projected to increase by a further third from where they are today. The largest projected increase in incidence rates are in people aged over 80.

From Cancer Research UK (2010a)

Age-specific melanoma mortality rates have also risen significantly over the last 30 years, albeit at a slower pace than incidence because earlier diagnosis has resulted in a bigger proportion of thinner tumours. Mortality has been greater in men, despite the higher female incidence, with male deaths almost double those seen in females. The greatest increase in mortality has been in men aged over 65, who usually present late with thick tumours that have a poorer prognosis. Despite the steady rise in mortality rates with age, substantial numbers of deaths occur in younger people. In 2008 in the UK, 110 people aged less than 40 died from melanoma and over half of all deaths were in people aged less than 70 (Cancer Research UK, 2010b).

1.1.2 Risk factors

Melanoma is almost exclusively a malignancy of fair-skinned individuals (Parkin et al., 1999). Increased risk for melanoma is associated with pigmentation phenotypes, number of melanocytic naevi, immunosuppression, a family history of the disease, a history of severe sunburn during childhood or adolescence and sunbed use (MacKie et al., 2009; Sekulic et al., 2008).

(i) Pigmentation phenotypes

There are a number of pigmentation risk factors, each associated with an approximate doubling of risk, including fair skin, blue or green eyes, blonde or red hair and sun sensitivity or inability to tan (Gandini et al., 2005b). Variants of the *MC1R* (melanocortin-1 receptor) gene are associated with the combination of red hair, freckling and sun sensitivity. However, the *MC1R* gene may also have a non-pigmentation effect (Raimondi et al., 2008). *TYR* (tyrosinase) gene variants and a haplotype spanning the *ASIP* (agouti signalling protein) locus are also associated with melanoma (Gudbjartsson et al., 2008).

(ii) Melanocytic naevus count

The strongest phenotypic risk factor for melanoma is the presence of increased numbers of melanocytic naevi, commonly referred to as moles (Chang et al., 2009b; Gandini et al., 2005a). There is a substantially increased risk (approximately seven-fold) associated with the presence of 101-120 naevi compared with less than 15. Some individuals are said to

have the atypical mole syndrome (AMS), which has been defined by one group as the presence of at least three of the following clinical features: (a) 100 or more common naevi >2 mm in diameter; (b) two or more atypical naevi; (c) one or more naevi on the buttock and/or two or more naevi on the dorsum of the feet; (d) one or more naevi on the anterior scalp; (e) one or more pigmented lesion of the iris (Newton Bishop et al., 1994). The AMS phenotype is a potent risk factor for melanoma. The terms atypical naevus and dysplastic naevus are often used interchangeably although their definitions are controversial and still evolving (Elder 2006b). Clinically, they are larger than common moles (>5 mm in diameter), exhibit pigment variegation (ranging from tan to dark brown to pink) and usually have irregular, notched and ill defined borders.

Twin studies have demonstrated that the number of naevi is predominantly genetically determined, with a smaller effect of sun exposure (Bataille et al., 2000). Genome-wide association studies have recently identified several loci that determine naevus number (Bishop et al., 2009; Falchi et al., 2009).

(iii) Immunosuppression

An increased incidence of melanoma has been observed in solid organ transplant recipients (Le Mire et al., 2006) and is *probably* emerging in people with human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), thus indicating an association between melanoma risk and immunodysfunction (Bunker & Gotch, 2010).

(iv) Family history and melanoma susceptibility genes

Individuals with a family history of melanoma have approximately double the risk of developing the disease (Gandini et al., 2005c). Rare families exist in which large numbers of melanoma cases arise. Most of these families have hereditary mutations in the *CDKN2A* (cyclin dependent kinase inhibitor 2A) gene and some very rare families have mutations in the *CDK4* (cyclin dependent kinase 4) gene (Lin et al., 2008; Meyle & Guldberg, 2009; Pho et al., 2006). In the majority of *CDKN2A* mutation-positive families in the UK and Australia, family members have an increased risk of melanoma alone but in mutation-positive families in North America and some parts of Europe, there is also an increased risk

of pancreatic cancer (Goldstein et al., 2006). Hereditary mutations are responsible for a very small proportion of melanoma. The frequency of *CDKN2A* mutations is 20-40% in families where there are three or more affected first degree relatives, and less than 5% if there are only two (Newton Bishop & Gruis, 2007).

(v) Ultraviolet radiation (UVR) exposure

The sun is the principal source of UVR. Risk of melanoma is most strongly linked to intermittent exposure to high-intensity sunlight, often resulting in sunburn, rather than to chronic exposure (Chang et al., 2009a; Gandini et al., 2005b). Migration studies suggest that severe episodic sunburn in early life correlates most strongly with melanoma risk (Whiteman et al., 2001). Excessive exposure to UVR is the main modifiable risk factor so far identified for melanoma and thus strategies in primary prevention have focussed on education that promotes sun protection.

(vi) Sunbeds

The use of sunbeds, a source of artificial UVR, increases the risk of melanoma especially when used before the age of 35 (The International Agency for Research on Cancer Working Group on artificial ultraviolet light and skin cancer, 2007). It has been estimated that sunbed use causes 100 deaths a year from melanoma in the UK (Diffey, 2003). Legislation was passed in 2010 banning the use of sunbeds in under-18s in England and Wales.

1.1.3 Clinicopathological features

Four main subtypes of melanoma are recognised clinically and histologically (Cummins et al., 2006; Sekulic et al., 2008; Thompson et al., 2005): superficial spreading melanoma (SSMM); nodular melanoma (NMM); lentigo maligna melanoma (LMM); acral lentiginous melanoma (ALMM). Examples are illustrated in Figure 1.2. There are also several uncommon variants that constitute less than 5% of cases, for example desmoplastic and naevoid melanoma. The term melanoma *in situ* (MMIS) is used when melanoma cells are confined to the epidermis with no invasion into the dermis. Criteria for the histological diagnosis of melanoma are based on architectural and cytological signs (Elder, 2006a).

(i) Superficial spreading melanoma (SSMM)

SSMM accounts for 70% of melanoma. It usually occurs at sites of intermittent, intense sun exposure (on the trunk in men and on the legs and back in women) and is most frequently seen in individuals aged 30-50. It usually appears as a macule that slowly progresses to a plaque, often comprising multiple colours and pale areas of regression (Figure 1.2a&b). SSMM has a radial growth phase (RGP), during which the lesion is predominantly in the epidermis and increases in diameter, followed by a vertical growth phase (VGP) in which the lesion extends downwards into the dermis and exhibits increased metastatic potential.

(ii) Nodular melanoma (NMM)

NMM accounts for 15-30% of melanoma (Figure 1.2e). It usually appears as an exophytic brown-black and frequently eroded or ulcerated tumour, occurring most commonly on the legs and trunk. Since it has a predominantly VGP, NMM has a tendency toward greater depth of invasion and is associated with a worse prognosis than the other subtypes.

(iii) Lentigo maligna melanoma (LMM)

LMM accounts for less than 5% of melanoma (Figure 1.2c&d). It is typically located on chronically sun-damaged skin (such as the head, neck or arms) of fair-skinned older individuals. Its benign precursor lesion, lentigo maligna, is a tan macule that grows slowly in a radial fashion and may eventually display a palpable component of VGP clinically signalling progression to LMM. Histologically, it is characterised by a lentiginous proliferation of atypical melanocytes at the dermo-epidermal junction and features of chronic sun exposure (solar elastosis).

(iv) Acral lentiginous melanoma (ALMM)

ALMM occurs on the palms, soles or beneath the nail plate (subungual variant, Figure 1.2f) and is the least common subtype of melanoma, accounting for 2-8% of cases in fair-skinned persons. However, it accounts for 29-72% of melanoma in dark-skinned individuals, such as African-American, Asian and Hispanic persons (Gloster & Neal, 2006).

Figure 1.2: Melanoma subtypes

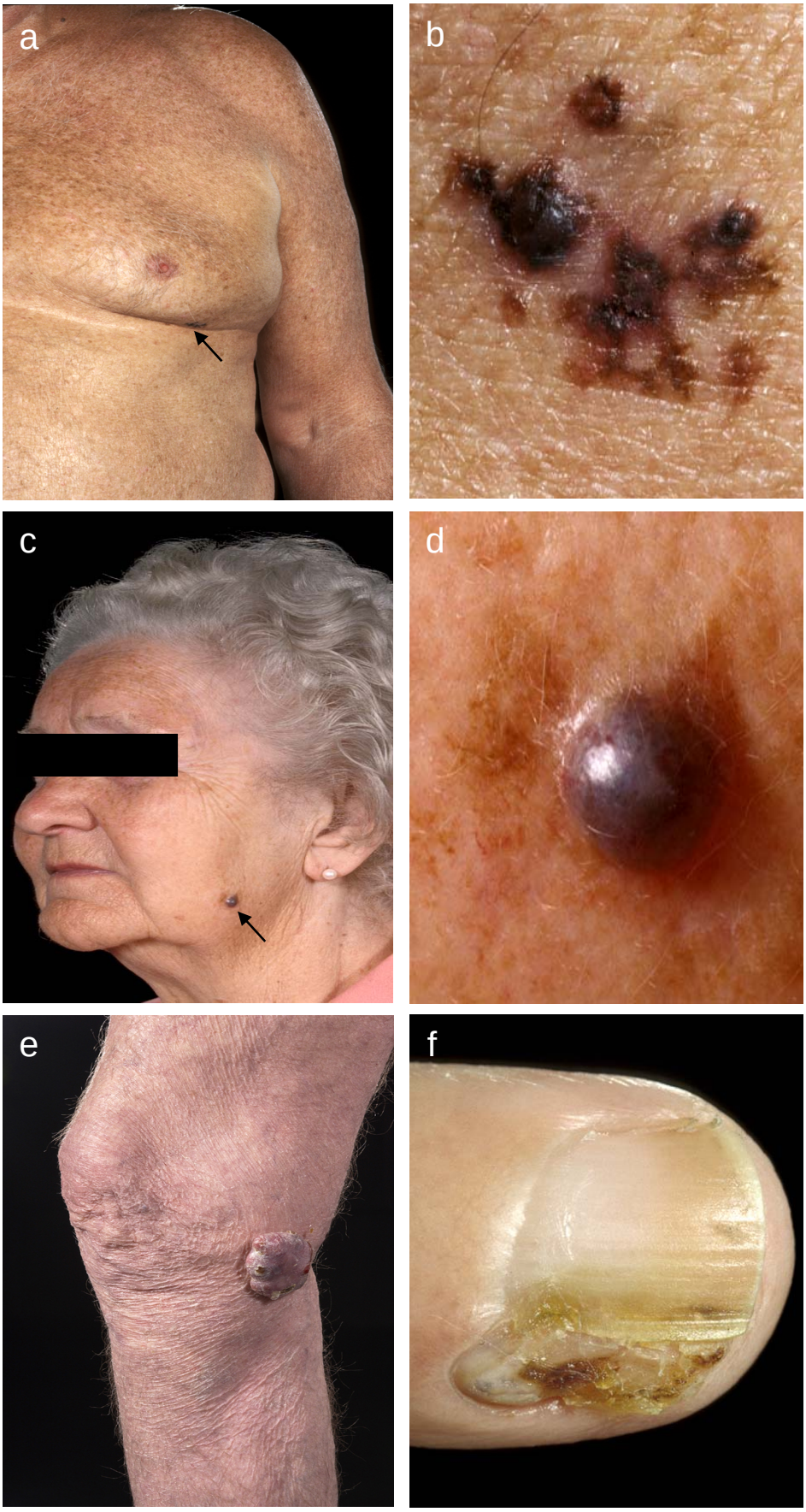


Figure 1.2: Legend

Photographs of patients showing the four main melanoma subtypes (courtesy of Professor C. B. Bunker and Medical Illustration, Chelsea and Westminster Hospital). Patient consent was obtained for the use of these photographs.

(a) Superficial spreading melanoma (SSMM) on the trunk of a male (arrow), Breslow thickness 0.8 mm.
(b) A close-up image of the SSMM. The lesion has an irregular indented outline and variable pigmentation, with shades of brown and black. There are paler areas of regression and nodular areas, indicating vertical invasion.
(c) Lentigo maligna melanoma (LMM) on the cheek of an elderly female (arrow), Breslow thickness 3.9 mm.
(d) A close-up image of the LMM. The lesion comprises an irregular tan coloured macule with a large central bluish-black nodule, indicating vertical invasion.
(e) Nodular melanoma (NMM) on the lower leg of an elderly male, Breslow thickness 13 mm. The lesion is a large red exophytic nodule with little pigmentation (amelanotic) and areas of erosion.
(f) Acral lentiginous melanoma (ALMM), subungual variant, Breslow thickness 3 mm. There is variable brown pigmentation under the nail and on the skin of the lateral nail fold. The nail plate is split and distorted.

1.1.4 Prognostic factors

A number of clinical and histopathological factors correlate with prognosis.

(i) Age

Prognosis worsens with increasing patient age at the time of diagnosis (Payette et al., 2009). In a large American study comprising 17,600 patients, each 10-year increase in age was associated with a decline in both five and 10-year survival rates (Balch et al., 2001). It is thought that prognosis worsens with age probably because other factors known to worsen prognosis are more frequent in the melanomas seen in older patients. One study found that as age increased, so did the tumour thickness, presence of ulceration, regression and proportion of men (Chao et al., 2004).

(ii) Sex

Female sex confers a better prognosis than male sex. Women tend to present with thinner tumours compared with men, which could account for the underlying protective effect of female sex. It has also been suggested that the prognosis is better because women present with melanoma at a younger age than men and tend to have more limb lesions than trunk lesions compared with men (Payette et al., 2009). However, male gender is associated with an adverse outcome that persists even after adjustment for other prognostic variables (de Vries et al., 2008; Lasithiotakis et al., 2008). In a large Dutch study comprising 10,538 patients, the relative excess risk for male patients to die from melanoma was 1.87 after adjusting for time period of diagnosis, region, age, Breslow thickness, melanoma subtype, body site, nodal and metastatic status (de Vries et al., 2008). The gender effect on survival remains unresolved and further studies are required. In particular, it is difficult to attribute this to hormonal influences as the adjusted risk estimates are similar among patients below 45 or above 60 years of age (de Vries et al., 2008).

(iii) Anatomic location

A number of studies have demonstrated that melanomas located on the extremities confer a better prognosis than lesions on the trunk (Payette et al., 2009). However, lesions located more distally on the extremities, such as the foot, are associated with a worse prognosis

(Hsueh et al., 1999; Talley et al., 1998). Head and neck melanomas, particularly scalp, neck and ear lesions, have also been shown to carry worse prognoses than those located elsewhere (Lachiewicz et al., 2008). However, some studies have not demonstrated that anatomic location carries prognostic significance particularly when Breslow thickness was taken into account (Hoersch et al., 2006; Payette et al., 2009).

(iv) Histopathological characteristics

Depth of tumour invasion (Breslow thickness), ulceration and mitotic rate (histologically defined as mitoses/mm^2) are the most important prognostic factors (Balch et al., 2001; Balch et al., 2009; Payette et al., 2009; Ivan 2011). Of these, Breslow thickness (measured vertically in millimeters from the top of the granular cell layer of the epidermis to the deepest point of tumour involvement) has been shown to be the factor that best correlates with prognosis (Breslow, 1970). Level of dermal invasion (Clark's level) is only of independent prognostic significance for thin (<1 mm thick) tumours. Increased tumour thickness confers a higher metastatic potential and a poorer prognosis. Approximate five year survival rates are: 95-100% for tumours <1 mm thick; 80-96% for tumours 1-2 mm thick; 60-75% for tumours 2.1-4 mm thick; and 50% for tumours >4 mm thick (Roberts et al., 2002). Therefore, accurate diagnosis at an early stage is of utmost importance. However, despite the strong prognostic value of the Breslow thickness thin melanomas occasionally metastasise and patients with thick melanomas occasionally have long survival periods. The presence of ulceration microscopically, defined as the loss of epidermis overlying the tumour, is also associated with a worse prognosis.

Melanoma proliferation, defined by the mitotic rate, has recently been found to be a powerful and independent predictor of survival. Data from America have demonstrated a highly significant correlation between increasing mitotic rate and declining survival rates (Balch et al., 2009; Thompson et al., 2011). Mitotic rate has also been found to be the most important prognostic factor in thin (<1.0 mm) melanomas (Kesmodel et al., 2005).

1.1.5 Treatment

In 1840, the British surgeon Samuel Cooper recognised that advanced melanoma was untreatable when he commented, "the only chance for benefit depends upon the early removal of the disease" (Cooper, 1840). More than one and a half centuries later, this situation remains largely unchanged. Surgical excision is the only curative treatment for melanoma. Excision margins are guided by the Breslow thickness (Roberts et al., 2002). As tumour depth increases, excision margins widen because prognosis directly relates to Breslow thickness. Treatment for metastatic disease includes further surgery to remove metastatic deposits, single agent chemotherapy with dacarbazine and palliative radiotherapy (Marsden et al., 2010; Roberts et al., 2002). Although there are few effective treatments for advanced disease, very recently a number of molecularly targeted therapies (discussed in section 1.1.6 iii) have been subject to clinical trials and are showing promise.

1.1.6 Pathogenesis

Melanoma arises from melanocytes, pigmented cells that are predominantly found in the skin and eyes (Gray-Schopfer et al., 2007). Melanocytes produce melanins, the pigments responsible for skin and hair colour. Cutaneous melanocytes originate from neural-crest progenitors that migrate to the skin during embryogenesis and reside in the basal layer of the epidermis and in the hair follicles. Melanocyte homeostasis is regulated by epidermal keratinocytes. The latter secrete factors in response to UVR that regulate melanocyte survival, differentiation, proliferation and motility, stimulating melanocytes to produce melanin and resulting in the tanning response. Mutations in critical growth regulatory genes, the production of autocrine growth factors and the loss of adhesion receptors can contribute to altered intracellular signalling in melanocytes. Consequently melanocytes can escape their tight regulation by keratinocytes and proliferate, leading to the formation of a benign naevus.

The development of melanoma has been described in the literature as a stepwise progression from cutaneous melanocyte to benign naevus and dysplastic naevus stages to MMIS and finally invasive melanoma (Miller & Mihm, 2006). However, a melanocytic naevus precursor is not required for most melanomas, the majority of which arise *de novo*

(i.e. in normal skin). Melanoma occurs in conjunction with a pre-existing naevus in only about 20-40% of cases and less than 50% of the naevi associated with melanoma are dysplastic (Bevona et al., 2003; Elder 2006b). It has been suggested that transformed melanocyte stem cells, melanocyte progenitors or de-differentiated mature melanocytes may give rise to melanomas (Grichnik, 2008).

The sequence of events resulting in the transformation of normal melanocytes to melanoma cells, frequently referred to as melanomagenesis, is not well understood. It likely involves stepwise progressive genetic mutations that alter cell proliferation, differentiation, and death and impact on susceptibility to the carcinogenic effects of UVR (Bennett, 2008).

(i) Ultraviolet radiation (UVR)

UVR can have direct mutagenic effects on DNA by: stimulating cells to produce growth factors (such as stem cell factor, fibroblast growth factor, and transforming growth factor α); reducing cutaneous immunity; promoting reactive oxygen species of melanin that cause DNA damage and suppress apoptosis (Hussein, 2005; Pfeifer et al., 2005). Both UVB (280–320 nm wavelength) and UVA (320–400 nm) can damage DNA, often producing UV landmark mutations, C to T or CC to TT via cyclobutane dimers (Pfeifer et al., 2005). When these mutations affect the function of oncogenes, tumour suppressor genes or important housekeeping genes, transformation of melanocytes to melanoma may occur.

There is strong epidemiological and molecular evidence that implicates UVR exposure in the pathogenesis of melanoma (Ivry et al., 2006; Rigel, 2008). The increased melanoma incidence in patients affected with xeroderma pigmentosum, a genetic disorder where UVR-induced DNA damage cannot be repaired, supports a causal role for sun exposure in melanoma development. Additionally, DNA repair capacity is impaired in patients who develop melanoma on sun-exposed sites (Wei et al., 2003). Strong evidence links sun exposure to the development of melanoma from comparative studies of migrant populations and native residents in geographic areas with high ambient solar radiation, especially fair skinned individuals of European origin (Ivry et al., 2006; Whiteman et al., 2001). Melanomas are observed in higher density in sun-exposed rather than sun-protected areas.

Even in individuals predisposed to melanoma by inheritance of *CDKN2A* mutations, incidence is much higher in those living at lower latitudes (Bishop et al., 2002). This suggests close interactions between genetic susceptibility and UVR exposure in melanomagenesis. Although it is appreciated that the key to understanding the process by which melanocytes are transformed into melanoma lies in the interaction between genetic factors and UVR, the nature of this relationship has remained obscure (Maddodi & Setaluri, 2008). For example genes that are mutated in melanoma, such as *BRAF* and *N-RAS*, do not show typical UV "fingerprint" mutations (Hocker & Tsao, 2007) and little is known about the long-term UVR-induced changes in the melanocyte transcriptome, changes that could significantly contribute to melanoma susceptibility.

It is generally thought that some genetically susceptible individuals are at higher risk when they have greater intermittent UVR exposure (MacKie et al., 2009). However, to date, there is no convincing evidence for interactions between genes and environment in the aetiology of melanoma. An unanswered question is why some people are more likely to develop melanoma than others in ostensibly phenotypically homogenous populations. It could be argued that the incidence of melanoma in fair-skinned individuals in Queensland, Australia, should be even higher than epidemiologically recorded if phenotypic determinants and UVR are the most important aetiological factors. It is likely that there are other as yet undetermined genetic elements that contribute to the UVR-induced transformation of melanocytes.

(ii) Molecular pathways

Cancer development is a multi-step process in which genetic and epigenetic events contribute to the emergence of a malignant clone of cells (Thompson et al., 2005). Accumulation of mutations in critical genes can alter normal programmes of cell proliferation, differentiation and death. Several genetic influences have been identified in melanomagenesis, including mutations or transcriptional variations in tumour suppressor genes, such as *CDKN2A*, *PTEN* and *p53* and in oncogenes, such as *BRAF*, *NRAS* and *AKT3* (Bennett, 2008; Polsky & Cordon-Cardo, 2003; Sekulic et al., 2008). Although numerous genes are mutated in melanoma, many of these loci are uniquely and critically positioned

within three key cell signalling networks (Figure 1.3): the growth-promoting RAS signalling network; the tumour-constraining CDKN2A network; the downstream regulator of apoptosis p53/Bcl-2 network (Hocker et al., 2008).

The RAS signalling network regulates cell growth, survival and invasion through two distinct cascades namely the RAS/RAF/MEK/ERK, also known as the MAPK (mitogen-activated protein kinase), and the RAS/PI3K/AKT signalling pathways (Gray-Schopfer et al., 2007). The RAS/MAPK pathway is a key regulator of melanocyte proliferation. Activating mutations in one of two key MAPK pathway genes, *BRAF* or *NRAS*, are seen in a high proportion of melanomas and benign naevi. The enzyme BRAF is a member of the RAF family of serine/threonine kinases. The *BRAF* gene is mutated in 40-60% of melanomas, the most frequent mutation being a glutamic acid (E) for valine (V) substitution at position 600: V600E (Davies et al., 2002). ^{V600E}BRAF stimulates constitutive ERK (extracellular signal-regulated kinase) signalling, stimulating proliferation and survival and providing essential tumour growth and maintenance functions. In some melanomas mutational activation of growth-factor receptors, such as c-KIT (a transmembrane receptor tyrosine kinase), can also result in hyperactivation of these pathways (Curtin et al., 2006; Roskoski, 2005). The tumour suppressor protein PTEN is a negative regulator of the RAS/PI3K pathway. Inactivation of *PTEN* by deletion or mutation, although rare, leads to constitutive activation of this pathway (Tsao et al., 2004). Increased AKT activity prolongs cell survival through the inactivation of the BAD protein and increases cell proliferation (del Peso et al., 1997).

Activating mutations in *BRAF* and *NRAS* can trigger a profound cellular growth arrest, known as oncogene-induced senescence (Mooi & Peeper, 2006). This is thought to protect cells from the acquisition of further mutations and to block progression to malignancy. In melanoma, it has been proposed that the senescence response has been lost, compared with benign naevi where the process is intact (Michaloglou et al., 2005). The observation that *BRAF* is mutated in up to 80% of benign naevi, yet most naevi remain indolent for many years and only rarely progress to melanoma, indicates that oncogenic *BRAF* alone is insufficient for melanoma initiation (Pollock et al., 2003).

Figure 1.3: Key signalling networks and associated genetic alterations in melanoma

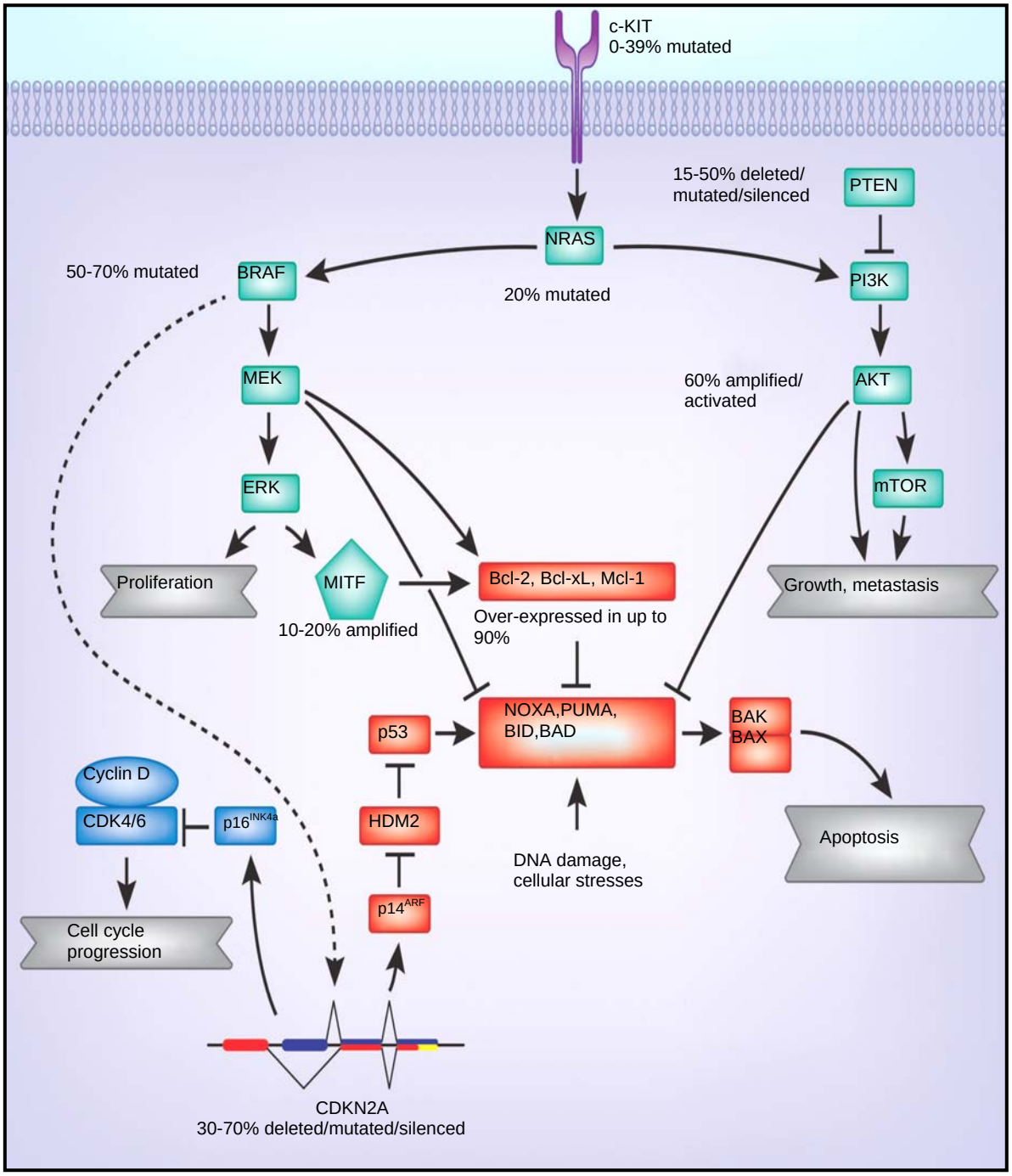


Figure 1.3: Legend

A simplified illustration of three of the major signalling networks involved in melanoma tumourigenesis, survival and senescence is depicted. Genetic alteration frequencies/type(s) in melanoma (%) are indicated.

Multiple signalling pathways are initiated at the cell membrane via activation of c-KIT, the stem cell factor receptor tyrosine kinase that triggers many downstream events, including activation of the RAS signalling network (green) comprising the RAS/RAF/MEK/ERK (MAPK) and RAS/PI3K/AKT pathways. Both pathways have been implicated in melanoma proliferation, survival and progression. The RAS/PI3K/AKT pathway is inhibited by the lipid phosphatase PTEN. Once activated AKT has several different enzymatic substrates many of which contribute to tumour proliferation and survival, such mTOR. The *MITF* gene is a key player in melanocytic development but may also function as an oncogene in melanoma. *MITF* gene transcription and post-translational modifications are strongly influenced by multiple upstream pathways, including c-KIT activation.

The *CDKN2A* locus encodes the tumour suppressor proteins $p16^{INK4a}$ and $p14^{ARF}$, which are thought to contribute to senescence and tumour growth restriction. The $p16^{INK4a}$ protein is an inhibitor of CDK4 and CDK6 and thus prevents progression of the cell cycle. The $p16^{INK4a}$ protein is implicated in senescence induced by oncogenes including activated *BRAF*. In melanoma, it has been proposed that the senescence response has been lost (dashed arrow).

The p53/Bcl-2 signalling network (red) is a major contributor to melanoma apoptosis and chemosensitivity and is regulated by many of the oncogenic melanoma pathways. The Bcl-2 superfamily includes pro-apoptotic (BAX, BAK, BAD, BID, NOXA, PUMA) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1) members. In response to irreversible DNA damage, p53 becomes activated and induces the expression of pro-apoptotic members of the Bcl-2 family, which eventuates in apoptosis. The $p14^{ARF}$ protein can activate p53 by inactivating HDM2, an ubiquitin ligase that targets p53 for degradation by the proteasome.

Adapted from Hocker et al. (2008)

One of the senescence barriers in human cells involves the $p16^{INK4a}$ tumour suppressor protein encoded from the *CDKN2A* locus, which also encodes the tumour suppressor $p14^{ARF}$ (Mooi & Peeper, 2006). The $p16^{INK4a}$ protein is an inhibitor of the cyclin-dependent kinases CDK4 and CDK6, and prevents S-phase (synthesis phase) cell cycle entry by maintaining the retinoblastoma (RB) protein in an activated state (Sharpless et al., 2003). The $p16^{INK4a}$ protein has been found to be inactivated in the vast majority of melanomas through mutation, deletion or promoter hypermethylation of *CDKN2A* (Bennett, 2008). Genomic alterations affecting other components of the $p16^{INK4a}$-CDK4/6-RB senescence barrier have also been found in melanoma, but at significantly lower frequencies than *CDKN2A* alterations (Bennett, 2008; Hocker et al., 2008). The $p14^{ARF}$ protein activates the tumour suppressor protein p53 by inactivating HDM2, an ubiquitin ligase that targets p53 for degradation by the proteasome (Meyle & Guldberg, 2009). Loss of $p14^{ARF}$ leads to HDM2-mediated inactivation of p53. The p53 protein, often referred to as the "guardian of the genome", arrests the cell cycle allowing for repair of damaged DNA or the induction of apoptosis.

Downstream genetic elements such as the p53/Bcl-2 network, which is one of the most crucial regulators of melanoma cell apoptosis, are thought to underlie resistance to apoptosis and chemotherapy (Hocker et al., 2008). As most chemotherapeutic drugs function by inducing apoptosis in malignant cells, resistance to apoptosis is thought to be the main cause of drug resistance in melanoma.

In summary, it is likely that melanomagenesis involves molecular changes that (a) initiate clonal expansion, (b) overcome cell senescence and (c) reduce apoptosis.

(iii) A molecular classification of melanoma and its therapeutic implications

Recent findings have fuelled an enthusiastic attempt to formulate a molecular classification of melanoma (Fecher et al., 2007; Smalley et al., 2009; Thompson et al., 2009). Curtin et al. showed that four clinical subgroups of disease (melanoma arising from non-chronically sun-damaged skin, melanoma arising from chronically sun-damaged skin, melanoma arising from mucosal surfaces, and melanoma arising from acral surfaces) are characterised

by unique combinations of genome-wide aberrations in DNA copy number and oncogenic alterations (Curtin et al., 2005). Non-chronically sun-damaged melanomas are associated with frequent activating mutations in *BRAF* and a relative increase in frequency of *NRAS* mutations. Conversely, melanomas arising from mucosal and acral surfaces as well as skin with chronic sun damage do not usually show oncogenic mutations in *BRAF* and *NRAS* but do have augmented copy numbers of *CDK4* and *cyclin D1* and mutations in *KIT*. These recent findings have suggested that molecular subtyping of melanoma, rather than the conventional histopathological categorisation, might have greater practical utility with respect to drugs that specifically target receptor tyrosine kinases, downstream kinases and other signalling proteins.

The identification of activating mutations in *BRAF* in 40-60% of melanoma has led to the hypothesis that kinase inhibitors targeting BRAF might have therapeutic use. Clinical trials with sorafenib, the first agent with potential inhibitory activity against RAF family kinases, did not demonstrate significant clinical responses (Gajewski 2011; Romano et al., 2011). Trials with agents aimed at blocking RAS pathway signalling at other levels, including farnesyltransferase inhibitors (targeting RAS proteins directly) and MEK inhibitors (targeting the kinase downstream from RAF), were also disappointing. However, two recent studies with the oral BRAF inhibitor vemurafenib (PLX4032), a potent kinase inhibitor with specificity for the *BRAF V600E* mutation within cancer cells, have shown dramatic results (Chapman et al., 2011; Flaherty et al., 2010). Vemurafenib frequently induced tumour regression in patients with *BRAF V600E*–mutant metastatic melanoma and also improved overall survival. The KIT inhibitor imatinib has also been investigated and there is some evidence that it can induce regression in some of the small proportion of melanomas driven by mutations in *KIT* (Carvajal et al., 2011).

In time it is hoped that melanoma tumours will be routinely screened for the presence of a panel of specific predictive biomarkers, including mutations in *BRAF* and *KIT,* to enable allocation of individual patients to the most effective therapies (Romano et al., 2011).

1.2 Retroviruses

1.2.0 Summary

Retroviruses are a diverse group of human and animal viruses united by a distinct mechanism of replication whereby a DNA copy of the viral RNA genome (provirus) is integrated into the chromosomal DNA of the host cell. Retroviruses possess the enzyme reverse transcriptase (RT) and have the ability to reverse the usual direction of information flow in biological systems. Historically, transcription was thought only to occur from DNA to RNA, but RT transcribes RNA to DNA. The term "retro" (Latin, *retro*=backwards) in retrovirus refers to this reversal. The replication strategy of retroviruses is designed for long-term persistent infection, allowing the virus to be transmitted both vertically (from parent cell to daughter cells via the provirus) and horizontally (from cell to cell via virions). The discovery of HIV and HTLV (human T-lymphotropic virus) and the catastrophic impact of AIDS on human health have significantly raised the profile of retroviruses as human pathogens. Retroviruses have long been associated with cancer in animals. Fundamental insights into molecular pathways of carcinogenesis have been gained through the study of animal retroviruses, including the discovery of oncogenes. Thus far, only HTLV-1 amongst human retroviruses has been shown to be directly oncogenic. An aetiological role for viruses in human cancers has important implications. Firstly, the prevention of infection may reduce the risk of developing cancer and secondly, molecular and functional studies may uncover novel targets for cancer treatment. To this end, great effort has been directed towards identifying novel retroviruses involved in human cancer.

1.2.1 Background and an historical perspective

Although retroviruses were not named until 1974 (Baltimore, 1975), the diseases with which they are now associated had been observed in animals many years earlier. Bovine leukaemia and Jaagsiekte in sheep (also referred to as ovine pulmonary adenocarcinoma), caused by bovine leukaemia virus (BLV) and Jaagsiekte sheep retrovirus (JSRV) respectively, were recognised in the 19th century (Weiss, 2006). Vallée and Carré (1904) demonstrated that equine anaemia was infectiously transmitted by a filtrate and the

aetiological agent, termed equine infectious anaemia virus (EIAV), was subsequently shown to be a retrovirus.

Oncogenic retroviruses have been studied since the turn of the 20th century when Ellermann and Bang (1908), searching for an infectious cause of leukaemia, showed that erythroid leukaemia in chickens could be transferred from one chicken to another by cell-free tissue filtrates. This was followed by Rous's demonstration in 1911 of the transfer of sarcoma in chickens through filtrates and subsequent isolation of the infectious agent (Rous, 1911), since termed Rous sarcoma virus (RSV). This groundbreaking work later earned Rous a share of the Nobel Prize for Physiology or Medicine in 1966. Subsequently, Bittner observed the vertical transmission of murine breast cancer by adoptive nursing and postulated that a cancerous agent, or "milk factor", could be transmitted by mothers with breast cancer to young mice via the mother's milk (Bittner, 1936). This factor in murine breast milk was later found to be mouse mammary tumour virus (MMTV), a retrovirus (Coffin et al., 1997).

RSV particles were shown to contain RNA (Crawford & Crawford, 1961), hence oncogenic retroviruses were named RNA tumour viruses. The observation of the heritability of the RSV-transformed phenotype, even in the absence of viral replication, led Temin to postulate that the RSV genome made a DNA copy in the infected cell which then integrated into host chromosomal DNA (Temin, 1963; Temin, 1964). This was termed the DNA provirus hypothesis and the concept of reverse transcription was proposed. Temin's hypotheses went against the central dogma of molecular biology, originally proposed by Francis Crick in 1958, which states that DNA is transcribed into RNA that is then translated into proteins (Crick, 1970), and were thus met with scepticism. However in 1970 Temin and Baltimore independently discovered RT, an RNA-dependent DNA polymerase responsible for reverse transcription (Baltimore, 1970; Temin & Mizutani, 1970). Thus the possibility that genetic information could be passed on in this way was finally accepted. In 1975, Temin and Baltimore shared the Nobel Prize in Physiology or Medicine for the discovery of RT.

In 1980, HTLV-1 became the first infectious human retrovirus to be discovered (Poiesz et al., 1980). It has been shown to cause adult T-cell leukaemia/lymphoma (ATLL) or neurological disease (HTLV-associated myelopathy/tropical spastic paraparesis) in a minority of infections (Taylor, 2007). A related virus, HTLV-2, was subsequently reported in a patient with hairy cell leukaemia (Kalyanaraman et al., 1982). Subsequently HIV types 1 and 2, which both cause AIDS, were identified in 1983 and 1986 respectively (Barre-Sinoussi et al., 1983; Clavel et al., 1986).

1.2.2 Structure and genomic organisation

Retroviruses are enveloped viruses with single-stranded, positive-sense RNA genomes (Coffin et al., 1997). The components of a retrovirus virion and the genomic organisation of a provirus (the DNA form of a retrovirus) are illustrated in Figure 1.4. A virion contains two copies of the RNA genome and molecules of host cell RNA that have been packaged during assembly. These molecules include transfer RNA (tRNA) that are bound to each copy of the virus RNA, through base pairing with the primer binding site, and function to prime reverse transcription. Each retrovirus binds a specific tRNA.

A number of proteins are associated with the virus RNA including the nucleocapsid (NC) protein, which coats the RNA, and enzymatic proteins such as RT, DNA-dependent DNA polymerase, integrase (IN) and protease (PR). Encasing the RNA and its associated proteins is the capsid, constructed from a lattice of capsid (CA) proteins. A layer of matrix (MA) protein lies between the capsid and the envelope. Two proteins are associated with the latter: a transmembrane (TM) protein bound to a glycosylated surface (SU) protein.

The genes encoding the proteins are organised in three main regions of the genome: *gag* (*g*roup-specific *a*nti*g*en), *pol* (*pol*ymerase) and *env* (*env*elope). Retroviruses are designated as either simple or complex, depending on the complexity of their genome. Simple retroviruses, such as RSV, usually only have the three structural genes whereas complex retroviruses, such as HTLV and HIV, encode additional regulatory proteins derived from multiply spliced mRNA. The functions of retroviral genes, proteins and non-coding sequences are summarised in Table 1.1.

Figure 1.4: Retrovirus structure, genomic organisation and replication

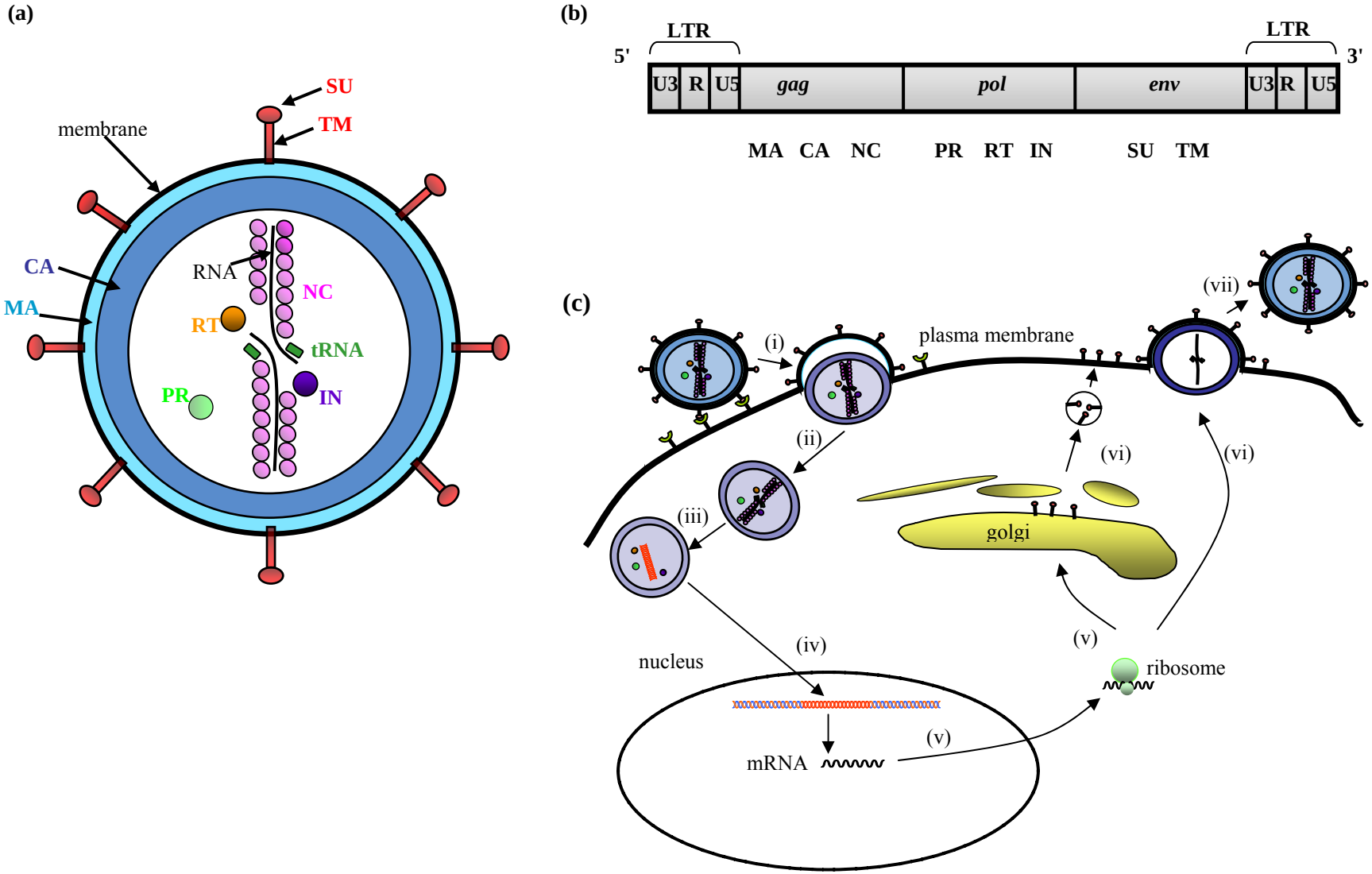


Figure 1.4: Legend

(a) Generalised structure of a retrovirus virion. The capsid, which is made up of capsid (CA) proteins, contains two copies of the RNA genome associated with nucleocapsid (NC) proteins, the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN), and cellular tRNA molecules. The capsid, surrounded by a layer of matrix (MA) protein, is contained within a lipid envelope comprising the surface (SU) and transmembrane (TM) envelope glycoproteins.

(b) The DNA form of a retrovirus genome (provirus). Retroviral genomic RNA differs from provirus by the absence of long terminal repeats (LTRs), which are built during reverse transcription. Each LTR is composed of three distinct regions, namely the repeated region (R) and unique sequences at 5' (U5) and 3' (U3) ends. The *gag* gene encodes the internal structural proteins (MA, CA and NC), *pol* encodes the retrovirus enzymes (PR, IT, IN) and *env* encodes the envelope proteins (SU, TM). These genes are present in all infectious retroviruses. Some retroviruses, for example HTLV-1, have genes that encode additional proteins with special functions in the retroviral life cycle or in pathogenesis.

(c) Retrovirus replication. (i) Retroviruses infect their target cells by adsorption to specific cell surface receptors, leading to conformational changes in the envelope and receptor molecules thereby triggering fusion of the viral and cell membranes. (ii) Fusion releases the viral core into the cytoplasm. (iii) Reverse transcription is initiated, primed by the bound tRNA and catalysed by RT, during which the single-stranded RNA genome is converted into a double-stranded DNA form and the LTRs are formed. (iv) The DNA subsequently becomes integrated into the chromosomal DNA of the cell to form the provirus, catalysed by IN. (v) Viral genes and proteins are expressed using the host's cellular machinery for transcription and translation. Some retroviruses, such as HTLV-1, also encode accessory proteins that can regulate these processes. (vi) Assembly of retroviral capsid occurs either in the cytoplasm prior to budding, or at the plasma membrane concomitant with budding. (vii) Once released, PR is activated and the viral polyproteins are cleaved into their mature forms. This maturation step is required for infectivity.

Table 1.1: Functions of retroviral genes, proteins and non-coding sequences

Abbreviation	Full name	Function(s)
Genes		
gag	*g*roup-specific *a*nti*g*en	encodes the internal structural proteins, namely MA, CA and NC
pol	*pol*ymerase	encodes the retrovirus enzymes, namely PR, RT and IN
env	*env*elope	encodes the envelope proteins, namely SU and TM
Proteins		
MA	matrix	Lines the envelope
CA	capsid	protects the core; most abundant protein in virion
NC	nucleocapsid	protects the RNA genome; forms the core
PR	protease	essential for Gag polyprotein cleavage during maturation
RT	reverse transcriptase	reverse transcribes the RNA genome; also has endonuclease activity
IN	integrase	required for integration of the provirus into host chromosomal DNA
SU	surface	outer envelope glycoprotein; major virus antigen; responsible for viral attachment and penetration
TM	transmembrane	inner component of the mature envelope glycoprotein
Non-coding sequences		
-	primer binding site	Used by the virus to begin reverse transcription
R	repeat sequence	provides the sequence homology for strand transfer during reverse transcription of the RNA genome
U3	unique sequence at 3' end of genome	forms the 5' end of the provirus after reverse transcription; contains the promoter elements responsible for transcription of the provirus
U5	unique sequence at 5' end of genome	First part of the genome to be reverse transcribed, forming the 3' end of the provirus genome

1.2.3 Replication

The defining features of retrovirus replication are (a) reverse transcription of the viral RNA after the infection of a target cell, catalysed by RT and (b) integration of the DNA product into the cellular chromosomal DNA, catalysed by IN (Coffin et al., 1997). After integration, the DNA provirus replicates by directing the synthesis of viral proteins and particles using the host's cellular mechanisms for transcription and translation. Capsid assembly occurs either in the cytoplasm or at the plasma membrane. New progeny virions are released by budding. Figure 1.4c summarises the retrovirus replication cycle.

1.2.4 Classification

The International Committee on the Taxonomy of Viruses has classified retroviruses into seven genera, based on phylogenetic and sequence analysis of the RT (Linial et al., 2005). This classification is summarised in Table 1.2.

1.2.5 Retroviruses and cancer

Viruses are the aetiological agents in approximately 15% of human cancers (Table 1.3). In the skin, human papilloma virus (HPV) has been implicated in non-melanoma skin cancer (Akgul et al., 2006; Molho-Pessach & Lotem, 2007) and a novel human polyomavirus, the Merkel cell polyomavirus (MCPyV), has been associated with Merkel cell carcinoma (Feng et al., 2008). The latency period can be many years because the development of cancer is complex and involves multiple steps in addition to virus infection.

Following the identification of retroviruses that cause cancers in animals, great effort has been directed toward identifying related viruses in human cancer. The only human retrovirus that is directly associated with cancer is HTLV-I, which causes ATLL (Matutes, 2007). Although not oncogenic *per se*, HIV-1 and HIV-2 lead to lymphoma, Kaposi's sarcoma (KS) and cervical carcinoma by allowing the DNA viruses underlying these tumours to exert their oncogenic effects in the immunosuppressed host (Boccardo & Villa, 2007; Carrillo-Infante et al., 2007; Damania, 2007; Grulich et al., 2007; Parkin, 2006; Talbot & Crawford, 2004).

Table 1.2: Classification of exogenous retroviruses

Genus & subfamily	Previous nomenclature	Genome Structure	Species infected	Examples (in-text abbreviations in parentheses)
Orthoretrovirinae:				
Alpharetrovirus	Avian C-type oncoretrovirus	Simple	Birds	Avian leukosis viruses Rous sarcoma virus (RSV)
Betaretrovirus	B-type oncoretrovirus D-type oncoretrovirus	Simple	Mice Primates Sheep	Mouse mammary tumour virus (MMTV) Mason-Pfizer monkey virus Jaagsiekte sheep retrovirus (JSRV)
Gammaretrovirus	Mammalian C-type oncoretrovirus	Simple	Mice Cats Primates Birds	Murine leukaemia virus (MLV) Feline leukaemia viruses Gibbon ape leukaemia virus Reticuloendotheliosis virus
Deltaretrovirus	C-type oncoretrovirus	Complex	Cattle Primates	Bovine leukaemia virus (BLV) Human T-lymphotropic viruses (HTLV)
Epsilonretrovirus	None	Simple	Fish	Walleye dermal sarcoma virus
Lentivirus	Lentivirus	Complex	Primates Sheep Cats Horses	Human immunodeficiency viruses (HIV) Simian immunodeficiency viruses Maedi/visna virus Feline leukaemia virus Equine infectious anaemia virus (EIAV)
Spumaretrovirinae:				
Spumavirus	Foamy virus	Complex	Primates Cats Cattle	Human foamy virus and simian foamy virus Feline foamy virus Bovine foamy virus

Modified from Voisett et al. (2008)

Table 1.3: Viruses associated with human cancer

Virus	Abbreviation	Cancer	% virus-positive*
human papillomavirus	HPV	cervical cancer	100
		vulval cancer	40
		penis cancer	>40
		anal cancer	90
hepatitis B virus	HBV	liver cancer	50
hepatitis C virus	HCV	liver cancer	25
Epstein-Barr virus	EBV	Burkitt's lymphoma	>90
		Hodgkin's lymphoma	>50
		post-transplant lymphoma	>80
		nasopharyngeal carcinoma	100
human herpesvirus 8	HHV-8	Kaposi's sarcoma (KS)	100
		primary effusion lymphoma	100
		multicentric Castleman's disease	>50
human T-cell lymphotropic virus type 1	HTLV-1	adult T cell leukaemia/lymphoma	100

Viruses are the aetiological agents in approximately 15% of all cancers. In some instances, not all cases of the tumour carry the virus but the virus accelerates a process that otherwise happens infrequently (for example, HHV-8 and multicentric Castleman's disease). In other scenarios, every case of the tumour involves the virus (HPV and cervical cancer; EBV and nasopharyngeal carcinoma; HHV-8 and KS).

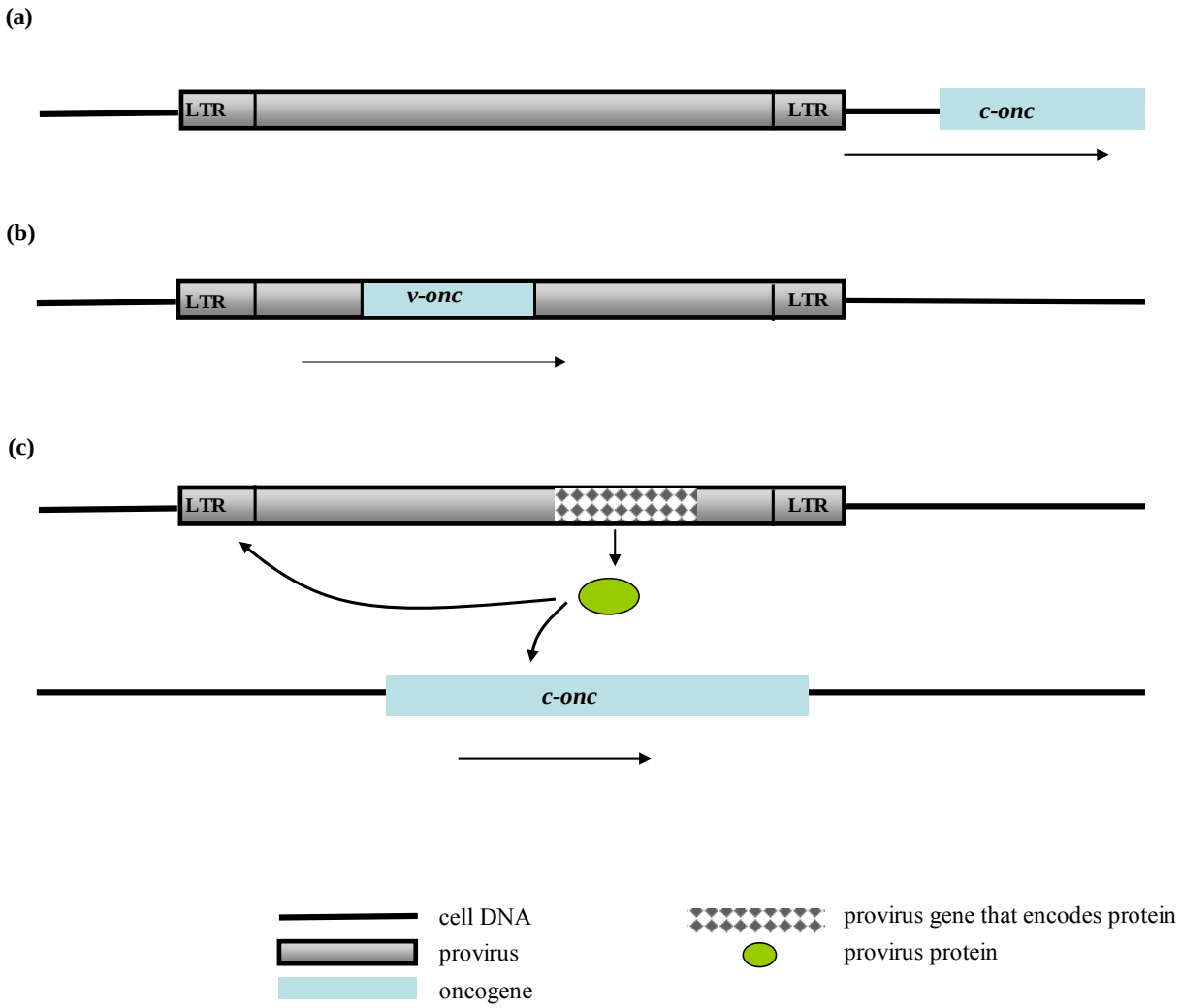
Modified from Cancer Research UK (2010c)

Crucial insights into the molecular biology of cancer have been gained through the study of animal retroviruses. Many years following the identification of RSV, oncogenic transformation by this virus was found to be due to an additional gene present in its genome that was not needed for virus replication. This gene was termed *v-src* (viral sarcoma) and was the first so-called "oncogene" to be characterised (Duesberg & Vogt, 1970). In 1976, Varmus and Bishop identified a gene with a sequence homologous to *v-src* in uninfected chickens and subsequently showed the gene to be present in many other organisms including humans (Martin, 2004; Stehelin et al., 1976). Varmus and Bishop's work uncovered a fundamental principle of cancer biology, namely that almost all known oncogenes are altered forms of normal genes. The term proto-oncogene is sometimes used to describe the normal cellular gene. In 1989, Varmus and Bishop were awarded the Nobel Prize for Physiology or Medicine for their contributions to the discovery of oncogenes.

The mechanisms of cell transformation by retroviruses are summarised in Figure 1.5. The two general mechanisms by which retroviruses induce tumours, discerned by early studies in mice and chickens, are insertional mutagenesis and oncogene capture (Maeda et al., 2008; Voisset et al., 2008). Insertional mutagenesis arises by retroviral integration at a site in the host DNA adjacent to a cellular oncogene (*c-onc*). Examples of the latter include genes for growth factors, protein kinases and transcription factors. The promoter and enhancer elements in the viral long terminal repeat (LTR) then activate the expression of *c-onc*, thus enhancing cell proliferation and subsequent tumourigenesis. Tumours appear only after a long latent period of months or years. Oncogene capture involves the insertion of *c-onc* sequences into the retroviral genome by recombination during reverse transcription. The resulting retrovirus (now containing *v-onc*) is usually replication-defective due to the disruption of essential genes. Co-infection of the same cell with a helper (wild-type) retrovirus is required for transmission. An exception is RSV where the genome retains enough of the structural gene sequences to remain capable of replication. Oncogenesis is a result of the constitutive expression of the "captured" oncogene, *v-onc*, under the control of the viral LTR. Such viruses are known as acutely-transforming retroviruses because of the fast growth of tumours, usually within weeks, in the animals in which they occur. In addition to these two well-characterised mechanisms of oncogenesis,

a small number of retroviruses encode their own oncogenic protein. For example, the Tax protein of HTLV-1 promotes cellular proliferation by transactivating the expression of a number of *c-onc*.

Figure 1.5: Mechanisms of cell transformation by retroviruses



Legend:

The provirus integrates into the genome of the host cell and this can result in cell transformation by three different mechanisms: (a) the integrated provirus activates an adjacent cellular oncogene (*c-onc*) via regulatory sequences in the provirus long terminal repeat (LTR); (b) the provirus carries a "captured" cellular oncogene (*v-onc*) constitutively expressed under the control of the LTR; (c) the provirus-encoded protein acts in *trans* not only to activate the LTR, but also cellular genes including *c-onc*.

1.3 Human endogenous retroviruses

1.3.0 Summary

The principal feature of the retroviral life cycle is integration of viral genetic information into the genome of the host cell, forming the provirus and thereby a permanent association between cell and retrovirus. Integration into the chromosome of a germ cell enables retroviruses to colonise the germ line of their hosts where they can persist as stable integrated proviruses, known as endogenous retroviruses (ERVs), for multiple generations. Human ERVs (HERVs), remnants of ancestral exogenous retroviral infections that became fixed in the germ line DNA, possess a similar genomic organisation to present day exogenous retroviruses but are not infectious (replication competent). Only members of the HERV-K (HML 2) provirus family have ORFs for all viral genes and are therefore the most likely to be biologically active. Additionally some members of this provirus family are present only in a proportion of the human population and are termed insertionally polymorphic HERVs. HERVs may be of benefit to the host and have been proposed to play important roles in a number of physiological processes including formation and functioning of the human placenta. However, HERVs might also be harmful and be involved in certain diseases. HERV-K has been associated with a number of human cancers through observation of expression of HERV-K mRNA, proteins and RVLPs in cancer tissues, and the presence of anti-HERV-K antibodies in patients**.** Animal models have provided fascinating insights into the potential mechanisms of HERVs in tumourigenesis. Thus far, evidence for a pathogenic role mediated by insertional mutagenesis in humans is lacking. Putative mechanisms include suppressing anti-tumour immune responses or malignant transformation induced by expression of potentially oncogenic accessory proteins, such as the HERV-K encoded Rec and Np9 proteins. It has been suggested that insertionally polymorphic HERVs are more likely to cause disease than non-polymorphic HERVs but, thus far, no significant associations with disease have been established.

1.3.1 Background and origin

Nearly 50% of the human genome is derived from ancient transposable elements (TEs), sequences of DNA that can move from one position to another within the genome of a single cell in a process termed transposition (Coffin et al., 1997). These mobile genetic elements are classified by their mechanism of transposition and structural organisation. Class I TEs (the retroelements), include the full-length HERVs, the retrotransposons, the retroposons and the solitary LTRs. These use an RNA-mediated mode of transposition and encode a RT. In contrast, class II TEs use a DNA-mediated mode of transposition. Previously considered as junk, selfish or parasitic DNA, it is now thought that these elements have participated in shaping the human genome during evolution, through increasing genome plasticity and conferring beneficial effects on the species (Cordaux & Batzer, 2009; Deragon & Capy, 2000; Makalowski & Toda, 2007; Oliver & Greene, 2009). Thus, rather than being a fixed structure, the genome of a eukaryotic cell can harbour sequences that move from one site on a chromosome to a completely different site. This phenomenon of plasticity is considered important because it allows rapid changes in the genome. Furthermore retroelements may carry regulatory sequences, for example promoters, to new sites in the genome and thus alter the expression of existing adjacent genes.

ERVs were discovered in the late 1960s and early 1970s with the identification of avian leukosis virus in the domestic fowl, and murine leukaemia virus (MLV) and MMTV in the laboratory mouse (Weiss, 2006). Bittner also observed that mammary tumour predisposition could be transmitted by the male in certain mouse strains and, subsequently, MMTV itself (in an endogenous form) was found to be the inherited factor (Bentvelzen & Daams, 1969; Bentvelzen et al., 1970). Exogenous and endogenous retroviruses sometimes co-exist in the same species, such as MLV and MMTV together with their respective endogenous counterparts.

ERVs were not discovered in humans until the early 1980s (Bonner et al., 1982; Callahan et al., 1982; Martin et al., 1981; O'Connell et al., 1984; Westley & May, 1984). Commonly referred to as HERVs, they integrated into the genome following ancient germ-line

infections by exogenous retroviruses. Thus HERVs are transmitted vertically as proviruses (in a Mendelian fashion) rather than the horizontal transmission characteristic of an infectious retrovirus (Bannert & Kurth, 2006). During evolution, HERVs have been amplified by repeated events of reintegration of reverse-transcribed mRNA (retrotransposition) and thus spread throughout the genome. It is currently estimated that HERV sequences make up about 8% of the human genome, representing around 4,000 proviruses and thousands more solo LTRs (Bannert & Kurth, 2006).

Studies have shown that HERVs are of great antiquity, for the majority are also present in the genomes of both apes and Old World monkeys suggesting that they originated more than 30 million years ago. HERV LTRs can serve as a "molecular clock" estimate for the time of integration of the provirus (Hughes & Coffin, 2004; Johnson & Coffin, 1999). Assuming that the two LTRs are identical at the time of integration and that they show mutation rates similar to those seen in introns, the approximate time of insertion into a genome can be calculated. The longer the provirus has been resident in the genome of its host, the more divergent are its LTRs.

There are over 20 HERV families and these are further subdivided into distinct groups and subgroups, each representing an independent evolutionary lineage (Belshaw et al., 2005; Tristem, 2000). Although many of these HERV colonisations took place more than 10 million years ago, a significant number took place during the human period of evolution. HERVs have shown to be useful tools in studying evolution and the plasticity of primate genomes (Buzdin, 2007; Sverdlov, 2000).

Over time, most HERVs have undergone recombinational deletion. This involves homologous recombination between the flanking LTRs of a full-length HERV, leaving behind a relic structure in the genome termed a solo LTR as depicted in Figure 1.6 (Belshaw et al., 2007). HERVs remained replication competent until they were inactivated, either by recombinational deletion or by random mutation, and could not be transcribed from the host DNA to replicate and infect another cell (Boeke & Stoye, 1997).

1.3.2 Classification

HERVs have been divided into three classes (I, II and III) based on their sequence similarity to animal gammaretroviruses, betaretroviruses, or spumaviruses, respectively (Gifford & Tristem, 2003; Nelson et al., 2003). There is no standard nomenclature for HERVs, but one system refers to the single letter amino acid code for the tRNA specificity of the primer binding site used to initiate reverse transcription (Bannert & Kurth, 2004; Lower et al., 1996). Therefore, if it were an infectious virus HERV-K would use lysine and HERV-W would use a tryptophan tRNA (Table 1.4).

1.3.3 Genomic organisation

Most HERV copies exist as solo LTRs but full-length HERV proviruses have a genomic structure akin to the proviruses of exogenous retroviruses, comprising *gag*, *pol* and *env* genes flanked by two LTRs (Figure 1.6a). The majority of HERV sequences are defective due to mutations, deletions or termination signals within coding sequences but some members of the HERV-K (HML 2) family have ORFs for all viral genes (Buzdin, 2007).

1.3.4 Biological roles

It has been postulated that HERVs have persisted and been retained during evolution because they performed and continue to perform useful biological functions (Ryan, 2004; Ryan, 2009). It is thought that HERVs, along with the other TEs, have contributed to genome plasticity (Jern & Coffin, 2008; Nelson et al., 2003). In addition, proviral LTRs serve as transcription regulators, alternative promoters and polyadenylation signals for several cellular genes (Brosius, 1999; Buzdin, 2007; Schon et al., 2001). It has been suggested that HERVs may confer resistance to viral infections by a number of mechanisms including retroviral receptor blockade (by HERV products) or interference of replication by antisense mRNA (Buzdin, 2007). Although there is no direct evidence for this in humans, at least two genes derived from ERVs have been shown to prevent virus spread through receptor interference or inhibition of proviral integration in mice (Best et al., 1996). Another possibility is that HERV peptides could prime an immune response to an undesirable viral agent (Nelson et al., 2003).

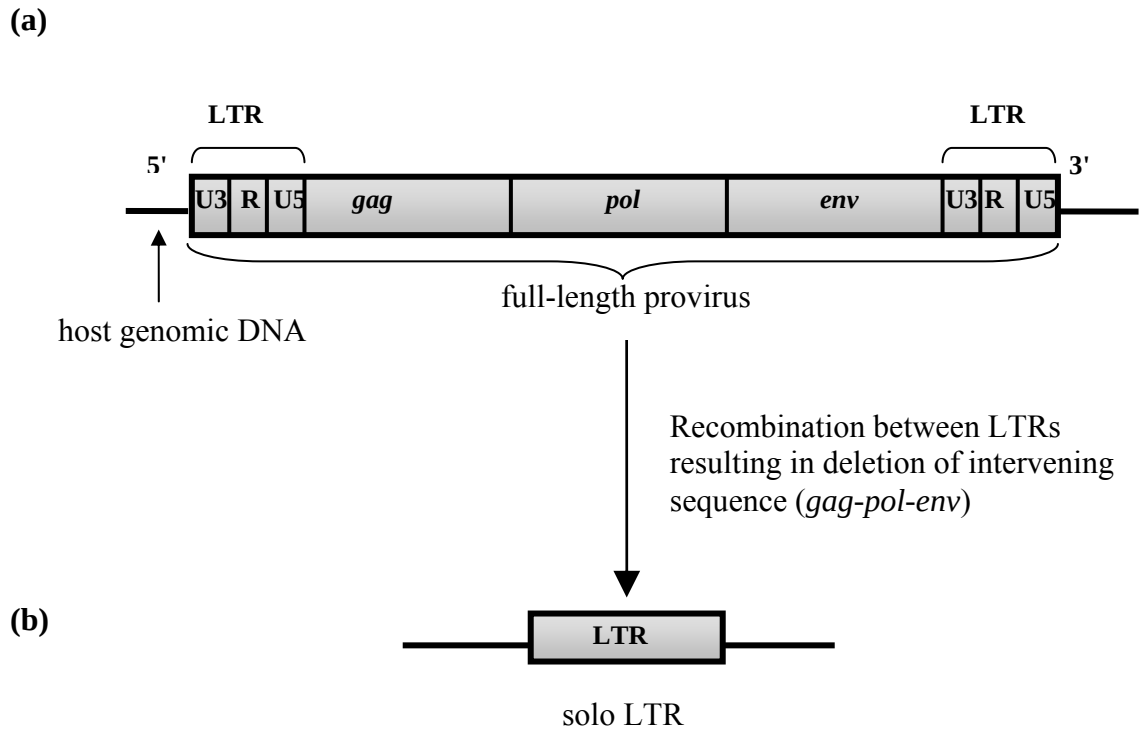
Table 1.4: Classification of HERVs

Class I	Class II	Class III
Group 1, HERV-HF	Group 1, HERV-K (HML-1)	HERV-L
HERV-H (RTLV-H, RGH)	HERV-K (HML-1.1)	
HERV-F	Group 2, HERV-K (HML-2)	
Group 2, HERV-RW	HERV-K10	
HERV-R (ERV9)	HERV-K-HTDV	
HERV-W	Group 3, HERV-K (HML-3)	
HERV-P (HuERS-P, HuRRS-P)	HERV-K (HML3.1)	
Group 3, HERV-ERI	Group 4, HERV-K (HML-4)	
HERV-E (4-1, ERVA, NP-2)	HERV-K-T47D	
51.1	Group 5, HERV-K (HML-5)	
HERV-R (ERV3)	HERV-K-NMWV2	
RRHERV.1	Group 6, HERV-K (HML-6)	
Group 4, HERV-T	HERV-K (HML-6p)	
HERV-T (S71, CRTK1, CRTK6)	Group 7, HERV-K (HML-7)	
Group 5, HERV-IP	HERV-K-NMWV7	
HERV-I (RTLV-1)	Group 8, HERV-K (HML-8)	
HERV-IP-T47D (ERV-FTD)	HERV-K-NMWV3	
Group 6, ERV-FRD	Group 9, HERV-K (HML-9)	
ERV-FRD	HERV-K-NMWV9	
	Group 10, HERV-K (HML-10)	
	HERV-KC4	

Classification systems for endogenous and exogenous retroviruses have developed separately and are poorly integrated. HERVs have been divided into three classes (I, II, and III) based on their sequence similarity to animal gammaretroviruses, betaretroviruses, or spumaviruses, respectively. Within each class are groups and subgroups. Class II HERVs show homology to mammalian betaretroviruses hence the use of HML in the nomenclature, which stands for *h*uman endogenous *M*MTV –*l*ike. One system of HERV nomenclature refers to the single letter amino acid code for the tRNA specificity of the primer binding site used to initiate reverse transcription.

Modified from Nelson et al. (2003).

Figure 1.6: Genomic organisation of a HERV provirus and solo LTR formation



Legend:

(a) Full-length HERV proviruses comprise *gag, pol and env* genes flanked by two long terminal repeats (LTRs). Retroviral genomic RNA differs from genomic DNA copy (provirus) by the absence of LTRs, which are built during the complex multistep process of reverse transcription. R, repeated region; U5 and U3, unique sequence at 5' and 3' ends respectively.

(b) Most HERVs exist in the form of solo LTRs that have arisen as a result of homologous recombination events between the identical LTRs flanking the proviral genes.

Certain HERV *env*-encoded proteins are involved in the formation and physiological function of the human placenta. The *env* genes of HERV-W and HERV-FRD encode the proteins syncytin-1 and syncytin-2 respectively, which fuse the trophoblast cells of the placenta into the confluent multinucleated syncytial layer (Blond et al., 2000; Mi et al., 2000). Syncytin-2 has also been shown to have immunosuppressive properties that may play a role in the maternal tolerance to foetal antigens (Mangeney et al., 2007). There is growing evidence that HERVs and their products are active during human germ cell formation and embryogenesis. It has previously been demonstrated that HERV-R (ERV-3), also involved in formation of the placenta, is highly expressed in many human foetal tissues as well as the sebaceous glands of normal skin (Andersson et al., 2002; Andersson et al., 1996).

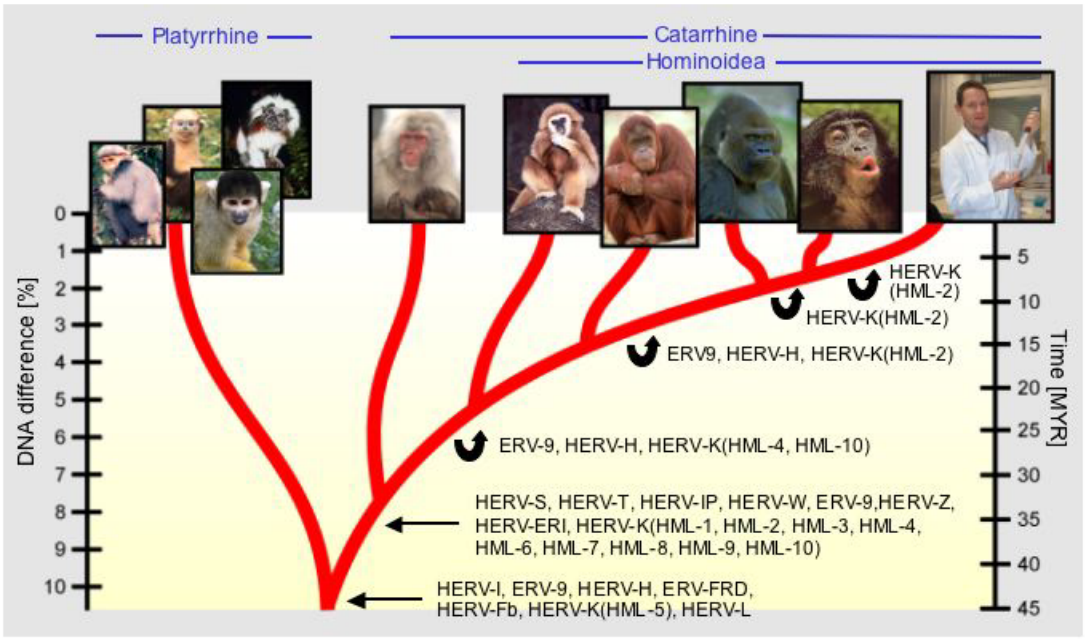
1.3.5 HERV-K family

(i) Members

The HERV-K family comprises 60 full-length proviruses and 2,500 solo LTRs of which 134 members, all within the HERV-K (HML-2) subgroup (Mamedov et al., 2004; Buzdin, 2007), integrated into the human genome after the divergence of humans and chimpanzees (Figure 1.7).

Some of the full-length human-specific proviruses contain nearly intact ORFs with few or no mutations (Barbulescu et al., 1999; Mayer et al., 1999; Reus et al., 2001; Turner et al., 2001) and encode functional proteins in vitro (Berkhout et al., 1999; Dewannieux et al., 2005; Harris et al., 1997; Kitamura et al., 1996; Magin et al., 1999; Mueller-Lantzsch et al., 1993; Yang et al., 1999). Thus the HERV-K family is often referred to as the most "biologically active" family of HERVs (Tonjes et al., 1996). However, although laboratory engineered HERV-K (HML-2) proviruses are infectious (Dewannieux et al., 2006; Lee & Bieniasz, 2007), no infectious (replication competent) HERV proviruses have been identified in the human population thus far.

Figure 1.7: The evolutionary history of HERV-K



Legend:

HERV integration and amplification events during primate evolution. HERVs are remnants of ancient germ line infections with exogenous retroviruses that have become genetically fixed and vertically transmitted through primate evolution. Presumed integration/expansion events for each HERV subclass are indicated with arrows (Greenwood et al., 2005). The numbers to the left indicate the percentage difference between DNAs of primates and those to the right give the estimated number of millions of years (MYR) since they diverged from a common ancestor. A major invasion and expansion of ERVs occurred after the platyrrhine (New World monkeys) lineage separated from the catarrhine lineage (Old World monkeys and apes/hominoidea). The majority of HERVs (including members of the HERV-K family) are present in the genomes of both apes (including gibbons, orangutans, gorillas, chimpanzees and humans) and Old World monkeys suggesting they originated more than 30 million years ago. The HERV-K (HML-2) subgroup is the only group known to contain human-specific members i.e. HERVs that integrated after the divergence of humans and chimpanzees.

From Leib-Mösch (2010)

(ii) Insertional polymorphisms

Insertionally polymorphic HERVs are proviruses that are present only in a proportion of the human population i.e. some individuals have the insertion (provirus) while other individuals have the empty pre-insertion site (no provirus). There are high levels of insertional polymorphism in the HERV-K (HML-2) family (Belshaw et al., 2005). HERV-K113 and HERV-K115, both full-length insertionally polymorphic proviruses, are evolutionarily recent additions to the human genome, estimated to have become integrated 200,000 and 1.2 million years ago respectively (Turner et al., 2001). Moyes et al. found the frequency of HERV-K113 and HERV-K115 in 174 individuals from Africa to be 21.8 and 34.1% respectively, compared with 4.16 and 1% in 96 people in the UK and 0% for both proviruses in 54 people from Papua New Guinea (Moyes et al., 2005). Both proviruses have ORFs for all their genes, apart from a frameshift mutation in the *gag* gene of HERV-K115. Hence HERV-K113 can potentially encode all elements necessary for a functional retrovirus. To date, 10–13 further insertional polymorphisms of HERV-K (HML-2) have been reported (Belshaw et al., 2005; Hughes & Coffin, 2004; Macfarlane & Simmonds, 2004; Moyes et al., 2007).

(iii) Splicing patterns

The polyadenylated full-length transcript derived from HERV-K can be spliced further, resulting in different splice forms depicted in Figure 1.8a (Buzdin, 2007). Unspliced mRNA encodes the Gag and Pol proteins. The Gag protein is further cleaved to generate MA, CA and NC proteins. Pol is the retroviral RT. Spliced mRNA encodes the Env protein and double-spliced mRNA encodes the accessory proteins Rec or Np9.

Two forms of HERV-K provirus exist (Bannert & Kurth, 2004). Type 2 proviruses are complete whereas type 1 proviruses have a 292 base pair (bp) deletion at the boundary of the *pol* and *env* genes (Figure 1.8b). Type 2 proviral transcripts, 1.8 kb (kilobases) long, encode the accessory protein Rec (previously known as cORF) that has functional homology to lentiviral RNA-binding nuclear export proteins such as the HIV-encoded Rev and HTLV-encoded Rex proteins (Lower et al., 1995; Magin et al., 1999). Rec binds to unspliced or partially spliced viral transcripts and facilitates their transfer to the cytoplasm

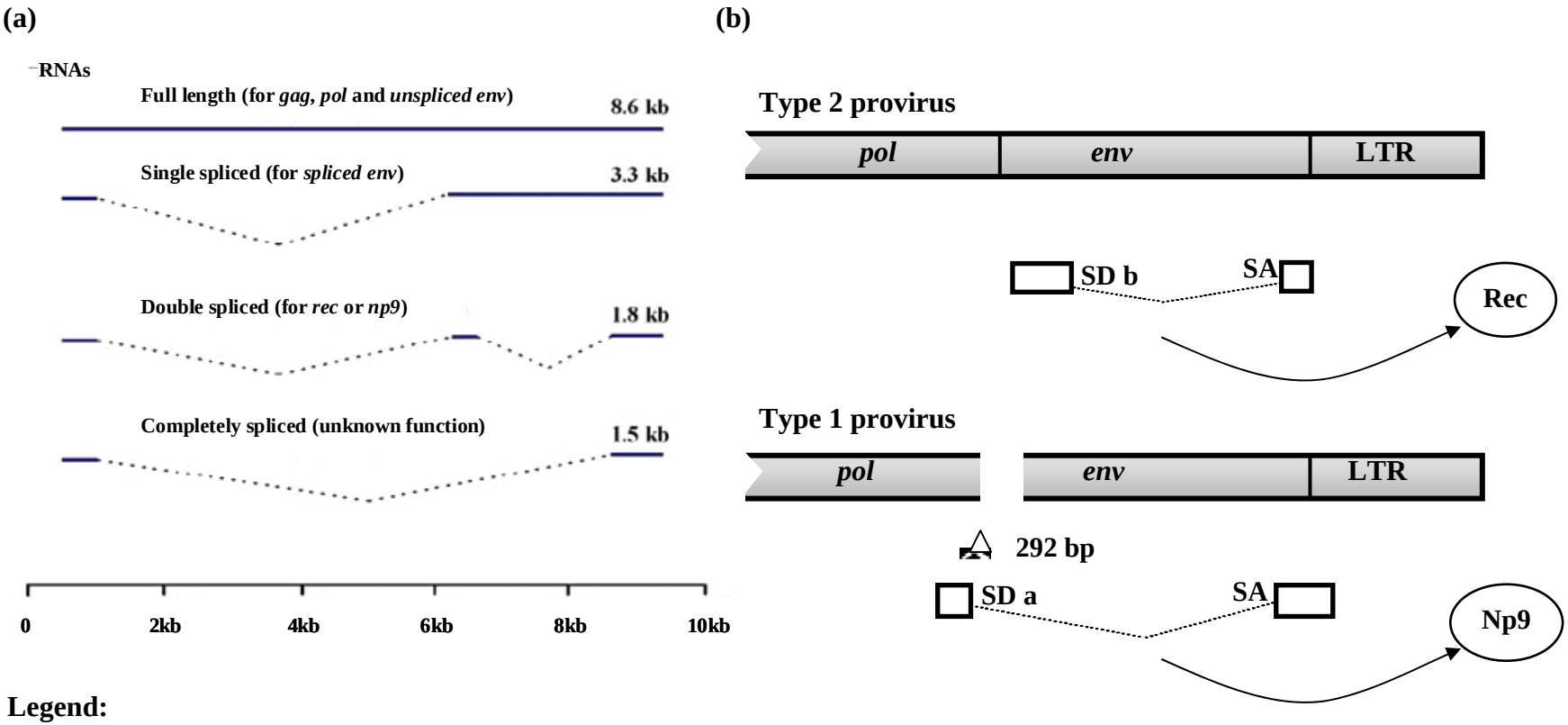
where they can be translated. Rec is specifically localised to the nucleolus, suggesting that it plays a role in the HERV life cycle. Type 1 provirus-specific double-spliced mRNA derived protein, Np9, is also localised to the nucleus. Although expression of Np9 has been observed in a number of tissues and cell lines, the molecular function remains elusive (Armbruester et al., 2002).

1.3.6 HERVs and disease

HERVs have been implicated in the pathogenesis of a number of human diseases, in particular multiple sclerosis, Type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus (SLE), Sjogrens syndrome, schizophrenia and certain cancers (Nelson et al., 2003; Nelson et al., 2004; Ryan, 2004; Voisset et al., 2008). Regarding skin disease, there is evidence to suggest that HERVs are associated with psoriasis, alopecia areata (AA) and CD30-positive lymphoproliferative disorders, summarised in Table 1.5. The evidence associating HERVs with melanoma is considered in detail later (section 1.4).

Several pathogenic mechanisms for HERVs in disease have been postulated. These include disruption or modulation of expression of host genes, through the LTR promoter activity, at or near their integration site; suppression or stimulation of both the adaptive and innate immune response by the Gag or Env proteins (which have also been speculated as potential autoantigens); and detrimental cellular effects induced by accessory proteins such as Rec and Np9 (Moyes et al., 2007; Voisset et al., 2008). In addition LTRs may act as "biological transducers", as HERV expression can be altered through hormonal and environmental signals (Nelson et al., 2004). For example, Hohenadl et al. demonstrated the activation of HERV transcription in human epidermal keratinocytes by UVB irradiation and in UVB-provoked or lesional skin samples of lupus erythematosus patients (Hohenadl et al., 1999). The authors suggested that this may contribute to the pathogenesis of the disease, where inappropriate antigenic presentation of UVB-induced viral and cellular proteins could stimulate autoantibody production. However, evidence that these pathogenic mechanisms occur in vivo is lacking and, thus far, there is no conclusive proof of a *causative* role for HERVs in any human disease. Nevertheless, it is possible that HERVs are aetiological co-factors in disease.

Figure 1.8: HERV-K transcripts



Legend:

(a) HERV-K transcripts. Full-length mRNA encodes Gag and Pol proteins. Single-spliced mRNA encodes Env protein, double-spliced mRNA encodes Rec or Np9 protein and completely spliced mRNA is of unknown function. Modified from Buzdin (2007).
(b) Diagram illustrating the *pol*, *env* and 3' long terminal repeat (LTR) regions of HERV-K type 1 and 2 proviruses. The proviruses differ in a 292 bp deletion that contains the splice donor (SD) site used to generate *rec* transcripts (SDb). An alternative upstream SD site (SDa) is used for *np9* messages. SA, splice acceptor.

Table 1.5: Association of HERVs with skin disease

Disease	Findings	References
Psoriasis	Association of murine leukaemia virus (MLV)-like group of HERVs with psoriasis reported. Anti-MLV antibodies detected with a higher frequency in sera from patients with psoriasis compared with control sera. IgG response to Gag and Env dramatically increased in psoriasis.	Moles et al., 2003
	Expression of HERV-E transmembrane envelope glycoprotein detected by immunohistochemistry in lesional epidermis of patients with psoriasis and atopic dermatitis. Pattern of expression in keratinocytes different in both skin diseases and positive staining of dermal CD4+T cells observed in psoriatic skin only.	Bessis et al., 2004
	Elevated expression of mRNA derived from HERV-W, HERV-K, and HERV-E together with new variant (related to ERV-9/HERV-W) detected in lesional versus non-lesional psoriatic skin. New variant rarely observed in normal or atopic dermatitis skin samples, thus implicated in psoriasis pathogenesis. However, absent from psoriatic blood and sera.	Moles et al., 2005
	RT proteins detected in normal and psoriatic skin with increased number of RT-positive cells in the psoriatic lesions. RT activities two-three times higher in psoriatic protein extracts than in normal skin protein extracts. Authors concluded that active ERV elements can produce functional RT proteins and suggested that a basal level of ERV activity exists in normal skin and that keratinocyte hyperproliferation and/or inflammation promotes this activity.	Moles et al., 2007

Disease	Findings	References
Systemic lupus erythematosus (SLE)	Expression and autoantigenicity of HERVs, in particular HRES-1 and ERV-3, demonstrated in patients with SLE in a number of studies. Postulated that HERVs may lead to autoimmunity directly, by encoding autoantigens, or indirectly, by affecting expression of genes regulating immune responses and tolerance. If expressed, HERVs likely targets of cross-reactivity for virally induced immune responses. Such cross-reactivity, i.e. molecular mimicry between self-antigens and viral proteins has been proposed as a trigger of autoimmunity.	Anders et al., 1996; Banki et al., 1992; Bengtsson et al., 1996; Brookes et al., 1992; Hohenadl et al., 1999; Li et al., 1996; Magistrelli et al., 1999; Perl et al., 1995
Alopecia areata (AA)	Presence of serum antibodies to human intracisternal A-type particle retrovirus proteins found to be significantly associated with AA. Antibodies detected in a high frequency of AA patients compared with normal blood donors.	La Placa et al., 2004
CD30-positive lymphoproliferative disorders (LPD)	Retrovirus-like particles (RVLPs) observed by electron microscopy in lesional tissue and tumour cell lines from primary cutaneous CD30-positive LPD. HERV transcripts related to ERV 9 demonstrated in lesional tissue and cell lines. RT activity detected in the cell line supernatants.	Kempf et al., 2003

In the last two decades, several studies have provided some evidence that HERVS may be involved in certain skin diseases. SLE is included because many patients will have skin involvement. HERVs have also been associated with melanoma and this evidence is summarised in section 1.4.

1.3.7 HERVs and cancer

(i) Human cancers associated with HERV-K

A clear-cut role for HERVs in human cancer has not been established, but there is mounting evidence that HERV-K may be oncogenic**.** HERV-K transcripts, proteins and RVLPs with homology to HERV-K have been reported in several cancers including melanoma, testicular germ cell tumours (TGCTs), breast cancer, myeloproliferative disorders and ovarian cancer, summarised in Table 1.6.

Antibodies to HERV-K have been detected in patients with melanoma, breast cancer and TGCTs. HERV-K Gag and Env antibody titres have been observed to decrease with effective treatment of TGCTs (Boller et al., 1997; Kleiman et al., 2004; Sauter et al., 1996; Sauter et al., 1995). In one study, anti-TM antibodies were universally found in TGCT patients but not in healthy controls (Sauter et al., 1996). T-cell reactivity to HERV-K peptides has been reported in patients with a history of seminoma and a minority of healthy controls (Rakoff-Nahoum et al., 2006). Breast cancer patients have also been shown to mount cellular and humoral immune responses against HERV-K, characterised by the presence of increased levels of antigen-specific T-cell proliferation, cytokine production, HERV-K–specific cytolytic T-lymphocyte (CTL) activity, and the presence of anti-HERV-K Env IgG in patient sera (Wang-Johanning et al., 2008). However, anti-HERV-K antibodies have also been reported in some patients with autoimmune diseases and healthy individuals (Badenhoop et al., 1999; Herve et al., 2002).

(ii) Putative mechanisms for HERV tumourigenesis

A number of different mechanisms whereby HERVs could be involved in tumourigenesis have been suggested, summarised in Figure 1.9. Specific examples relevant to melanoma are discussed later (section 1.4). It has been postulated that HERVs could be involved in tumourigenesis as a result of expression of mRNA, functional proteins or VLPs (virus-like particles)/RVLPs. However, it remains unknown whether HERV expression is merely an epiphenomenon or indicates a direct contribution to the process of tumourigenesis. Furthermore, a number of recent studies have demonstrated that HERV transcripts, including HERV-K mRNA, are also expressed in many normal human tissues (Flockerzi et al., 2008; Muradrasoli et al., 2006; Seifarth et al., 2005).

Table 1.6: Human cancers associated with HERV-K

Cancer	Evidence	References
Melanoma	mRNA and protein expression; RVLPs; anti-HERV-K Ab	Buscher et al., 2006; Buscher et al., 2005; Hahn et al., 2008; Hirschl et al., 2007; Humer et al., 2006; Muster et al., 2003; Schiavetti et al., 2002
Testicular germ cell tumours (TGCTs)	mRNA and protein expression; RVLPs; anti-HERV-K Ab	Bieda et al., 2001; Boller et al., 1997; Boller et al., 1993; Gotzinger et al., 1996; Herbst et al., 1999; Herbst et al., 1998; Herbst et al., 1996; Kleiman et al., 2004; Rakoff-Nahoum et al., 2006; Roelofs et al., 1998; Sauter et al., 1996; Sauter et al., 1995
Breast cancer	mRNA and protein expression; anti-HERV-K Ab	Contreras-Galindo et al., 2008; Frank et al., 2008; Wang-Johanning et al., 2003; Wang-Johanning et al., 2001; Wang-Johanning et al., 2008
Ovarian cancer	mRNA and protein expression	Wang-Johanning et al., 2007
Myeloproliferative disorders	mRNA and protein expression; RVLPs	Brodsky et al., 1993; Contreras-Galindo et al., 2008; Depil et al., 2002

HERV-K has been associated with a number of human cancers because of the expression of HERV-K mRNA, proteins and retrovirus-like particles (RVLPs) in cancer tissue and the presence of anti-HERV-K antibodies (Ab) in patients.

Figure 1.9: Putative mechanisms for HERV tumourigenesis

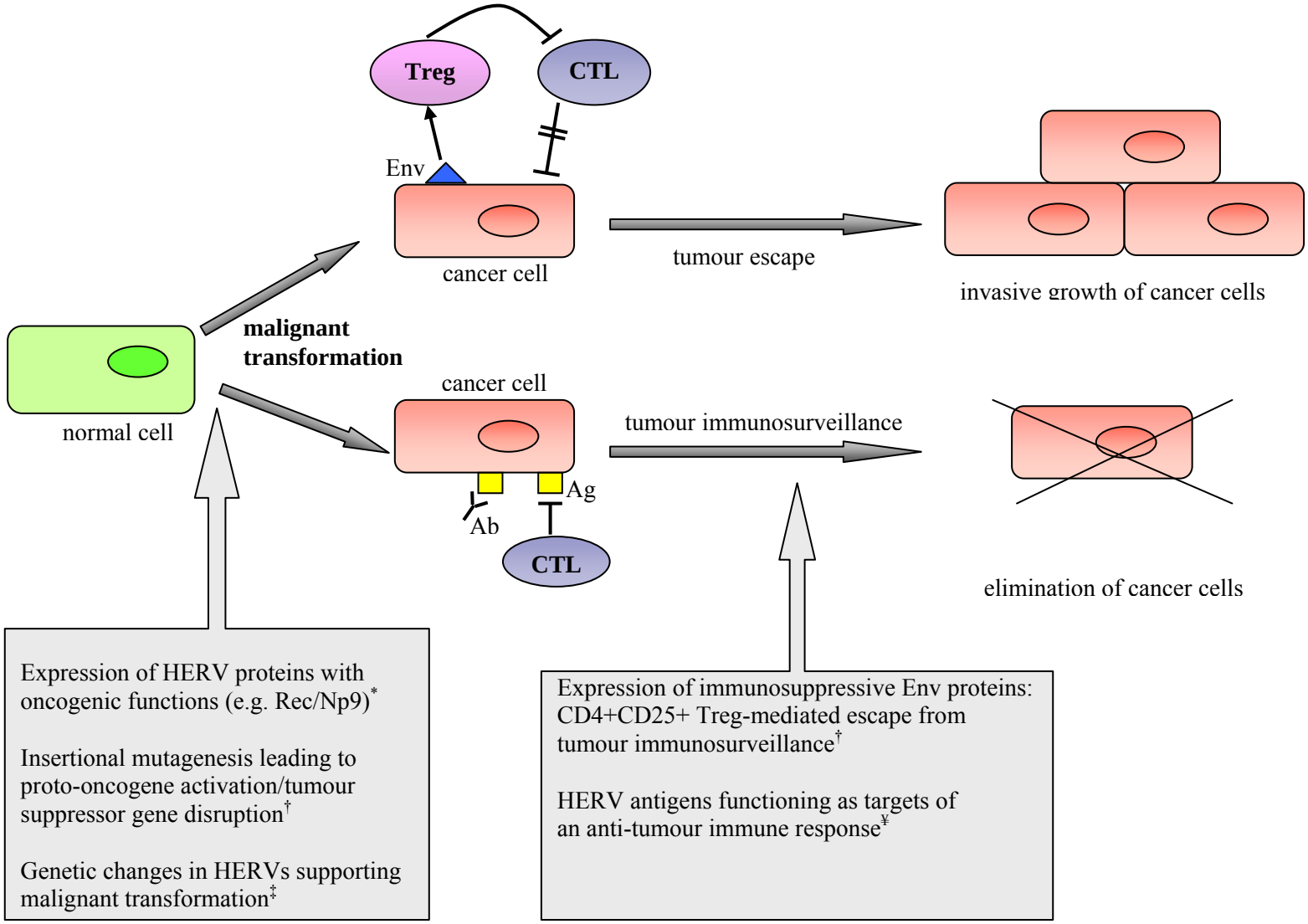


Figure 1.9: Legend

Malignant transformation is the process by which a normal cell (green) turns into a cancer cell (red) and the potential mechanisms whereby HERVs contribute to this process are illustrated (grey boxes). Malignant cells are normally controlled by the immune system (tumour immunosurveillance) and cancers may arise from a population of transformed cells that have developed the ability to escape immunosurveillance. Expression of HERV antigens (HERV-Ags, yellow squares) on cancer cells may trigger an anti-tumour immune response involving antibodies (Ab) and CD8+ CTL, which ideally results in the elimination of cancer cells. However, expression of immunosuppressive HERV Env (blue triangle) proteins on cancer cells may give rise to a $CD4^{+}CD25^{+}$ regulatory T cell (Treg)-mediated suppression of anti-tumour CTL, leading to escape of cancer cells from immunosurveillance and subsequent invasive growth.

Modified from Ruprecht et al. (2008)

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* Some evidence for relevance in human cancer

† Evidence in animal models only

‡ Hypothetical mechanism

¥ Some evidence for anti-HERV immune responses in human cancers but functional relevance unclear

Murine models have provided strong evidence for a causal role of ERVs in tumourigenesis, such as leukaemias induced by endogenous MLV and mammary tumours caused by endogenous MMTV. Notably, in both cases, there are exogenous variants that also induce tumours. Leukaemogenesis in the affected mouse strains is a multistep process necessitating the generation of leukaemogenic recombinant viruses, known as mink cell focus forming (MCF) viruses, from at least three endogenous MLV proviruses. *De novo* integration of MCF recombinant viruses can activate proto-oncogenes, eventually causing leukaemia (Fan, 1997). Expression of endogenous MMTV related proviruses in lactating mammary glands of affected mouse strains may result in the release of infectious virus, followed by reinfection, novel proviral insertions, and activation of cellular proto-oncogenes (Boeke & Stoye, 1997).

A common characteristic of animal models is the presence of replication-competent ERVs that are able to form novel proviral insertions that induce tumours by insertional mutagenesis. In the case of MMTV such a replication-competent ERV is apparently inherited, whereas in other cases replication competency is dependent upon recombination events between different ERV proviral loci. It is clear that tumourigenic properties of ERVs in animal models are dependent on retroviral movement, i.e. *de novo* insertions of proviral copies in somatic cells of the host. However, no infectious (i.e. replication competent) HERVs have been identified in the human genome thus far, and evidence for novel somatic cell proviral HERV insertions in present-day humans is lacking.

Belshaw et al. (2004 & 2005) have suggested that the absence of infectious HERVs in the published human genome sequence does not exclude their existence. They have proposed that there are HERV-K (HML-2) elements in the human germ line that are still active and infectious in present day humans, and that such elements may cause disease in the individuals carrying them (Belshaw et al., 2005; Belshaw et al., 2004). Since these elements may be harmful to their hosts, they are unlikely to reach high allele frequencies in the human population as a whole. Only if an infectious HERV-K (HML-2) element acquires inactivating mutations it may, in its "neutral" state, reach high allele frequencies and eventually become fixed in the population. Robust evidence for the current mobility of HERV elements and resultant pathogenic functions is required. The presence of novel integrations of HERV-K

(HML-2) elements in genomic DNA of malignant somatic cells in addition to corresponding empty pre-integration sites in genomic DNA of normal somatic cells in the same individual would constitute such evidence.

There is evidence that HERV-K-encoded proteins, notably Rec and Np9, may be involved in tumourigenesis. Both proteins bind the promyelocytic leukaemia zinc finger (PLZF) protein that plays a role in spermatogenesis (Boese et al., 2000; Denne et al., 2007). Since PLZF can act as a transcriptional repressor, impairment of its function through binding of Rec or Np9 might promote cell proliferation through the activation of proto-oncogenes such as c-*myc*. In turn, this could result in the up-regulation of c-*myc* regulated genes, such as *p53, PCNA* and *IkBa*. Phenotypically, this is associated with increased proliferation and reduced apoptosis of cells stably co-expressing Rec and PLZF compared to cells stably expressing PLZF alone (Denne et al., 2007). Interestingly, mice transgenic for *rec* show changes in the testis typical of early seminoma (Galli et al., 2005).

In immunocompetent individuals the tendency to develop cancer is attenuated by the immune system, a phenomenon known as tumour immunosurveillance. The evasion of immunosurveillance (termed tumour escape) is an important step towards uncontrolled growth of transformed cells and tumour formation (Dunn et al., 2002; Garcia-Lora et al., 2003). Immunosuppressive functions of HERV Env proteins have been implicated in the inhibition of an anti-tumoural immune response, through aberrant expression in cancers in animal models (Ruprecht et al., 2008). Subversion of immunosurveillance is thought to be mediated by $CD4^+CD25^+$ regulatory T-cells (Tregs) and is discussed later (section 1.4.3) in the context of murine melanoma. It is uncertain whether such mechanisms are relevant to human cancer. Further work is required to clarify whether immunosuppressive HERV Env proteins are expressed in human cancers and if such expression is related to Treg-mediated tumour immune escape.

Conversely, there is some evidence to suggest that HERV proteins may function as tumour antigens and, by eliciting an anti-tumoural immune response, might exert a protective effect (Ruprecht et al., 2008). Additionally, such immune responses may have the potential to serve

as tumour markers. Tumour associated aberrantly expressed HERV epitopes could elicit an anti-tumour immune response when subject to immunosurveillance. However, whether the production of anti-HERV-K antibodies contributes to the immunological control of tumour growth remains obscure. Further studies are needed to determine the functional consequences of HERV-directed immune responses in terms of tumour control and the biological usefulness of such responses

It has been suggested that insertionally polymorphic HERVs are more likely to cause disease than non-polymorphic HERVs because their recent insertion might have disrupted host genes and, as evolutionarily recent integrations, they are more likely to have retained functional coding sequences that might modify cellular proliferation or immune responses (Moyes et al., 2007). However, thus far, no significant associations of polymorphic HERVs with disease have been established (Burmeister et al., 2004; Moyes et al., 2008; Otowa et al., 2006)

1.4 HERVs and Melanoma

1.4.0 Summary

Since the 1970's, evidence that a retrovirus might be involved in melanomagenesis has been accumulating (Table 1.7). RVLPs and the expression of HERV mRNA and proteins have been demonstrated in melanoma tissue. In addition, antibodies to HERV proteins have been observed in melanoma patients. In vitro and mouse models have provided intriguing insights into the potential mechanisms of HERVs in melanomagenesis. Although there is theoretical and laboratory evidence to specifically implicate HERV-K in melanomagenesis, an aetiopathogenic role has not yet been proven. It is possible that expression of HERV-K mRNA and proteins, and formation of RVLPs are epiphenomena associated with tumourigenesis. Work so far has focused on melanoma cell lines and metastasis tissue thus further comparative investigations of HERV-K expression in primary melanoma and normal skin are required. Further analyses of the potentially oncogenic HERV-K proteins Rec and

Np9 are necessary to ascertain whether they are important in the causation of melanoma and have potential as novel therapeutic targets.

1.4.1 RVLPs in melanoma

Following the discovery of VLPs in hamster melanoma (Epstein & Fukuyama, 1970), evidence has been sought for a viral aetiology in human melanoma. RVLPs were first detected in human melanoma by electron microscopy (EM) and biochemically in the 1970s (Balda & Birkmayer, 1973; Balda et al., 1975; Birkmayer et al., 1974; Birkmayer et al., 1972; Hehlmann et al., 1978; Parsons et al., 1974; Parsons et al., 1976).

Initially VLPs and RT activity were found in lymph node metastases of human melanoma (Birkmayer et al., 1972). Subsequently more detailed studies followed. In particular, the biochemical features of oncogenic RNA viruses were sought, namely a single-stranded high molecular weight RNA and RT activity (Balda et al., 1975; Birkmayer et al., 1974). Balda et al. prepared virus particle-enriched fractions from primary and metastatic melanoma tissue and examined them by the "simultaneous detection test", for the presence of high-molecular-weight RNA associated with a RT (Balda et al., 1975). The authors determined the density of the particles that had a positive simultaneous detection test and found that most of the positive particles showed density characteristics of RNA tumour viruses (retroviruses).

More recently, RVLPs have been demonstrated in melanoma cell line supernatants by EM (Buscher et al., 2005; Muster et al., 2003). They were shown to have RT activity and package sequences with homology to HERV-K108 and to contain Gag and Env proteins (Muster et al., 2003). No RT activity was detected in supernatants from cultured neonatal human melanocytes (NEHMs) suggesting that the production of particles containing RT is exclusive to melanoma. However, these RVLPs were found to be defective and non-infectious (Buscher et al., 2005). Another study that aimed to characterise particle-derived sequences found them to be defective and to vary between different melanoma cell lines, where even particles from the same cell line contained heterologous sequences (Hirschl et al., 2007).

Table 1.7: Summary of evidence associating HERVs with melanoma

Year	Finding	References
1970's	RVLPs observed in human melanoma tissue and melanoma cell lines by EM and biochemically	Balda & Birkmayer, 1973; Balda et al., 1975; Birkmayer et al., 1974; Birkmayer et al., 1972; Hehlmann et al., 1978; Parsons et al., 1974; Parsons et al., 1976
2002	A HERV-K antigen, presented in association with HLA-A2, on the autologous tumour cells of a melanoma patient shown to be targeted by CTLs.	Schiavetti et al., 2002
2003	RVLPs in supernatants of human melanoma cell lines shown to have RT activity, package sequences with homology to HERV-K108, and contain Gag and Env proteins. Expression of the HERV-K108 *pol* gene and of Gag, Rec and Env proteins demonstrated in primary and metastatic melanoma tissue.	Muster et al., 2003
2005	Expression of full-length HERV-K mRNA and spliced *env* and *rec* mRNA demonstrated in metastatic melanoma tissue and cell lines. HERV-K TM and HERV-K Gag protein expression detected in primary and metastatic melanoma samples by immunohistochemistry. RVLPs in melanoma cell line supernatants shown to be defective and non-infectious.	Buscher et al., 2005
	ERV expression shown to be required for murine melanoma tumour growth in vivo by RNA interference (RNAi) experiments. Proposed that ERVs promote melanomagenesis by a regulatory T-cell mediated subversion of immunosurveillance.	Mangeney et al., 2005
	Inhibition of endogenous RT, by RT inhibitors and RNAi, shown to antagonise melanoma growth in vivo in mouse experiments.	Sciamanna et al., 2005

Year	Finding	References
2006	Expression of spliced *env*, *rec* and *np9* mRNA demonstrated in metastatic melanoma tissue and melanoma cell lines by RT-PCR. TM and Rec, but not Np9, protein expression demonstrated in metastatic melanoma tissue by immunohistochemistry.	Buscher et al., 2006
	Antibodies directed against HERV-K TM and Env proteins reported retrospectively in sera from melanoma patients. However, HERV-K Rec and Np9-specific antibodies not detected.	Buscher et al., 2006; Humer et al., 2006
	Characterisation of the MelARV (melanoma-associated retrovirus) proviruses present in the murine primary B16 melanoma cell line and a metastatic sub-line revealed mobility of the element with new proviral insertions targeting critical genes that are likely to be involved in cell mobility and metastatic spread.	Pothlichet et al., 2006
2007	RVLP-derived sequences found to be defective and to vary between different melanoma cell lines. Particles from the same cell line contained heterologous sequences.	Hirschl et al., 2007
2008	Anti-HERV-K Gag and Env antibodies detected retrospectively in 16% of sera from melanoma patients but not in sera from healthy controls. Anti-HERV-K reactivity elevated in patients with ALMM, mucosal and uveal melanoma compared to patients with LMM, NMM and SSMM. Serological HERV-K reactivity shown to be an independent marker of reduced survival probability.	Hahn et al., 2008
2009	Human melanoma cells in vitro observed to undergo a transition from an adherent to a more malignant, non-adherent phenotype when exposed to stress conditions. Non-adherent cells showed increased proliferative potential, activation HERV-K expression and production of RVLPs. Down-regulation of HERV-K expression by RNAi prevented the transition from the adherent to the non-adherent growth phenotype.	Serafino et al., 2009

1.4.2 HERV-K mRNA and protein expression in melanoma

In 2003, expression of the *pol* gene and of Gag, Rec and Env proteins was demonstrated in primary and metastatic melanoma tissue (Muster et al., 2003). Surgical touch preparations of primary melanomas, lymph node metastases and cutaneous metastases, studied by *in situ* hybridization, showed high copy numbers of the HERV-K108 *pol* sequence in tumour cells. Lymph node and benign naevus tissue samples from healthy individuals were negative. Using immunofluorescence, retroviral proteins were found to be expressed in melanoma cell lines and all primary melanomas (9/9), lymph node and cutaneous metastases but only 1/25 benign naevi and none of the benign lymph nodes or cultured NEHMs.

Buscher et al. (2005) investigated the expression of HERV-K mRNA in metastatic melanoma tissue and melanoma cell lines by RT-PCR. Expression of full-length mRNA derived from Types 1 and 2 HERV-K proviruses was detected in all tissues and cell lines investigated. Expression of spliced *env* and *rec* was found in 45% of melanoma metastasis samples, and in 44% of melanoma cell lines. In cultured NEHMs, spliced *rec* was detected in the absence of spliced *env*. Expression of retroviral proteins was studied in a melanoma cell line by immunofluorescence and Western blot analyses, and in a number of metastatic and six primary melanoma samples by immunohistochemistry. Expression of HERV-K TM envelope protein was observed in the cell line. However, in contrast to the findings of Muster et al., HERV-K TM protein expression was detected in approximately 50% and HERV-K Gag in approximately 70-80% of primary and metastatic melanoma samples.

The expression of TM, Rec and Np9 mRNA and proteins has been studied in further detail (Buscher et al., 2006). As discussed, these proteins are of particular interest because Rec and Np9 are potentially oncogenic and TM is putatively immunosuppressive (section 1.3.7). Env-specific, Rec-specific and Np9-specific antisera were generated, characterised and used to study protein expression in metastatic melanomas and melanoma cell lines. Analogous to previous findings, spliced *env* and *rec* mRNA were detected by RT-PCR in 39% of metastatic melanoma samples and in 40% of melanoma cell lines, and *np9* mRNA was detected in 29% and 21%, respectively. Immunohistochemical analyses showed 38%

of the metastatic melanoma samples to be positive for TM protein, 14% for Rec but none for Np9.

1.4.3 Immunological evidence

Nearly three decades after the early EM studies, interest in the potential association of retroviruses with human melanoma was reignited when an HERV-K antigen, presented in association with the human leucocyte antigen (HLA) class I molecule HLA-A2 on the autologous tumour cells of a melanoma patient, was shown to be targeted by CTLs (Schiavetti et al., 2002). The antigenic peptide, comprising nine amino acids, was found to be encoded by a short ORF in the *env* region of a defective HERV-K (HML-6) provirus. The gene encoding the antigen, designated *HERV-K-MEL*, was expressed in most samples of cutaneous and ocular melanoma tested, but also in a majority of benign naevi and in some normal skin samples. The authors suggested that *HERV-K-MEL* is a source of antigens that are targeted by CTLs in melanoma patients and could therefore be used for vaccination. Intriguingly, a multicentre case-control study revealed a reduced risk of melanoma associated with BCG and/or Vaccinia vaccination in early childhood and/or with infectious diseases later in life (Pfahlberg et al., 2002). Consequently, Krone et al. (2005) postulated that these vaccinations or infections, shown to have analogues of the *HERV-K-MEL* epitope, generate populations of cross-reactive T-cells that participate in immunosurveillance and repair or kill melanocytes expressing this antigen.

Conversely, it has been proposed that HERVs promote melanomagenesis by a Treg-mediated subversion of immunosurveillance (Mangeney et al., 2005). Using RNA interference (RNAi), Mangeney et al. demonstrated that knocking down the melanoma-associated retrovirus (MelARV), an ERV that is spontaneously induced in B16 murine melanoma (Li et al., 1999), resulted in the rejection of tumour cells in immunocompetent mice. Re-expression of the sole MelARV *env* gene in the knocked-down cells partially ablated tumour rejection thus indicating that the *env* gene contributes to tumour immune escape. The authors hypothesised that ERV expression by the B16 melanoma is directly responsible for Treg induction, thus resulting in tumour progression. Supporting this, adoptive transfer of Tregs restored tumour progression in the knocked-down cells

suggesting that they are a key component of the ERV-mediated effect. Interestingly, several of the fully coding HERV *env* genes are immunosuppressive in vivo in a murine model and, the authors have postulated, might promote tumour escape in humans (Mangeney et al., 2001).

Regarding human melanoma, there is evidence that the expression of HERV-K proteins may induce humoral immune responses. Buscher et al. (2005) detected antibodies specific for HERV-K TM protein in 22% of sera from 60 metastatic melanoma patients by Western blot analyses. Sera from 20 normal blood donors and patients with AA were negative. However, HERV-K Rec and Np9-specific antibodies were not detected in the sera of metastatic melanoma patients nor in sera from control normal blood donors and AA patients (Buscher et al., 2006). Humer et al. (2006) confirmed that some melanoma patients have antibodies to HERV-K Env. Following prediction analyses of potential epitopes on HERV-K, they identified an immunodominant epitope on the Env protein that is recognised by antibodies from sera of melanoma patients. The prevalence of antibodies was significantly higher in sera from 81 melanoma patients compared with 95 control sera from healthy individuals.

In a recent study to investigate the prognostic relevance of serological anti-HERV-K reactivity in melanoma, HERV-K Gag and Env antibodies were detected retrospectively in 51 of 312 randomly selected and blinded sera from melanoma patients and in none of 70 sera from healthy controls (Hahn et al., 2008). Anti-HERV-K reactivity was elevated in patients with ALMM, mucosal and uveal melanoma (melanoma subtypes that occur at sun-protected sites) compared with patients affected by LMM, NMM and SSMM. Patients with HERV-K antibodies had a significantly decreased disease-specific overall survival. Serological HERV-K reactivity was shown to be an independent marker of reduced survival probability in most melanoma patients. However, *prospective* studies are needed to support the possibility that serological HERV-K reactivity provides additional prognostic information to that of established melanoma markers.

1.4.4 Other studies

Sciamanna et al. (2005) investigated whether high levels of endogenous RT activity are directly linked to cell transformation. They inhibited RT activity in melanoma cell lines using two methods; pharmacological inhibition by two RT inhibitors (nevirapine and efavirenz) and down regulation of expression of RT-encoding LINE-1 (long interspersed elements) by RNAi. Both methods reduced proliferation, induced morphological differentiation and reprogrammed gene expression. These effects were reversible upon discontinuation of the RT inhibition, suggesting that RT contributes to an epigenetic level of control. Additionally, inhibition of RT activity in vivo antagonised tumour growth in mouse experiments. In further experiments, HERV-K interfered melanoma cells were found, in vivo, to have reduced tumourigenic potential after inoculation in nude mice (Oricchio et al., 2007). Based on previous studies, the authors suggested that HERV-K expression may favour tumour progression by reducing immunosurveillance in mice.

Characterisation of the MelARV proviruses present in the primary B16 melanoma cell line and also a sub-line selected in vivo for increased metastatic activity, revealed mobility of the element with new proviral insertions targeting critical genes and changing their transcriptional profile (Pothlichet et al., 2006). The genes specifically targeted by new proviral insertions in the metastatic tumour cells, namely *itga2* (integrin α2) and *dok 5* (docking protein 5), are likely to be involved in cell mobility and metastatic spread. Integrins are cell membrane proteins that play a vital role in cell adhesion and signal transduction. Fascinatingly, the development of the VGP in human melanoma is associated with loss of expression of cell adhesion and extracellular matrix molecules including *itga2* (Haqq et al., 2005). Thus MelARV can act both at the genetic level, by insertional mutagenesis, and at the epigenetic level promoting immunosuppression of the host. The authors have proposed that these properties may also be relevant to human melanoma although there is no firm evidence to support this.

Serafino et al. (2009) recently showed that HERV-K plays a role in the transition of human melanoma cell lines from an adherent to a non-adherent growth phenotype, the latter comprising a number of morphological and molecular alterations characteristic of malignant cells. These changes, initially observed to occur spontaneously in cell cultures,

were induced experimentally by exposing cells to starvation conditions. Not only were the phenotypic changes associated with massive production of HERV-K-related VLPs, they were dependent on HERV-K expression because the changes were reversed when HERV-K expression was down-regulated by RNAi. The authors suggested that these findings implicate the activation of HERV-K expression as an important contributory element to morphological and functional cell changes during melanoma progression.

1.5 Challenges to molecular research on primary melanoma

1.5.0 Summary

A significant challenge in dermatology research is the lack of availability of fresh or snap-frozen and cryopreserved tissue. This is particularly true of primary melanoma. However histopathology archives, historical collections of FFPE tissue specimens representing an immense range of dermatological disease, are an excellent resource for retrospective studies. Data generated from such studies can result in the identification of novel biomarkers for disease classification, diagnosis and prognosis and illuminate potential therapeutic targets. In the last decade, great strides have been made in techniques to extract nucleic acids of sufficient quality and quantity from FFPE tissue for use in downstream applications. The use of modern mRNA profiling techniques, such as real-time qRT-PCR and microarray analyses, has greatly advanced the molecular understanding of disease and recent studies have demonstrated that real-time qRT-PCR can provide valuable gene expression data from archival tissue specimens.

1.5.1 Primary melanoma tissue for research

Most suspect melanocytic lesions, unless very large or in a difficult anatomical location, are diagnosed and treated by excisional surgery. This provides the optimal specimen for full histological evaluation of a suspected melanoma including the Breslow thickness (Calonje, 2000). Thus a major challenge in molecular melanoma research is the lack of availability of fresh or cryopreserved tissue.

1.5.2 RNA extraction from archival tissue

The process of tissue handling and processing from patient to paraffin block comprises three major phases, namely "warm ischemia", fixation and tissue processing (Chung et al., 2008). Warm ischemia refers to the time of transfer of a tissue specimen from an operation room (or removal of blood supply) to formalin pot. Following fixation in formalin, the specimen is placed on a tissue processor where it is serially dehydrated in graded alcohols, the alcohols replaced with xylene in a process referred to as clearing and the specimen impregnated by paraffin.

(i) RNA degradation in archival tissue

The reliable quantification of gene expression in FFPE tissue is limited by the quality and quantity of mRNA that can be extracted from such samples (Farragher et al., 2008). RNA is very sensitive to degradation by ribonucleases (RNAses), stable and active enzymes ubiquitous in living organisms, making its recovery from archival material problematic. Furthermore, although fixation confers chemical stability on tissue thereby preserving its morphological and histochemical properties, the properties of nucleic acids in the tissue are altered by fixatives. The latter can cause covalent modification of mRNA by the addition of mono-methylol groups to the bases and strand breakage, resulting in low-yields. In addition, fixation causes cross-linkage between nucleic acids and proteins making subsequent RNA extraction, reverse transcription and quantification difficult (von Ahlfen et al., 2007).

(ii) Factors that influence the degree of RNA degradation

The degree of RNA degradation in archival tissue is affected by several factors (Chung et al., 2008; Farragher et al., 2008; Hewitt et al., 2008; von Ahlfen et al., 2007). These include pre-fixation factors (for example, tissue type, amount and duration of warm ischaemia), fixation-related factors (for example, pH, temperature and duration of fixation, as well as type of fixative) and post-fixation factors (for example, duration of tissue processing, temperature and duration of tissue storage).

It has been demonstrated that substantial RNA degradation may occur during the warm ischaemia phase (Chung et al., 2008). This phase may vary from minutes to hours, for example small biopsies are directly placed in fixative and transported to the pathology laboratory. Larger specimens, including excisional biopsy specimens and removed organs, are usually held at room temperature and subsequently transported to the pathology laboratory where they are dissected and sectioned into appropriate sizes for fixation. Endogenous factors, such as the presence of degradative enzymes, also impact on RNA quality in tissue especially in pancreas, liver and stomach. A recent study demonstrated that RNA degradation due to warm ischemia may be reduced by cooling the specimen and fixing it at 4°C (Bussolati et al. 2011).

Fixation time affects RNA quality and it has been observed that fixation times exceeding 48 hours are detrimental (Chung et al. 2008). The length of fixation can vary greatly and is often dependent on the time of day of receipt of the specimen. For example, biopsy samples acquired on a Friday afternoon usually remain in formalin (at room temperature) for over 48 hours before being processed by the pathology laboratory. Notably, a study with archival melanoma tissue observed a 85-99% decrease in mRNA levels for both β-actin and MART-1 (melanoma antigen recognized by T cells-1) in FFPE tissue compared with matched frozen tissue even after one day of fixation (Abrahamsen et al., 2003).

Although a number of alternative fixatives, such as Bouin, Carnoy, acetone and alcohol, have been used as substitutes for formalin they frequently induce tissue artefacts that can make microscopic histopathological analysis difficult. Moreover, these fixatives are not suitable for immunohistochemistry reactions. Thus formalin has remained the preferred fixative and the last few decades have seen the introduction of buffered formalin preparations. Data on the different buffers is limited but phosphate-based buffers (pH 7.0) appear to be superior for RNA recovery (Chung et al. 2008). It has been suggested that formalin should be buffered to a neutral pH as prolonged tissue hypoxia reduces pH in tissues locally, causing degradation of nucleic acids (Farragher et al., 2008; Hewitt et al., 2008).

Tissue processing protocols are standardised to a limited extend within laboratories and routinely categorised as short and long run protocols, which are used for biopsies and excisional specimens respectively (Hewitt et al., 2008). Biopsy specimens are routinely processed using both protocols but, because of their larger volume, excisional specimens are excluded from short protocols as they will not be impregnated adequately. The diversity of protocols used is extensive and may include differences in time for different steps of dehydration, clearing and impregnation. Chung et al. (2008) found that shorter tissue processing times were associated with lower quality RNA possibly because inadequate dehydration results in residual water being trapped in the tissue, which can result in RNA degradation.

Degradation of RNA over time continues whilst the RNA is stored in the paraffin block through processes such as oxidation (Cronin et al. 2004; Ribeiro-Silva et al. 2007), but is influenced by storage conditions. It has been suggested that paraffin blocks should be stored in a controlled-temperature environment, protected from excessive heat, humidity, dryness and light in order to optimise nucleic acid recovery (Hewitt et al., 2008). RNA recovery can be improved by disposing of the first sections of the block (that have been exposed to air) and using deeper sections for the isolation of RNA (von Ahlfen et al., 2007). A number of studies have demonstrated a reduction in the quantity and quality of nucleic acids recovered from older tissues (Cronin et al., 2004; von Ahlfen et al., 2007). However it is unclear whether this is secondary to the storage time/conditions, the original quality of tissue processing, or changes over the years in the reagents and processes used in fixation.

1.5.3 Quantification of gene expression in archival tissue

(i) The principles of real-time qRT-PCR

Conventional RT-PCR is a well established method for converting and amplifying a single-stranded RNA template to yield abundant dsDNA product (Farrell, 2005). During the reverse transcription step, RT synthesises single-stranded DNA complementary to the RNA template (complementary DNA or cDNA). This is termed first strand synthesis. PCR

relies on thermal cycling, comprising cycles of repeated heating and cooling of the reaction to a defined series of temperature steps, for DNA melting (physically separating the two strands in a DNA double helix) and enzymatic replication of the DNA. During the first round of thermal cycling, Taq polymerase (a thermostable DNA polymerase originally isolated from the bacterium *Thermus aquaticus*) synthesises single-stranded DNA complementary to the cDNA from nucleotides using DNA primers thus generating dsDNA template. This is termed second strand synthesis. During subsequent rounds of thermal cycling, the dsDNA template is exponentially amplified. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Real-time qRT-PCR enables both detection and quantification of a specific sequence in a cDNA sample as absolute copy number, or relative expression when normalised to a housekeeping gene (Arya et al., 2005; Farrell, 2005; Logan et al., 2009). The amplified DNA is quantified as it accumulates in the reaction after each amplification cycle. Two common methods for quantification are (a) fluorescent dyes, such as SYBR® Green, that intercalate with dsDNA and (b) modified DNA oligonucleotide reporter probes, such as Taqman, that fluoresce when hybridized with a cDNA (Figure 1.10). The use of three oligonucleotides (two primers and one probe) in the latter method results in higher specificity and sensitivity by greatly reducing the background signals arising from non-specific PCR amplification of sequences other than the intended target (Heid et al., 1996; Logan et al., 2009). Real-time qRT-PCR has a number of advantages over conventional RT-PCR including speed, reduced risk of contamination (because nucleic acid is amplified, detected and quantified in a single closed-tube reaction as PCR proceeds) and the ability to quantify more accurately the amount of starting material present (Valasek & Repa, 2005).

(ii) Real-time qRT-PCR for gene expression analysis of FFPE tissues

The development of real-time qRT-PCR has had a major impact on the study of the molecular basis of disease. Several studies have demonstrated the feasibility of measuring gene expression in archival tissue (Abrahamsen et al., 2003; Antonov et al., 2005; Chung et al., 2006; Farragher et al., 2008; Hoshida et al., 2008; Ribeiro-Silva et al., 2007; Siebolts et

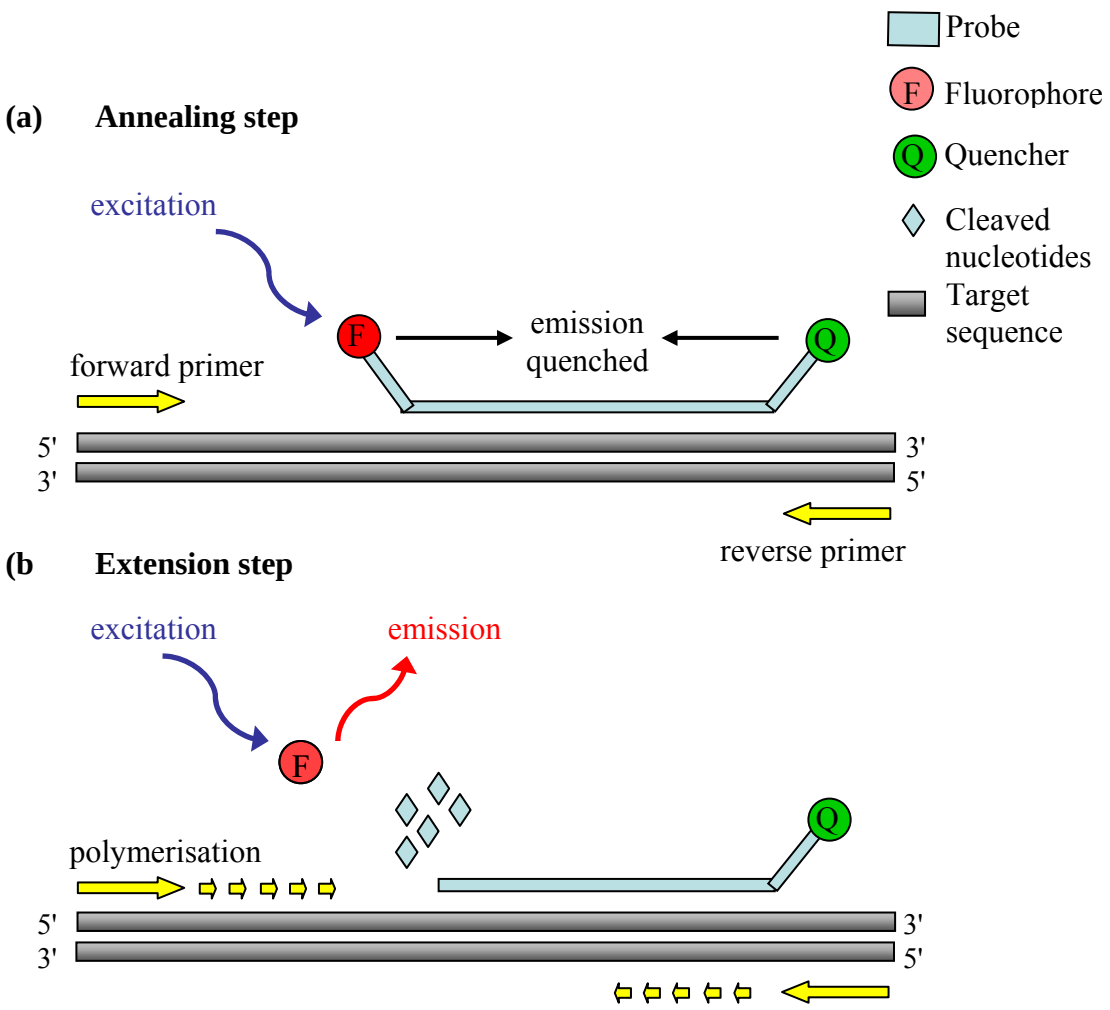
al., 2009; Yang et al., 2006). Genome-wide microarray analysis in archival tissue has, however, proven more difficult. One study found that only a few tissue blocks were of sufficient quality for microarray analysis, and gene signatures derived from FFPE samples contained fewer transcripts than those derived from frozen material (Penland et al., 2007).

Real-time qRT-PCR is an ideal method for the quantification of gene expression in samples where the number of target molecules is small and the amount of RNA available for analysis is limited, such as RNA derived from archival tissue. As the quantification of a target mRNA is measured relative to that of a reference housekeeping gene mRNA, accuracy and specificity is maintained despite any RNA degradation. A great advantage of real-time qRT-PCR is the ability to use very short RNA fragments for amplification and it is thus well suited to amplify cDNAs transcribed from the potentially highly degraded total RNA extracted from archival specimens.

Abrahamsen et al. (2003) specifically investigated the feasibility of mRNA analysis in FFPE metastatic melanoma tissue (lymph nodes) using real-time qRT-PCR. MART-1, β-actin, and β2-microglobulin mRNAs were consistently detected in FFPE metastatic melanoma, although the total mRNA detected was markedly reduced in fixed compared with fresh tissues. Quantification of MART-1 was, however, possible if this was expressed relative to a housekeeping gene. The PCR product from FFPE tissues could be increased up to 100-fold amplifying short (less than 136 bp) compared with long amplicons.

Real-time qRT-PCR has been used to measure gene expression in archival *primary melanoma* tissue in only one study prior to the start of this work (Ichihashi & Kitajima, 2001).

Figure 1.10: Diagram illustrating the principle of Taqman probes in real-time qRT-PCR



Legend:

(a) Annealing step: Taqman probes are sequence-specific oligonucleotides with a fluorophore, located at the 5' end, and a quencher moiety, located at the 3' end. During the annealing step of PCR, the Taqman probe and the PCR primers anneal to the target sequence. At this stage, the fluorophore is prevented from fluorescing because of its close proximity with the quencher.

(b) Extension step: Taq DNA polymerase extends the primers and when it reaches the probe, its 5' to 3' exonuclease activity cleaves the fluorophore from the probe. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

1.6 Hypotheses and aims

1.6.0 Summary

This work was conceived to investigate the role of HERV-K in melanomagenesis. The principal objective was to establish the feasibility and reliability of HERV-K expression from FFPE tissue. Such expression would then be correlated with clinical melanoma subtype and histological prognostic features. The strategy taken was to use a specific and sensitive novel real-time qRT-PCR methodology in clearly defined clinicopathological subtypes of archival primary melanoma. It is envisaged that the findings might afford important new insights into the role of HERV-K in melanomagenesis and illuminate the molecular classification of melanoma. Ultimately it is hoped that the results of molecular studies such as this will provide information useful for screening, prevention, prognostication and treatment of melanoma.

1.6.1 Hypotheses

In this work, it has been hypothesised that HERV-K gene expression is associated with primary melanoma and varies according to the clinicopathological subtype and Breslow thickness. Additionally, it has been postulated that primary melanoma is associated with the expression of novel HERV-K elements additional to those identified as present within the human genome project.

1.6.2 Aims

The general aim of this work was to undertake a detailed investigation of HERV-K gene expression in primary melanoma. The specific aims were to:

(i) investigate the expression of HERV-K mRNA in human melanoma cell lines to corroborate previously published findings and to establish positive controls for the investigation of HERV-K gene expression in archival clinical tissue (Chapter 3).

(ii) determine the feasibility of detecting HERV-K expression in FFPE tissue using a real-time qRT-PCR methodology (Chapter 4).

(iii) quantify the expression of full-length and spliced HERV-K mRNA in primary melanoma, benign naevi and normal skin and to correlate expression in primary melanoma with clinicopathological subtype and Breslow thickness (Chapter 5).

(iv) characterise and compare expressed HERV-K sequences in melanoma and normal skin to determine if expression of particular sequences is associated with malignancy (Chapter 6)

1.6.3 Strategy

Human-specific HERV-K (HML2) full-length proviruses (namely HERV-K 101-104, 107-109, 113 and 115) were specifically investigated because, as evolutionary recent integrations with nearly intact ORFs, they are more likely to be transcriptionally active and potentially pathogenic. The shorter term HERV-K has been used throughout this thesis for simplicity.

HERV-K mRNA expression was initially studied in two human melanoma cell lines, principally to establish positive controls for the investigation of expression in clinical tissue. Archival FFPE tissue specimens were used in this investigation because of the lack of availability of fresh primary melanoma tissue for research. Consequently, a large part of this work involved the development of a novel real-time qRT-PCR based methodology to investigate HERV-K expression in archival tissue. RNA was extracted from archival primary melanoma and control normal skin and benign naevus samples. HERV-K gene expression was quantified using the real-time qRT-PCR that was developed. Expressed HERV-K *env* sequences were cloned for sequence analysis from a number of melanoma and normal skin samples.

Chapter 2:
Materials and Methods

2.0 Abstract

This work involved research on human melanoma cell lines and human subjects. Informed consent for the use of melanoma and control benign naevus FFPE specimens was obtained from patients. Control normal skin FFPE specimens were obtained from a tissue bank. Clinical and histopathological features of melanoma cases were obtained from patient records and histopathology reports. RNA was extracted from cell lines, putative viral particles from cell supernatants and FFPE tissue. To verify that RNA was derived from melanoma tissue, tissue sections adjacent to the sections from which RNA was extracted were stained with haematoxylin and eosin (H&E) and the histology confirmed. To investigate HERV-K mRNA expression, a number of molecular techniques were used including conventional RT-PCR, real-time qRT-PCR and cloning. Data were analysed using sequencing software and conventional statistical tests.

2.1 Human melanoma cell lines and maintenance

2.1.1 Description of cell lines

Table 2.1 describes the cell lines used in this work and their provenance. The human melanoma cell lines A375 and WM2664 were a gift from Professor R. Marais (Cancer Research UK Centre for Cell and Molecular Biology). The 293T cell line was a gift from Miss S. Hughes (Section of Infectious Diseases, Imperial College).

Table 2.1: Cell lines

Cell line	Type	Description	Reference
A375	adherent	human melanoma cells of metastatic origin	Giard et al., 1973
WM2664	adherent	human melanoma cells of metastatic origin	Herlyn et al., 1985
293T	adherent	adenovirus-transformed human kidney epithelial cells stably expressing the Simian virus 40 antigen	DuBridge et al., 1987

2.1.2 Cell culture

The cell growth medium was Dulbecco's Modified Eagle's Medium (DMEM: GIBCO Invitrogen) containing 10% heat-inactivated (at 56°C for 1 hour) foetal calf serum (FCS: Scientific Laboratory Services), 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO Invitrogen). Cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 and passaged twice weekly at 80-100% confluence. The monolayer was washed once with phosphate-buffered saline (PBS: Sigma-Aldrich) before trypsin-ethylene diamine tetra acetic acid (EDTA: GIBCO Invitrogen) was added. Following incubation at room temperature for 3-5 minutes and when the cells had detached from the plastic surface of the tissue culture flask (Starstedt), they were resuspended in growth medium at a ratio of 1:4 and re-seeded into fresh flasks.

2.1.3 Freezing and thawing cells

For long-term storage, the cell lines were frozen as follows. The cells of a 75 cm^2 flask were harvested by incubation with 3 ml trypsin-EDTA then resuspended in 10 ml DMEM. The cells were pelleted by centrifugation at 400 RCF for 3 minutes in a 15 ml Falcon tube (Invitrogen) and resuspended in 5 ml freezing medium (DMEM containing 10% dimethyl sulfoxide (DMSO: Sigma-Aldrich) and 30% FCS). The cells in freezing medium were aliquoted on ice in 1 ml volumes into cryotubes (Merck) and frozen slowly overnight at minus 80°C in a cryofreezing container (Jencons) containing isopropanol (Sigma-Aldrich), in order to prevent the formation of ice crystals within the cells during freezing. The cells were then stored in liquid nitrogen.

Frozen cells were thawed in a 37°C water bath before being quickly decanted into a 15 ml Falcon tube containing 13 ml DMEM. The cells were pelleted by centrifugation at 400 RCF for 5 minutes, resuspended in 5 ml DMEM, transferred to a 25 cm^2 flask and incubated at 37˚C. The medium was replaced after 24 hours.

2.2 Study subjects

2.2.1 Ethical approval

Ethical approval was obtained for this work from the Royal Marsden Local Research Ethics Committee (Appendix 1). Informed signed written consent for the use of FFPE tissue was obtained and patients were given the option to consent for the use of their tissue for future studies.

2.2.2 Melanoma cases

(i) Inclusion and exclusion criteria

Cases identified with primary cutaneous melanoma were recruited. Patients less than 16 years of age and cases of metastatic melanoma (satellite lesions, nodal disease or distant metastases) were excluded.

(ii) Recruitment

A database recording the primary diagnoses of all tissues processed from 1992 onwards is held by the Histopathology Department at Charing Cross Hospital (CXH). All patients with a diagnosis of melanoma between January 2000 and September 2006 were identified. This time period was selected because tissue blocks prior to the year 2000 were stored at a different site. The histopathology reports were reviewed and 186 patients with *primary cutaneous* melanoma were invited by post to participate in the study. Copies of the invitation letter, patient information sheet and consent forms are included in Appendix 2. Fifteen invitations were returned unopened (presumably due to incorrect address). One-hundred and ten patients agreed to take part in the study, yielding a response rate of 64% (110/171).

2.2.3 Controls: benign naevi

(i) Inclusion and exclusion criteria

Subjects identified with benign naevi were recruited. Exclusion criteria were patients less than 16 years of age and patients with a history of melanoma. Patients with naevi where there were histological features of atypia (Mooi, 1997; Elder 2006b), namely

architectural disorder (asymmetry, subepidermal fibroplasia, a distorted rete ridge pattern with bridging between adjacent rete rides, shouldering, a lymphocytic infiltrate in the superficial dermis) and cytological atypia of melanocytes (characterised by pleomorphism, anisochromatism and variation in size, shape and staining intensity), were excluded.

(ii) Recruitment

All patients with a diagnosis of benign naevus between May and November 2006 were identified from the histopathology database at CXH. This time period was chosen because a large number of benign naevi are excised per month (approximately 50 cases). The histopathology reports were reviewed and 196 patients with *benign naevi* (without evidence of atypia) who matched the melanoma cases in terms of age and sex were invited, by post, to take part in the study. Six invitations were returned unopened because the address was incorrect. Seventy-eight patients agreed to take part in the study, yielding a response rate of 41% (78/190).

2.2.4 Controls: normal skin

Thirty-one anonymised samples of normal skin from patients without a history of melanoma were obtained from the Human Biomaterials Resource Centre (HBRC) at CXH. Since 2005, the HBRC has accrued samples of FFPE normal skin acquired from donors who have given informed consent for their tissue to be used for research. These samples have mostly been derived from skin removed during plastic surgery such as reduction mammoplasty and abdominoplasty, and limb amputation procedures. These samples were not specifically matched with the melanoma cases in terms of age and sex because of the small number of HBRC samples available.

2.2.5 Clinical features

(i) Melanoma cases

The hospital notes were reviewed to obtain the following clinical details:

Age at primary diagnosis

Site of melanoma

Current disease status (disease free, regional recurrence/satellites, nodal disease, distant metastases)
Previous history of melanoma
Family history of melanoma
Previous history of cancer

Where follow-up information was not available, because patients had been discharged or moved out of the area, the patient's General Practioner was contacted and asked whether the patient was fit and well, had relapsed or had died.

Although not part of this work, a questionnaire was also sent to recruited subjects to ascertain melanoma risk factors with the purpose of establishing a detailed database for future studies (Appendix 3). The questions were based on those used in previous case-control studies assessing melanoma risk, with some modifications (Naldi, 2005; Rafnsson et al., 2003; Westerdahl et al., 1996).

(ii) Controls: benign naevi

The histopathology database at CXH was reviewed to obtain the following clinical details:

Age at diagnosis
Site of naevus

(iii) Controls: normal skin

The following clinical details were provided by the HBRC for every sample acquired:

Sex
Age of patient at time of sample acquisition
Site of sample

2.2.6 Histopathological features

(i) Melanoma cases

The following information was obtained from the histopathology report:

Tumour subtype (SSMM, NMM, NMM, LMM, MMIS)

Breslow thickness (in mm)

Growth phase (RGP, VGP)

Presence or absence of ulceration

(ii) Controls: benign naevi

The type of benign naevus (junctional, intradermal or compound melanocytic naevus) was ascertained from the histopathology report.

2.2.7 Selection of tissue blocks

Where multiple blocks from the same sample were available, the choice of block for RNA extraction was based on examination of the original H&E stained slides. This enabled selection of the best block to obtain the maximum amount of melanoma or benign naevus tissue. The H&E slides were reviewed with Dr N. Francis, Consultant Histopathologist, CXH.

In some cases, extra-lesional skin was obtained to use as an internal control. In the melanoma cases, extra-lesional skin was derived from tissue blocks containing histologically verified uninvolved skin from the surgical excision margins. For benign naevus samples, histologically verified uninvolved skin was derived from tissue blocks containing the tips of the lesion.

2.2.8 Coding

The identities of samples were coded at the time of RNA extraction and were referred to by their code: MM=melanoma; BN=benign naevus and NS=normal skin.

2.3 RNA isolation

2.3.1 Precautionary measures

Special care must be taken when working with RNA because of the intrinsic chemical instability of RNA and the presence of RNAses (Farrell, 2005). Skin and dust particles, which harbour bacteria and fungi, are the most common sources of RNase contamination.

Several precautions were taken to create and maintain an RNase-free environment. RNA isolation was performed in a dedicated room. Sterile disposable gloves were worn at all times to prevent RNase contamination from the skin surface and were frequently changed. Bench surfaces, equipment and pipettes were cleaned with RNaseZap® (Ambion), an RNAse inactivating agent. Sterile RNase-free filter pipette tips (Gilson) and RNAse-free disposable plastic ware and reagents including water, provided in the commercial kits (Qiagen), were used. Purified RNA was stored at minus 70°C in buffer AVE (RNase-free water containing 0.04% sodium azide). During downstream procedures, such as cDNA synthesis, purified RNA was kept on ice to avoid degradation.

2.3.2 Cell lines

RNA was extracted from cell lines using the RNeasy® mini plus kit (Qiagen). Cells were harvested by trypsinisation, re-suspended in 14 ml DMEM and pelleted at 400 RCF for five minutes. The supernatant was completely aspirated and 600 µl buffer RLT Plus added to lyse the cells. The lysate was pipetted into a QIAshredder® spin column (Qiagen) and centrifuged for 5 minutes at 13,000 RPM in a microfuge to homogenise the lysate. The homogenised lysate was transferred to a genomic DNA (gDNA) eliminator spin column (Qiagen) and centrifuged for 45 seconds at 13,000 RPM to remove gDNA. A 600 µl aliquot of 70% ethanol was added to the flow-through to allow appropriate binding conditions for RNA. The sample was then applied to an RNeasy® spin column and centrifuged for 30 seconds at 13,000 RPM and the flow through discarded. 700 µl buffer RW1 wash buffer was added to the spin column, centrifuged for 30 seconds at 13,000 RPM and the flow through discarded. 500 µl buffer RPE was added to the spin column, centrifuged for 30 seconds at 13,000 RPM and the flow through discarded.

Again, 500 µl buffer RPE was added to the spin column, centrifuged for 2 minutes at 13,000 RPM and the flow through discarded. A 50 µl aliquot of buffer AVE was added directly to the spin column membrane and centrifuged for 1 minute at 13,000 RPM to elute the RNA. RNA was stored at minus 70°C.

2.3.3 Viral pellets

Putative viral particles from cell culture supernatants were purified and concentrated by ultracentrifugation. An aliquot (5-7ml) of 20% sucrose solution in PBS was placed in a thin wall polyallomer tube (Beckman Coulter). The supernatant was carefully overlaid onto the sucrose cushion and the tubes were centrifuged in a swinging bucket rotor (Sorvall AH-629) at 150,000 RCF in an Ultra Pro8 ultracentrifuge (Sorvall) for 90 minutes at 4°C. The supernatant and the sucrose solution were decanted and the virus pellet was air-dried for 10 minutes before storing at minus 70°C until use.

RNA was extracted from cell-free viral pellets using the QIAamp® Viral RNA kit (Qiagen). An aliquot (140 µl) of virus suspension was added to 560 µl cell lysis solution (buffer AVL) containing 5.6 µl carrier RNA and incubated for ten minutes. Ethanol (560 µl) was then added and the sample applied to columns and centrifuged at 8,000 RCF. Two further wash steps were carried out using 500 µl wash buffers AW1 and AW2. Finally, extracted viral RNA was eluted into 50 µl buffer AVE and stored at minus 70°C.

2.3.4 FFPE tissue

RNA was extracted from FFPE samples using the RNeasy® FFPE kit (Qiagen). Using a sterile scalpel, excess paraffin was trimmed off the sample block. Two tissue sections, 7 µm thick, were cut using a microtome and immediately placed into a sealed microcentrifuge tube. The microtome blade and scalpel were cleaned with RNaseZap® before each specimen was cut.

Tissue sections were processed whole. A 1 ml aliquot of xylene was added to the sections, vortexed vigorously for 10 seconds and centrifuged at 13,000 RPM in a microfuge for 4 minutes. The supernatant was removed using a Pasteur pipette. A 1 ml

aliquot of 100% ethanol was added to the tissue pellet to remove residual xylene, mixed by vortexing and centrifuged at 13,000 RPM for 4 minutes. The supernatant was removed using a Pasteur pipette and the pellet was allowed to dry in an incubator at 37°C for 20 minutes. The dried pellet was resuspended in 150 µl buffer PKD, 10 µl proteinase K added and incubated at 55ºC for 15 minutes to liberate RNA from the tissue. The sample was then incubated at 80ºC for 15 minutes to reverse partially formaldehyde modification of the released nucleic acids, and improve RNA yield and quality. Next, 320 µl buffer RBC was added to adjust binding conditions. The lysate was mixed thoroughly by pipetting, transferred to a gDNA eliminator spin column and centrifuged for 45 seconds at 13,000 RPM to remove gDNA. A 720 µl aliquot of 100% ethanol was added to the flow-through to allow appropriate binding conditions for RNA. The sample was then applied to an RNeasy® spin column, centrifuged for 30 seconds at 13,000 RPM and the flow-through discarded. A 500 µl aliquot of RPE wash buffer was added to the spin column, centrifuged for 30 seconds at 13,000 RPM and the flow through discarded. Another 500 µl aliquot of buffer RPE was added to the spin column, centrifuged for two minutes at 13,000 RPM and the flow through discarded. The RNeasy® spin column was then centrifuged at 13,000 RPM for 5 minutes with the lid open in order to dry the column membrane. A 30 µl aliquot of buffer AVE was added directly to the membrane and centrifuged for 1 minute at 13,000 RPM to elute the RNA. The RNA was stored at minus 70°C.

To verify that RNA had been extracted from tumour tissue, sections were taken from either side of the two sections from which the RNA was extracted and stained with H&E as for routine laboratory processing. The slides were examined, in the absence of the original histopathology report, with Dr N. Francis. This also enabled confirmation of the tumour subtype and growth phase. Some examples of the H&E stained tissue sections are shown in Chapter 5, Figure 5.1.

2.3.5 Spectrophotometric quantification of RNA

The concentration of RNA in the sample was determined by measuring the absorbance at 260 nm (A_{260}) in an Ultraspec 2000 UV/visible spectrophotometer (Pharmacia Biotech). An absorbance of one unit at 260 nm corresponds to 44 µg RNA per ml. Two µl of the RNA sample was diluted in 98 µl buffer AVE (1/50 dilution). The formulae used to calculate concentration and total amount of RNA in the sample were as follows:

$$\text{Concentration of RNA (ng/µl)} = A_{260} \times 44 \times 50$$

$$\text{RNA yield (ng)} = (A_{260} \times 44 \times 50) \times \text{total volume of RNA sample in µl}$$

2.4 PCR

2.4.1 Precautionary measures

PCR is an extremely sensitive technique, requiring only a few molecules of template DNA in a single reaction for amplification to several orders of magnitude. Therefore, it is essential to take adequate measures to avoid contamination from any DNA present in the laboratory environment (bacterial or human sources) or from PCR products from previous amplifications (carry-over contamination).

In this study, separate dedicated laboratory rooms were used for a) preparation and handling of pre-PCR reagents, b) setup of the PCR reaction and c) post-PCR processing, for example agarose gel electrophoresis or PCR product purification. Laboratory equipment, such as pipettes and microfuges, were not transferred between the rooms. Reagents, for example primers, were taken from stock solution and stored in small aliquots that could be discarded if contamination was suspected or observed. Sterile filter pipette tips were used for the setup of PCR reactions, to reduce both aerosol-borne contamination and the risk of transferring DNA between tubes. Laboratory gloves were frequently changed. A master mix of reagents was prepared for each set of reactions to reduce pipetting errors at low volumes, increase accuracy and limit the number of reagent transfers. For each set of PCR reactions, an additional identical reaction was set up but

nuclease-free water was added instead of template DNA. This negative control was performed alongside the experimental PCR reactions.

2.4.2 cDNA synthesis

For two-step RT-PCR, cDNA was synthesised using the Quantitect® Reverse Transcription Kit (Qiagen). This kit included a gDNA elimination step to remove residual gDNA contamination from the RNA extract. Contaminating gDNA can give rise to false positive results in RT-PCR, particularly if the primers do not span a splice junction, because of its ability to be an alternative template (Farrell, 2005).

The RNA sample was thawed on ice and the gDNA elimination reaction mix was prepared on ice. A 2 µl aliquot of gDNA "wipe-out" buffer and 2 µl RNase free water was added to 10 µl of template RNA. The reaction mix was incubated at 42°C for 2 minutes then placed on ice. The reverse transcription master mix, comprising 1 µl Quantiscript® RT, 4 µl Quantiscript® RT buffer and 1 µl RT primer mix (oligo dT and random primers), was prepared on ice. The gDNA elimination reaction mix (containing the template RNA) was then added to the reverse transcription master mix, containing the components necessary for first strand cDNA synthesis (20 µl total reaction volume). The sample was incubated for 15 minutes at 42°C, followed by three minutes at 95°C to inactivate the RT. cDNA was stored at minus 20°C.

2.4.3 One-step RT-PCR

Expression of HERV-K mRNA in the melanoma cell lines was initially investigated by one-step RT-PCR, as previously described (Buscher et al., 2005). One-step RT-PCR is a variant of standard two-step RT-PCR, in which all components of reverse transcription and PCR are mixed in one tube prior to commencing the reaction. Reverse transcription and PCR are subsequently carried out sequentially in one tube. The SuperScript III® One-Step RT-PCR System (Invitrogen) was used and the primers were synthesised by Invitrogen. Their sequences are shown in Table 2.2.

Table 2.2: Primers for the analysis of HERV-K mRNA expression by one-step RT-PCR

Primer	*Location*	Sequence
HPRT F*	*HPRT1*	5'-TGACACTGGCAAAACAATGCA-3'
HPRT R	*HPRT1*	5'-GGTCCTTTTCACCAGCAAGCT-3'
P1†	HERV-K LTR (5' UTR)	5'-GAGGCTGGCGGGATCCTC-3'
P2	HERV-K LTR	5'-GAAGGTACGCTCGAGCGTAATCATTGAG -3'
P3	HERV-K *gag*	5'-GAGCCATTACCGGCTCTGCTACATATTCG-3'
P4	HERV-K *pol*	5'-GTACCACTCCTCAGATGCAACTTAATCTAGC-3'
P5	HERV-K *env*	5'-GTGACATCCCGCTTACCATGTGATAAGTG-3'
P6	HERV-K *env*	5'-CGTCTAACCATGTCCCAGTGATGC-3'
P7‡	HERV-K *env*	5'-ACAAAACCGCCATCGTCATCATGGCCYG-3'

The designation of the primers, their location and nucleotide sequence are shown.

P, primer; F, forward primer; R, reverse primer; LTR, long terminal repeat; UTR, untranslated region.

* Designed by Dr S. Kaye (Section of Infectious Diseases, Imperial College)
† Buscher et al. (2005)
‡ Primer P7 was modified from the sequence published by Buscher et al. (2005) because the latter had a number of mismatches with published HERV-K sequences.

(i) Protocol

The reaction consisted of 2 µl template RNA, 12.5 µl Reaction Mix (a buffer containing 0.4 mM of each deoxyribonucleotide triphosphate (dNTP) and 2.4 mM $MgSO_4$), 0.5 µl Enzyme Mix (containing SuperScript III RT and Platinum® Taq Polymerase High Fidelity), 0.5 µl of each primer (10 µM) and RNase-free water to a final total volume of 25 µl. The PCR cycling conditions were as follows: 30 minutes at 55°C (reverse transcription), 2 minutes at 94°C (PCR hot start), 35 cycles of 94°C for 30 seconds (denaturing), 50°C for 30 seconds (annealing) and 68°C for 30 seconds (extension), then 68°C for 5 minutes (final extension).

False-positive results due to residual gDNA were determined by parallel experiments omitting the RT. For this, the AmpliTaq Gold® DNA Polymerase kit (Applied Biosystems) was used, as described in section 2.4.4.

(ii) Selection of a housekeeping gene

The housekeeping gene hypoxanthine phosphoribosyl-transferase (*HPRT*) was amplified as a positive control. This gene encodes the enzyme HPRT1 that plays a central role in the generation of purine nucleotides through the purine salvage pathway (Stout & Caskey, 1985). Ideally, a housekeeping gene that is expressed at a constant level in all cells at all times should be selected. However, no housekeeping genes exist for which the expression is constant in all tissues during different experimental conditions, or even within the same individual. In this work, *HPRT* was chosen because it has been demonstrated to be the best housekeeping gene to normalise gene expression data in tumour tissue including melanomas (discussed in more detail in Chapter 4, section 4.3.4).

2.4.4 Two-step RT-PCR

Two-step RT-PCR was used to investigate the expression of full-length (unspliced) HERV-K mRNA in A375 melanoma cells. The two-step method is more sensitive than one-step RT-PCR and can therefore improve yields of rare or low abundance targets. All primers were synthesised by Invitrogen and their sequences are shown in Table 2.3.

The reaction mix consisted of 2 µl template cDNA (section 2.4.2), 1 µl nucleotide mix (200 mM each), 5 µl PCR Gold® Reaction buffer (Applied Biosystems), 0.25 µl AmpliTaq Gold® Polymerase (Applied Biosystems), 0.5 µl of each primer (10 µM), 3 µl magnesium chloride (25 mM) and RNase-free water to a final total volume of 50 µl. The PCR cycling conditions were as follows: 8 minutes at 94°C (hot start), 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, then 72°C for 7 minutes. False-positive results due to residual DNA were determined by parallel experiments omitting the RT (termed "no-RT" controls).

Table 2.3: Primers for the amplification of full-length (unspliced) HERV-K mRNA by two-step RT-PCR

Primer	Location	Sequence
gag F	*gag* (1251-1273)	5'-CATGGTTTCCAGAACAAGGAACT-3'
gag R	*gag* (1382-1360)	5'-TGATTGGGCCATTATTAAAGCAG-3'
pol F	*pol* (5924-5946)	5'- ACAGGAGAAAGTACTTCCCATGT-3'
pol R	*pol* (6068-6045)	5'-TCTTAAGTCAGTGGAAAATTTCAC-3'
env F	*env* (7368 – 7391)	5'-ATTGCAGTCTTTCTACCCTTGGGA-3'
env R	*env* (7641 – 7663)	5'- ACCAGACTCCCAGACTATAACCT-3'

Designation of the primers, their location and nucleotide sequence are shown. The primers were designed Dr S. Kaye (Section of Infectious Diseases, Imperial College). The nucleotide positions of the primers with reference to HERV-K 108 (Gen Bank accession number AF164614) are indicated in parentheses. F, forward primer; R, reverse primer.

2.4.5 Real-time qRT-PCR

The development of a real-time qRT-PCR methodology to detect and quantify HERV-K gene expression in archival tissue is described in detail in Chapter 4. The Roche LightCycler® 1.0 Real-Time PCR System and LightCycler® software version 3 (for data analysis) were used. All primers and Taqman probes were synthesised by Eurogentec and their sequences are shown in Chapter 4, Table 4.1.

(i) Protocol

The reaction mix, consisting of 10 µl Quantitect® Probe Master Mix (Qiagen), 1 µl of each primer (10 µM), 0.4µl of Taqman probe (10 µM) and 5.6 µl RNase free water was dispensed into the reservoir of a pre-chilled 20 µl capillary (Roche Diagnostics) and 2 µl template cDNA added. The capillary was centrifuged for one minute at 300 RCF to sediment the reaction components before inserting in the Light Cycler®. The PCR cycling conditions were: initial activation step 15 minutes at 95°C, 50 cycles of 95°C for 0 seconds (denaturation) and 60°C for 80 seconds (combined annealing/extension step).

(ii) Experimental controls

Each run of PCR included a negative control (water) and a positive control (cDNA derived from A375 cells). No-RT controls were performed for each sample to exclude false-positive results due to residual gDNA. Samples were processed exactly as for RNA amplification but without addition of RT.

(iii) Determination of PCR inhibition

A 2.5 µl aliquot of test sample was spiked into a PCR reaction containing about 1000 copies bacteriophage λ DNA and a primer pair directed against λ DNA (λPC01 primer sequence 5′-GATGAGTTCGTGTCCGTACAACTGG-3′ and λPC02 primer sequence 5′-GGTTATCGAAATCAGCCACAGCGCC-3′, Applied Biosystems). The reaction mix consisting of 2 µl Master Mix (SYBR® Green, Roche), 0.5 µl of each primer, 0.5 µl λ DNA and 4 µl RNase free water was dispensed into a capillary and 2.5 µl of test sample added. The PCR cycling conditions were: initial activation step 10 minutes at 95°C, 45 cycles of 95°C for 10 seconds and 60°C for 10 seconds and 72°C for 30 seconds.

2.4.6 Agarose gel electrophoresis

PCR products were separated on a 2% agarose gel with a 200 bp DNA ladder (Promega). 2 g agarose and 100 ml of 1x buffer TAE (tris acetate EDTA) were mixed in a glass flask, heated in a microwave until boiling (2 minutes on full power) then allowed to cool to "hand warm" by running under a cold tap. In a fume cupboard, 5 µl ethidium bromide (10 mg/ml) was carefully added. The mixture was poured into a gel-casting tray and allowed to set for 30 minutes. When set, the combs and casting gates were removed and the gel transferred to an electrophoresis tank containing enough 1x TAE to cover the gel. 5 µl PCR product were mixed with 1 µl of 6x gel loading buffer by pipetting, and loaded into a well in the gel. Additionally, 0.5 µl DNA ladder, 1 µl loading buffer and 5 µl 1x TAE were mixed and loaded into one well in each row. The gel was run at 100 V for about 45 minutes, then visualised and photographed under UV light.

2.5 DNA sequencing

2.5.1 Purification of PCR product

Prior to sequencing, the PCR product was purified as follows: a 350 µl aliquot of water was applied to a Microcon column (Millipore), then PCR product was added, mixed by pipetting and the column centrifuged for 15 minutes at 500 RCF. The flow through was discarded and 35 µl water applied to the column membrane. The column was then inverted onto a collection tube and centrifuged for five minutes at 500 RCF. For each sequencing reaction, 1 µl of the appropriate gene specific primer was added to 9 µl purified PCR product.

2.5.2 Sequencing and analysis

Purified PCR products, mixed with the appropriate sequencing primer were sent for sequencing to the Medical Research Council Clinical Sciences Centre Genomics Core Laboratory at Hammersmith Hospital. Sequence editing was carried out using Sequencher® software (Version 4.7) and further analyses were done using Bio Edit and on-line tools. Sequences were aligned using Clustal W or BLASTn.

For cloning experiments, 20-24 clones were sequenced and aligned with published human genome sequences by BLASTn searching. Analyses were performed (Bioinformatics, Imperial College), including best match against human genome HERV-K sequences, together with any mutations.

2.6 Cloning

2.6.1 Background

The TOPO® TA® Cloning Kit for Sequencing (Invitrogen) was used to clone HERV-K *env* derived from A375 melanoma cells and FFPE skin tissue for sequencing. In TOPO® TA® cloning, Taq polymerase-amplified PCR product is directly inserted into a plasmid vector (pCR4-TOPO®). Taq polymerase has a non template-dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3′ end of PCR products. The vector, supplied in linear form, has single overhanging 3′ deoxythymidine (T) residues. This allows the PCR product (insert) to ligate with the vector (Figure 2.1). Vaccinia DNA topoisomerase I, used as both a restriction enzyme and a ligase, binds the insert to the vector (Shuman, 1994). During the transformation reaction, a proportion of competent *E. coli* cells take up a single plasmid molecule (Figure 2.1). The cells are then plated on a Petri dish containing agar and an antibiotic and incubated overnight. Because these steps are not always 100% efficient, there are three possible outcomes: the cell contains the plasmid with the insert; the cell contains the plasmid, but the plasmid does not contain an insert; or the cell does not contain a plasmid. Hence, a method for selecting *E. coli* cells is necessary to ensure that only colonies that contain a plasmid with the insert are processed and sequenced.

Since the pCR4-TOPO® vector contains ampicillin and kanamycin resistance genes, colonies can be grown on an agar plate containing either antibiotic thus preventing any bacterium that does not contain the plasmid from dividing (Figure 2.1). To prevent cells that contain a plasmid without an insert from growing, pCR4-TOPO® contains a lethal *E. coli* gene, *ccd*B, fused to the *lacZα* gene. Ligation of the PCR product disrupts

expression of the *lacZα-ccd*B gene fusion permitting growth of only positive recombinants upon transformation in *E. coli* cells (Bernard et al., 1994).

2.6.2 Generation of HERV-K unspliced *env* PCR product

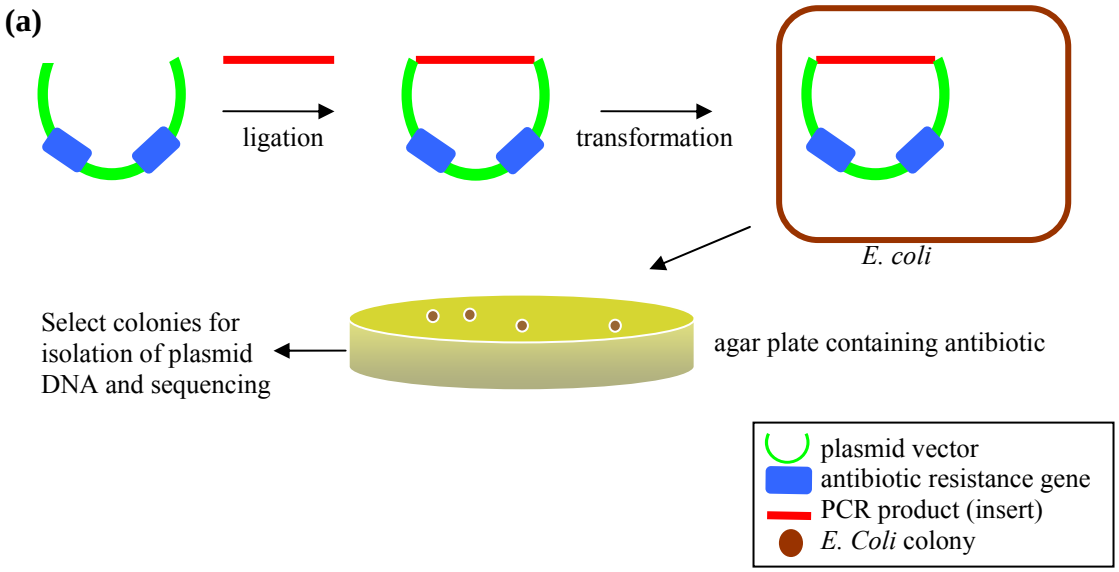
For cloning of HERV-K *env*, 1 µl of PCR product from the first round of amplification with the unspliced *env* primers was subjected to a second round of amplification using the same cycling conditions and primers. The PCR product from the second round of amplification was analysed by agarose gel electrophoresis. A no-RT control was performed for each sample and if positive, the corresponding sample was deemed ineligible for cloning due to contaminating gDNA.

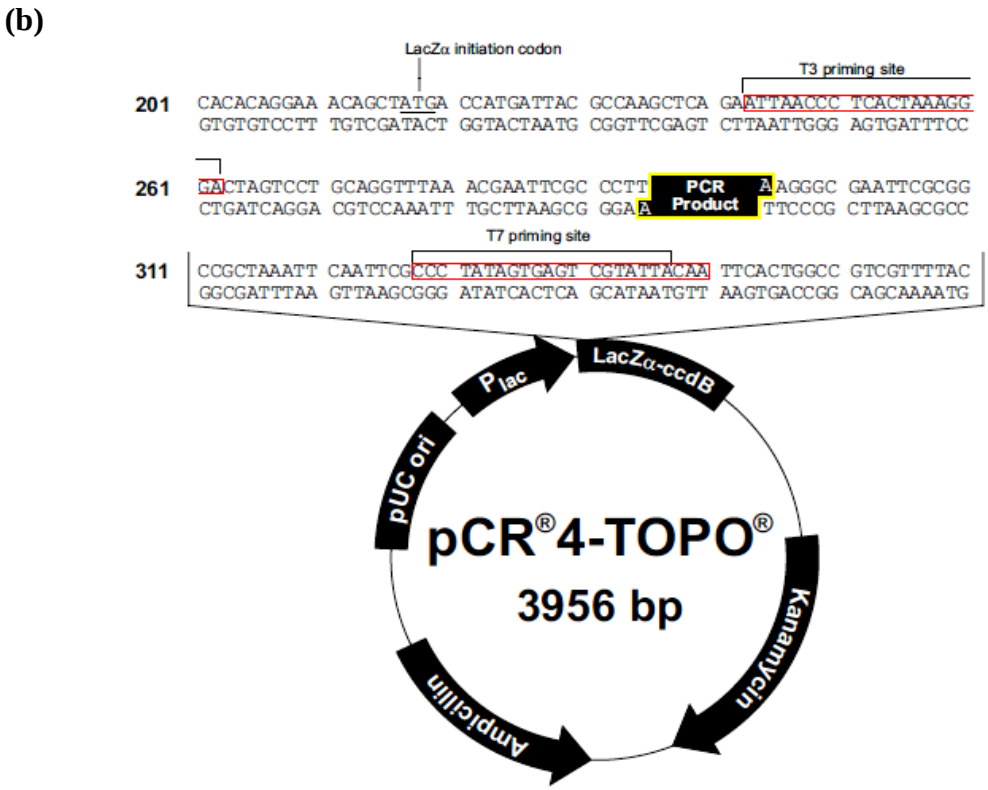
2.6.3 Cloning procedure

An aliquot (2 µl) of PCR product was added to 0.5 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and 0.5 µl of vector and incubated at room temperature for 30 minutes (TOPO® cloning reaction). A vector-only control reaction was also carried out with 2 µl water instead of PCR product.

The transformation of *E. coli* cells was achieved by adding half of the cloning reaction mix (1.5 µl) to a vial of One Shot TOP10® chemically competent *E. coli* cells (Invitrogen) and incubated on ice for 30 minutes. The remaining 1.5 µl was stored at -20°C for future transformation, if required. The cells were then heat-shocked at 42°C for 1 minute and immediately returned to ice for 2 minutes. A 250 µl aliquot of SOC medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, Invitrogen) was added to the vial, capped and shaken horizontally (250 RPM) at 37°C for 90 minutes in an Innova 4000 incubator shaker (New Brunswick Scientific). A 150 µl aliquot of the bacterial culture was spread on a pre-warmed Luria-Bertani (LB) agar plate containing 100 µg/ml ampicillin and incubated overnight at 37°C. The remaining culture was stored at 4°C for future plating.

Figure 2.1: Diagram illustrating cloning and map of plasmid vector pCR4-TOPO®





Legend:

(a) The plasmid vector contains ampicillin and kanamycin resistance genes. Only *E. coli* cells that receive plasmid can grow on the antibiotic-containing agar plate.
(b) The map (modified from the Invitrogen user manual) shows the features of the vector and the sequence surrounding the cloning site with nucleotide positions. A lethal *E. coli* gene, *ccd*B, is fused to the C-terminus of the LacZα fragment. Ligation of the PCR product disrupts expression of the *lac*Zα-*ccd*B gene fusion permitting growth of only positive recombinants upon transformation in *E. coli* cells. The vector also contains the priming sites T3 and T7 that are used during sequencing. P_{lac}, promoter for the *lac*Zα gene; and pUC ori, origin of replication in *E.coli*.

2.6.4 Small-scale plasmid preparation from bacteria

Twenty four bacterial colonies per agar plate were selected and each colony was transferred to a 10 ml Falcon tube containing 2 ml of LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl in 800 ml of distilled water) and 100 µg/ml ampicillin. These were cultured in a horizontal shaker (250 RPM) overnight at 37^{o}C. Bacterial cultures were transferred to 1.5 ml Eppendorf tubes, centrifuged for one minute at 8,000 RCF, supernatant removed and purification of plasmid DNA carried out.

DNA extraction was carried out using a QIAprep® Spin Miniprep kit (Qiagen). After the supernatant was removed, the bacterial pellet was resuspended by vortexing briefly with 250 µl buffer P1 (25 mM Tris-HCl and 10 mM EDTA buffer (pH 8.0) with 100 µg/ml RNase A). The bacterial suspension was lysed with 250 µl buffer P2 (200 mM NaOH, 1% SDS) and incubated at room temperature for five minutes. Neutralisation buffer N3 (350 µl) was added to the lysate which was then incubated for ten minutes on ice. Bacterial debris and gDNA were pelleted by centrifugation at 10,000 RCF for ten minutes. The plasmid DNA contained in the supernatant fluid was transferred to the spin column provided and centrifuged at 10,000 RCF for one minute, during which the plasmid DNA was bound to the column membrane while contaminants were eluted and discarded. Residual nuclease activity was removed by addition of 500 µl buffer PB followed by centrifugation at 10,000 RCF for one minute. The column was then washed with 750 µl buffer PE by centrifugation for one minute at 10,000 RCF. The flow-through was discarded and any residual wash buffer removed by further centrifugation for one minute at 10,000 RCF. Bound DNA was eluted by addition of 25-40 µl buffer EB to the membrane, followed by centrifugation at 10,000 RCF for one minute. The eluted DNA was stored at minus 20 ^{o}C until required.

T7 and T3 primers (Invitrogen) were used for sequencing. The sequence of the forward primer (T3) was 5'-ATTAACCCTCACTAAAGGGA-3' and the reverse primer (T7) 5'-TAATACGACTCACTATAGGG-3'. These bind specifically to the vector, allowing the *env* insert to be sequenced. For each sequencing reaction, 1 µl of the appropriate primer was added to 9 µl of purified plasmid DNA.

A number of samples were submitted, as glycerol stock plates, to Agencourt (Beverly, Massachusetts) for purification of plasmid DNA and sequencing. Media for glycerol stocks consisted of LB with 10% glycerol supplemented with ampicillin 50 µg/ml. Bacterial colonies were transferred into 96-well round bottom plates containing approximately 90 µl of media. Plates were covered with a lid, wrapped loosely with cellophane to minimise evaporation, incubated for twelve hours at 37ºC then immediately frozen at minus 80ºC. The glycerol plates were shipped to Agencourt on dry ice.

2.7 Statistics and data handling

The software package Statistical Package for Social Sciences (SPSS) version 16 was used to create a patient database and to perform statistical tests. The data were not normally distributed so non-parametric analyses were employed. Exact (2-sided) p values were calculated where sample sizes were small (Professor E. Kulinskaya, Statistician, Imperial College). The Kruskall-Wallace test was used to compare three or more unpaired groups and the Mann-Whitney test was used for two unpaired groups. The Wilcoxon matched pairs test was used to compare two groups of matched data. Spearman's rank correlation coefficient (Spearman's rho) was used to assess the relationship between two variables. Pearson's chi-square test or Fisher's exact test was used to compare two or more groups where the outcome variable was categorical and McNemar's test was used if the data were paired. P-values less than 0.05 were considered statistically significant.

Chapter 3:
Expression of HERV-K mRNA in human melanoma cell lines

3.0 Abstract

Introduction:

HERV-K transcripts and proteins have previously been demonstrated in human melanoma cell lines and RVLPs with homology to HERV-K have been reported in cell supernatants. The principal objectives of this work were to corroborate previously published data regarding HERV-K mRNA expression in human melanoma cell lines and to identify suitable positive controls for the investigation of expression in clinical material.

Methods:

RNA was extracted from the human melanoma cell lines A375 and WM2664 and expression of HERV-K mRNA investigated by RT-PCR. In addition, putative viral pellets were obtained from cell supernatants by ultracentrifugation and analysed for HERV-K viral RNA by RT-PCR.

Results:

Expression of full-length mRNA derived from type 1 and type 2 HERV-K proviruses was detected in both cell lines but spliced *rec* mRNA expression was observed in A375 cells only. Sequencing unequivocally confirmed the expression of *rec* mRNA in these cells. A375 cells were also observed to produce putative viral particles that contained sequences with homology to the *env* region of HERV-K. A two-step RT-PCR methodology was found to be superior to one-step RT-PCR because it circumvented false positive results due to contaminating gDNA.

Conclusions:

The findings here are concordant with previously published work. Melanoma cell lines have previously been shown to differ in their expression of spliced HERV-K mRNA and RVLPs with homology to HERV-K have been reported in some human melanoma cell line supernatants. Given the expression of both full-length and spliced *rec* mRNA in A375 cells, RNA derived from these cells was identified as a suitable positive control for experiments to investigate HERV-K gene expression in archival clinical tissue.

3.1 Introduction

HERV-K transcripts, proteins and RVLPs have previously been demonstrated in human melanoma cell lines. Expression of full-length mRNA derived from types 1 and 2 HERV-K proviruses has been observed in human melanoma cell lines and spliced transcripts in a proportion of these (section 1.4.2). Expression of HERV-K Gag, Env and Rec proteins has been demonstrated in melanoma cell lines by immunofluorescence and RVLPs with homology to HERV-K have been reported in cell supernatants (sections 1.4.1 and 1.4.2). In this study, the expression of HERV-K mRNA was investigated in the human melanoma cell lines A375 and WM2664 with the aims of corroborating previously published findings and identifying suitable positive controls for the investigation of HERV-K expression in archival clinical tissue. Additionally, cell supernatants were investigated for putative virus particles.

3.2 HERV-K mRNA expression in human melanoma cell lines

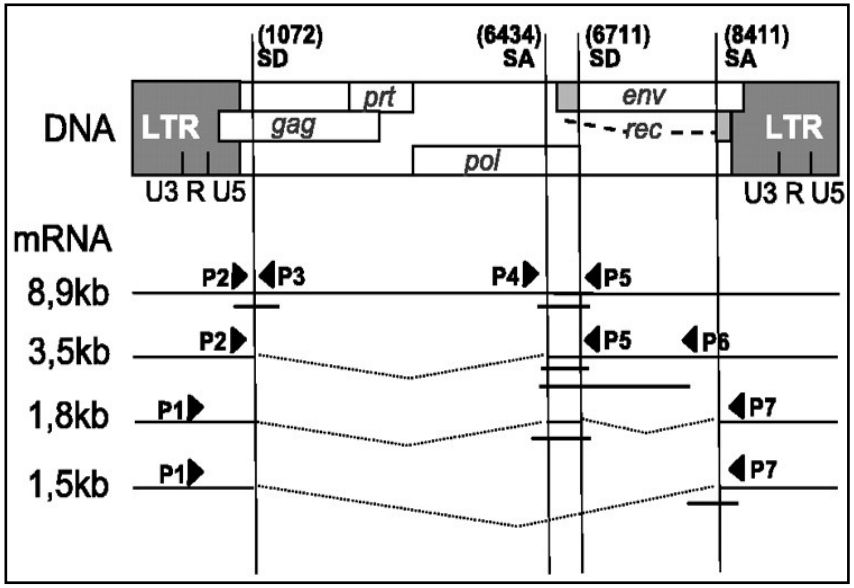
A set of seven previously published primer pairs (Table 2.2) was used to investigate the expression of HERV-K mRNA in the human melanoma cell lines A375 and WM2664 by RT-PCR. The locations of the primers are illustrated in Figure 3.1a. The primers were located before and behind the splice donor and acceptor sites, allowing full-length, spliced *env*, and double-spliced *rec* mRNA to be distinguished. Primers flanking the 292 bp deletion (between the *pol* and *env* genes, Figure 1.8b) were used to discriminate between type 1 and type 2 HERV-K proviruses.

Expression of full-length mRNA derived from type 1 and type 2 proviruses was observed in both cell lines (Figure 3.1b). WM2774 cells expressed full-length, but not spliced *env* or *rec*, mRNA. A375 cells expressed both full-length and *rec* mRNA, but not spliced *env*. Parallel experiments omitting the RT revealed the presence of residual gDNA contamination.

Expression of *rec* was confirmed by sequencing of the PCR product (Figure 3.2). This showed 100% homology with a published HERV *rec* mRNA sequence (Lower et al., 1995).

Figure 3.1: RT-PCR analysis of HERV-K expression in A375 and WM2664 cells

(a)



Primer pair	Gene	Type 1	Type 2
P2 + P3	*gag*	533 bp	533 bp
P4 + P5	*pol*	581 bp	873 bp
P2 + P5	spliced *env*	397 bp	689 bp
P2 + P6	spliced *env*	1523 bp	1815 bp
P1 + P7	*rec*	No product	616 bp

(b)

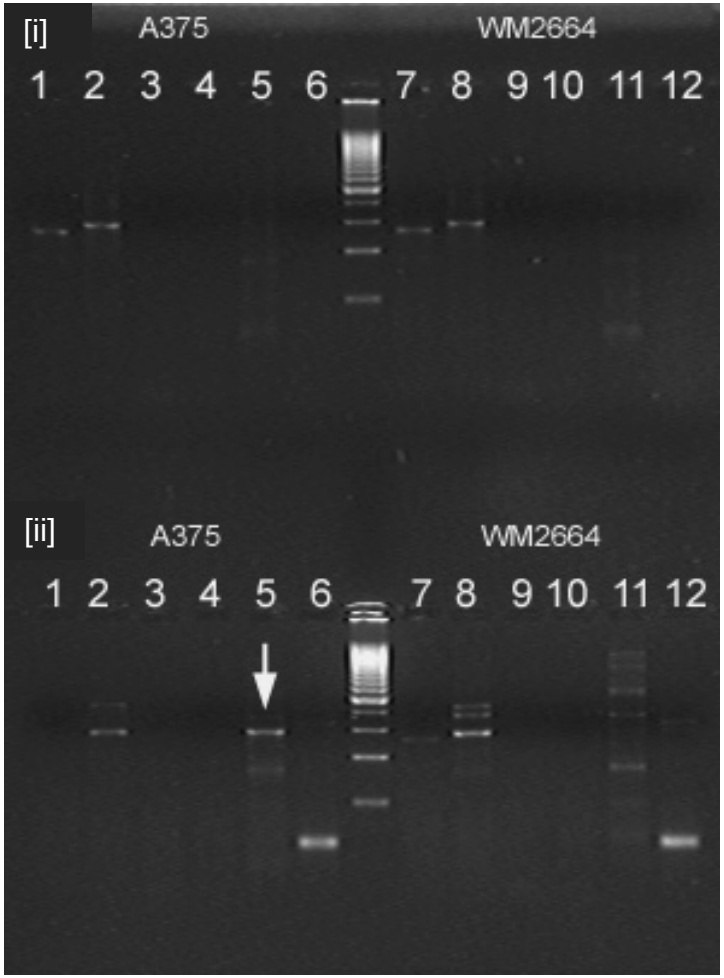


Figure 3.1: Legend

(a) The genomic organisation of a HERV-K provirus, the expression pattern of mRNA and the primer pairs (P1 to P7) used for RT-PCR analysis are shown (Buscher et al., 2005). The PCR product sizes for mRNA derived from type 1 and type 2 proviruses are indicated in the table. The spliced regions are indicated by dotted lines. P, primer; LTR, long terminal repeat; U3, 3' untranslated region; R, repeat region, U5, 5' untranslated region; SA, splice acceptor site (nucleotide position in brackets); SD, splice donor site.

(b) Expression of HERV-K mRNA was investigated in the human melanoma cell lines A375 and WM2664 by RT-PCR using primers P1-P7. The housekeeping gene *HPRT* was amplified as a positive control. The PCR products were analysed by agarose gel electrophoresis. A 200 bp molecular weight (MW) marker is located between lanes 6 and 7.
[i] False-positive results due to residual gDNA were determined by parallel experiments omitting the RT.
[i] & [ii] Lanes 1 and 7, *gag* (primers P2 + P3); lanes 2 and 8, *pol* (P4 + P5); lanes 3 and 9, spliced *env* (P2 + P5); lanes 4 and 10, spliced *env* (P2 + P6); lanes 5 and 11, *rec* (P1 + P7) and lanes 6 and 12, spliced *HPRT* (94 bp product).
The 581 and 873 bp bands in lanes 2ii and 8ii represent full-length transcripts derived from type 1 and type 2 proviruses respectively. The PCR product in lane 5ii (indicated by the arrow) was sequenced. The bands in lanes 1i, 2i, 7i and 8i represent residual gDNA contamination. Some non-specific bands are seen in lanes 5ii, 8ii and 11ii.

Figure 3.2: Nucleotide sequence alignment of PCR product corresponding to *rec* mRNA

```
emb|X82271.1|HERVCORF (Human endogenous retrovirus mRNA for central open reading
frame)*

Query†   CTCTATACTTTGTCTCTGTGTCTTTTTCTTTTCCAAATCTCTCGTCCCACCTTACGAGAA
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct‡   CTCTATACTTTGTCTCTGTGTCTTTTTCTTTTCCAAATCTCTCGTCCCACCTTACGAGAA
         (6433)§

Query    ACACCCACAGGTGTGTAGGGGCAACCCACCCCTACATCTGGTGCCCAACGTGGAGGCTTT
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    ACACCCACAGGTGTGTAGGGGCAACCCACCCCTACATCTGGTGCCCAACGTGGAGGCTTT

Query    TCTCTAGGGTGAAGGTACGCTCGAGCGTGGTCATTGAGGACAAGTCGACGAGAGATCCCG
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    TCTCTAGGGTGAAGGTACGCTCGAGCGTGGTCATTGAGGACAAGTCGACGAGAGATCCCG

Query    AGTACATCTACAGTCAGCCTTACGACATTTGAAGTTCTACAATGAACCCATCAGAGATGC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    AGTACATCTACAGTCAGCCTTACGACATTTGAAGTTCTACAATGAACCCATCAGAGATGC

Query    AAAGAAAAGCACCTCCGCGGAGACGGAGACATCGCAATCGAGCACCGTTGACTCACAAGA
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    AAAGAAAAGCACCTCCGCGGAGACGGAGACATCGCAATCGAGCACCGTTGACTCACAAGA

Query    TGAACAAAATGGTGACGTCAGAAGAACAGATGAAGTTGCCATCCACCAAGAAGGCAGAGC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    TGAACAAAATGGTGACGTCAGAAGAACAGATGAAGTTGCCATCCACCAAGAAGGCAGAGC

Query    CGCCAACTTGGGCACAACTAAAGAAGCTGACGCAGTTAGCTACAAAATATCTAGAGAACA
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    CGCCAACTTGGGCACAACTAAAGAAGCTGACGCAGTTAGCTACAAAATATCTAGAGAACA

Query    CAAAGGTGACACAAACCCCAGAGAGTATGCTGCTTGCAGCCTTGATGATTGTATCAATGG
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    CAAAGGTGACACAAACCCCAGAGAGTATGCTGCTTGCAGCCTTGATGATTGTATCAATGG

Query    TGTCTGCAGGTGTACCCAACAGCTCCGAAGAGACAGCGACCATCGAGAACGGGCCATGAT
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    TGTCTGCAGGTGTACCCAACAGCTCCGAAGAGACAGCGACCATCGAGAACGGGCCATGAT

Query    GACGATGG
         ||||||||
Sbjct    GACGATGG
```

Legend:

Nucleic acid sequence alignment using BLASTn. (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

* GenBank accession number X82271. Note that *rec* was formally designated *cORF* (central open reading frame)

† Query = sequence of the PCR product derived from A375 cells

‡ Sbjct = the Genbank sequence to which the query shows homology

§ Nucleotide position of first base with reference to HERV-K 108 (Gen Bank accession number AF164614)

3.3 Full-length HERV-K mRNA expression in A375 cells

3.3.1 Elimination of gDNA contamination

Residual gDNA in RNA extracts may give rise to false positive results when investigating the expression of full-length mRNA. In Figure 3.1b, it can be seen that parallel experiments omitting the RT revealed the presence of residual gDNA. Thus, to rectify this problem, reverse transcription was combined with a gDNA elimination step followed by conventional PCR (two-step RT-PCR, section 2.4.4).

3.3.2 Two-step RT-PCR analysis of full-length HERV-K expression

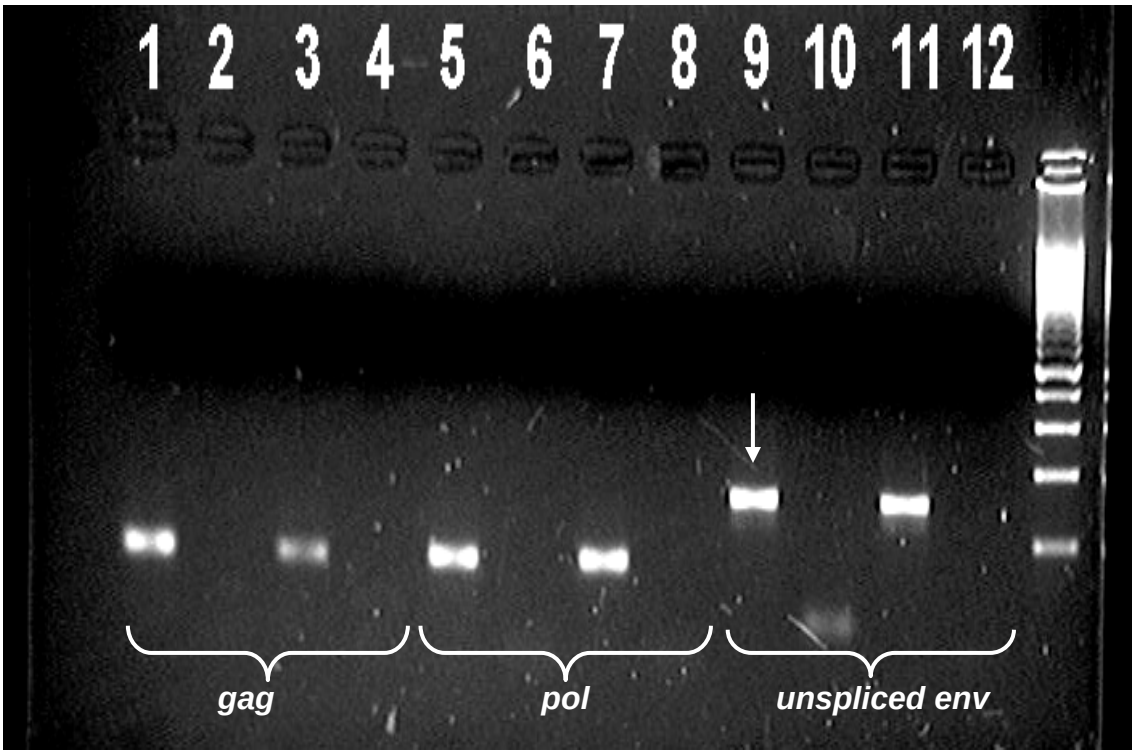
Expression of full-length mRNA (*gag, pol* and unspliced *env*) was investigated in A375 cells by two-step RT-PCR. The primers used in this experiment (Table 2.3) did not allow for distinction between mRNA derived from type 1 and type 2 proviruses. The primers used to amplify unspliced *env* did not span a splice junction and were designed to allow typing of expressed *env* sequences.

Parallel experiments using RNA as a template confirmed the presence of residual gDNA (Figure 3.3). Parallel experiments omitting RT demonstrated that gDNA was successfully eliminated in the cDNA synthesis reaction.

3.4 Characterisation of expressed *env*

To determine the type of HERV-K expressed, the PCR product corresponding to unspliced *env* mRNA was sequenced. The raw sequence chromatograms, shown in Figure 3.4, were strongly suggestive of expression of a mixture of mRNA species with or without a four base deletion, as previously described (Sugimoto et al., 2001). Specifically, the forward sequence was clean from base 1 to 109 (bases 7368 to 7476 of HERV-K108, accession number AF164614) then became garbled. In contrast, the reverse sequence was clean from base 296 to base 114 (bases 7663 to 7481 of HERV-K108) then became garbled. These findings are consistent with a frameshift caused by a four base deletion of bases 110-113. To analyse HERV-K *env* expression in A375 cells in more detail, the PCR product was cloned for sequence analysis. The findings are described in Chapter 6.

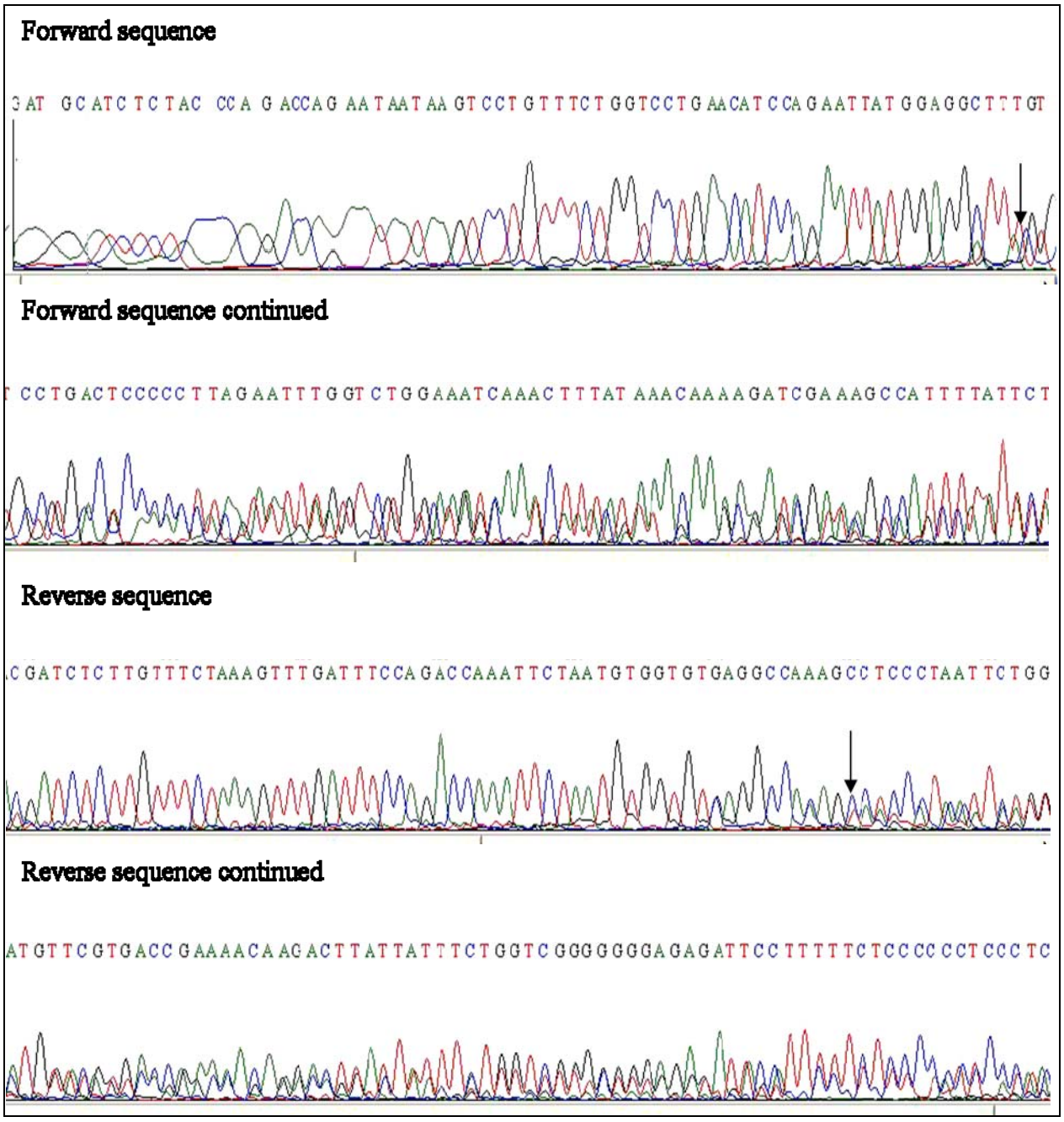
Figure 3.3: Two-step RT-PCR analysis of full-length HERV-K mRNA expression in A375 cells



Legend:

Expression of full-length HERV-K mRNA was investigated in A375 cells by two-step RT-PCR. The PCR products were analysed by agarose gel electrophoresis. A 200 bp MW marker is adjacent to lane 12. Lane 1, *gag* (132 bp); lane 5, *pol* (145 bp); lane 9, unspliced *env* (296 bp); lanes 3, 7 and 11, parallel experiments using RNA as a template were performed to determine the presence of residual gDNA in the RNA extracts; lanes 2, 6 and 10, parallel experiments omitting RT were performed to assess the effectiveness of gDNA elimination in the cDNA synthesis reaction (band in lane 10 is a non-specific band of low MW, of no significance); lanes 4, 8 and 12, negative controls (water template). The PCR product in lane 9 (indicated by the arrow) was sequenced.

Figure 3.4: Sequencing of PCR product corresponding to unspliced *env* mRNA



Legend:

Sections of the raw sequence chromatograms of forward and reverse sequences of the PCR product corresponding to unspliced *env* mRNA derived from A375 cells, illustrating the points at which the sequence becomes garbled (arrows).

3.5 Production of putative viral particles

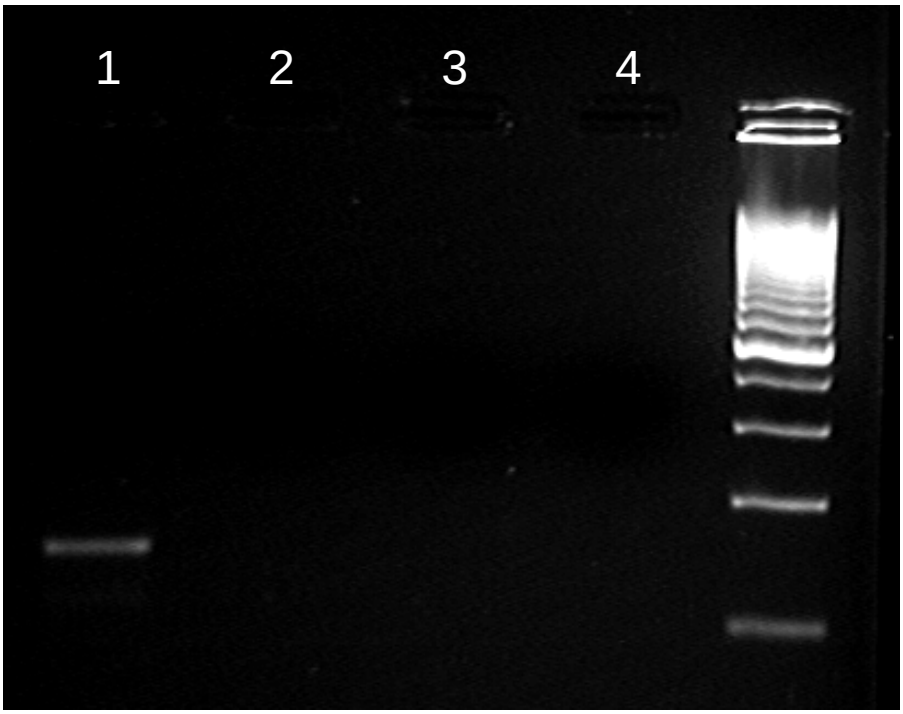
3.5.1 RT-PCR analysis of RNA from viral pellets

Putative viral pellets were obtained from the cell line supernatants by ultracentrifugation (section 2.3.3). RT-PCR analysis for HERV-K viral RNA was performed using the unspliced *env* primers (Table 2.3). There was evidence of viral particle release from A375 cells (Figure 3.5). Parallel experiments omitting the RT verified the absence of cellular DNA contamination.

3.5.2 Characterisation of viral pellets derived from A375 cells

In order to determine viral particle-associated sequences, the PCR product in lane one was sequenced. Analyses showed that the particles contained sequences with homology to the *env* region of HERV-K 101 and 109 (Figure 3.6).

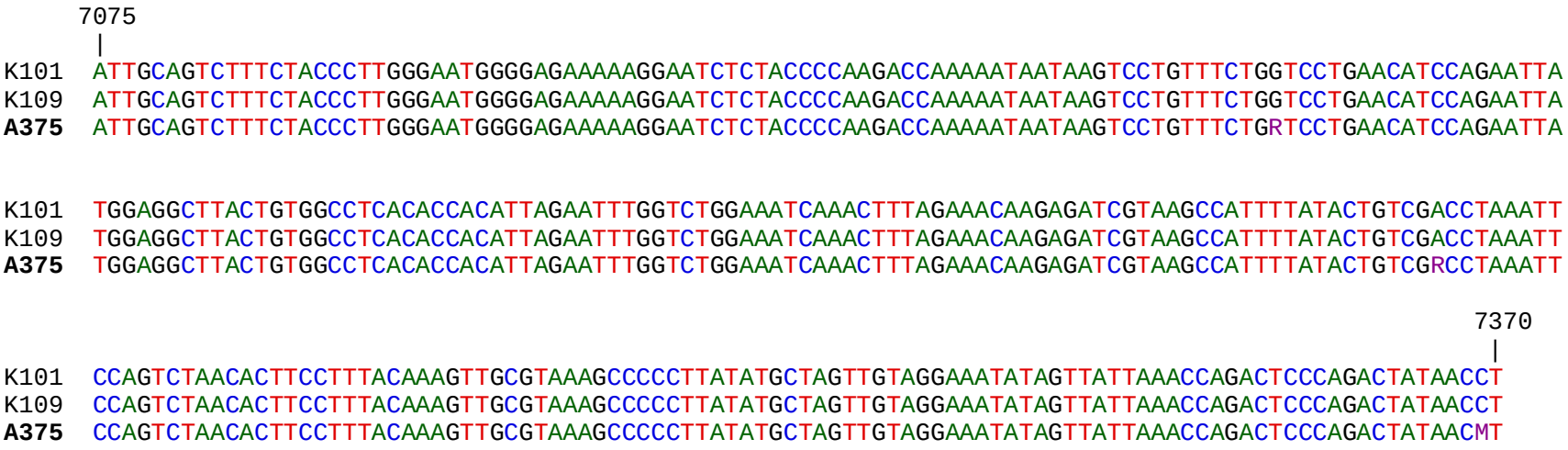
Figure 3.5: RT-PCR analysis of RNA from viral pellets obtained from human melanoma cell line supernatants



Legend:

Putative viral pellets were obtained from A375 and WM2664 cell supernatants by ultracentrifugation and RT-PCR analysis for HERV-K viral RNA performed. The PCR products were analysed by agarose gel electrophoresis. A 200 bp MW marker is adjacent to lane 4. Lane 1, viral RNA derived from A375 cell supernatant (296 bp product); lane 2, viral RNA derived from WM2664 cell supernatant (no product); lanes 3 & 4, RT omitted to verify the absence of cellular DNA contamination.

Figure 3.6: Nucleotide sequence alignment of A375 viral-particle derived sequence



Legend:

Nucleotide sequence alignment of A375 viral-particle derived sequence with the human specific HERV-K sequences K101 and 109, using ClustalW. The nucleotide positions of the first and last bases with reference to HERV-K 101 (Genbank accession number AF164609) are indicated. The letters R and M represent nucleotide ambiguity (R=G/A; M=A/C).

3.6 Discussion

The principal aims of this work were to corroborate previously published data regarding the expression of HERV-K mRNA in melanoma cell lines and to identify suitable positive controls for the investigation of expression in clinical material.

Expression of full-length mRNA derived from HERV-K type 1 and type 2 proviruses was detected in both human melanoma cell lines investigated. WM2774 cells expressed full-length HERV-K mRNA only. In contrast, expression of *rec* in the absence of spliced *env* was observed in A375 cells. However, it should be noted that spliced *env* mRNA was subsequently detected in A375 cells using real-time qRT-PCR probably due to the superior sensitivity of this method over conventional RT-PCR (section 4.3.1). Sequencing unequivocally confirmed the expression of *rec* mRNA in A375 cells.

These findings support those of Buscher et al. (2005) who studied five melanoma cell lines and observed expression of full-length HERV-K mRNA in them all. However, spliced *env* and *rec* mRNA were expressed in three cell lines only. Therefore, it appears that melanoma cell lines differ in their expression of spliced HERV-K mRNA. Given the expression of both full-length and *rec* mRNA in A375 cells, RNA derived from these cells was identified as a suitable positive control for experiments to investigate HERV-K gene expression in archival clinical tissue.

The ideal negative control would be a cell line that does not express HERV-K. However, it is likely that all cell lines express full-length HERV-K mRNA. Buscher et al. (2005) investigated normal human epidermal melanocytes and 17 cell lines established from various other tumours (including T-cell and B-cell lymphomas, ovarian, colorectal, breast, pancreas and brain) and found expression of full-length HERV-K mRNA in them all. Furthermore, since the start of this work HERV-K has been shown to be expressed in many normal tissues (discussed in Chapter 7).

Sequencing of unspliced *env* mRNA derived from A375 cells suggested expression of a mixture of mRNA species with or without a four base deletion as previously reported by Sugimoto et al. (2001). This finding prompted further investigation of the expressed sequences (Chapter 6). Sugimoto et al. (2001) studied HERV-K *env* mRNA expression in normal placental, foetal lung, and adult testicular tissue (in addition to peripheral leukocytes and a teratocarcinoma cell line) by RT-PCR and subsequent sequencing of cloned products. These workers identified eight distinct *env* sequences of HERV-K and found different transcriptional profiles between these tissues/cells. All tissues, peripheral leukocytes and the cell line expressed the four base deleted *env* sequence, which was found to be derived from a novel HERV-K gene designated ERVK5. However it is unlikely that functional Env protein would be generated because of the resultant frameshift.

Expression of Gag, Rec and Env proteins has previously been observed in human melanoma cell lines and was not investigated in this study (section 1.4.2). However, putative viral pellets were obtained from cell supernatants by ultracentrifugation and analysed for HERV-K viral RNA. The strategy employed for isolation of RVLPs in this study was specifically designed to avoid contamination with cellular debris. Putative viral particles were detected in A375 cell supernatants and sequence analysis revealed that these particles contain sequences with homology to the *env* region of HERV-K 101 and 109.

The existence of HERV-K-encoded RVLPs in vivo is well documented. RVLPs were first observed in cell lines derived from human germ-cell tumours and characterised by morphological features (Löwer et al., 1993; Boller et al., 1993). RVLPs have previously been demonstrated in melanoma cell line supernatants and melanoma tissue by EM but have been found to be defective and non-infectious (section 1.4.1). Data regarding particle-derived sequences from melanoma cell lines are limited although one study confirmed the sequences to be defective and also heterologous within the same cell line (Hirschl et al., 2007). To characterise specifically the sequences contained in particles derived from A375 cells, it would be necessary to clone and sequence the PCR product corresponding to viral pellet RNA. To confirm the assumption that particles are produced, cell supernatants could be examined by EM.

In conclusion, expression of HERV-K mRNA derived from type 1 and type 2 proviruses was detected in both of the human melanoma cell lines investigated but spliced *rec* mRNA was expressed in A375 cells only. Thus RNA derived from these cells was identified as a suitable positive control for experiments to investigate HERV-K gene expression in archival clinical tissue. A375 cells were also observed to produce putative HERV-K viral particles. These findings are concordant with previously published work. This work has also demonstrated that a two-step RT-PCR methodology is superior to one-step RT-PCR because it obviates false positive results due to contaminating gDNA.

Chapter 4:

Development of a methodology to study HERV-K mRNA expression in archival tissue

4.0 Abstract

Introduction:

A significant challenge in dermatology research is the lack of accessibility of fresh or cryopreserved tissue, particularly from primary melanoma. The last decade has seen vast improvements in techniques to extract nucleic acids of sufficient quality and quantity from archival FFPE tissue for use in downstream applications, such as PCR. The principal aim of this work was to determine the feasibility of detecting HERV-K expression in FFPE tissue using a real-time qRT-PCR methodology.

Methods:

TaqMan based real-time qRT-PCR methods to analyse the expression of HERV-K mRNAs were developed, using primers generating amplicons of 94 to 117 bp. The small target size minimised the effects of RNA fragmentation in FFPE tissue. RNA quality and normalisation of gene expression were controlled by quantification of the spliced mRNA of the housekeeping gene *HPRT*. Relative target gene expression was calculated using the formula $2^{-\Delta Ct}$ where ΔCt (Cycle threshold) = Ct *target gene* -Ct *HPRT*. RNA was extracted from 102 FFPE tissue blocks, comprising melanoma, benign naevus and normal skin specimens, stored from 42 to 3,129 days.

Results:

The *HPRT* gene was amplified in 97% of archival tissues analysed and these samples were accordingly deemed to be viable for the analysis of HERV-K mRNA expression. The assay developed had high intra- and inter-assay reproducibility. RNA yield was not affected by the age of the tissue and no PCR inhibition was identified.

Conclusions:

In this work, a sensitive, accurate and reproducible real-time qRT-PCR assay for the quantification of HERV-K gene expression levels in FFPE tissue samples has been developed. Quantification of *HPRT* (Ct *HPRT*) was used as an RNA quality control measure. This work emphatically demonstrates that archival tissue can be realistically used to study gene expression.

4.1 Introduction

Histopathology archives are an excellent resource for retrospective studies. In the last decade, DNA extracted from archival FFPE tissue has frequently been used in medical and dermatology research. For example, distinct sets of genetic alterations in melanoma have been identified by array-based comparative genomic hybridization of DNA extracted from archival specimens (Curtin et al., 2005). Data generated from such studies can result in the identification of novel biomarkers for disease classification, diagnosis and prognosis and illuminate potential therapeutic targets.

Improved techniques for extracting RNA from FFPE specimens and the advent of extremely sensitive fluorescence-based real-time qRT-PCR procedures have shown that it is possible to detect and quantify mRNA derived from FFPE tissue (section 1.5). The ability of real-time qRT-PCR to assay very small RNA fragments makes it particularly amenable to samples where RNA might be degraded such as FFPE tissue.

This principal aim of this work was to determine the feasibility of detecting HERV-K expression in FFPE tissue using novel real-time qRT-PCR methodology. A Taqman-based assay was chosen over other fluorescent dye methods, such as SYBR® green, because the use of three oligonucleotides (two primers and one probe) results in higher specificity and sensitivity (section 1.5.3). The design of the assay had to take into consideration the quality and integrity of the RNA (section 1.5.2). Several essential steps were required to ensure that the data generated were reliable and reproducible. The steps involved were: design of primers and Taqman probes applicable to RNA extracted from FFPE tissue; real-time qRT-PCR analysis of HERV-K expression in human melanoma cell lines; investigation of intra- and inter-assay reproducibility; extraction of RNA from FFPE tissue; replicate end-point dilution PCR to determine sensitivity and cut-off values; investigation of PCR inhibition.

4.2 Design of primers and probes

No methods specifically for the detection and quantification of HERV-K gene expression in archival tissue have previously been developed and published. In this study, it was intended to investigate the expression of full-length (*pol*, unspliced *env*)

and spliced HERV-K mRNA (spliced *env*, *rec* and *np9*) in addition to the housekeeping gene *HPRT*. The following strategy regarding primer and probe design was used:

(i) both the forward (F) and reverse (R) primers were located in the conserved regions of human specific HERV-K (HML2) sequences (types 101-115), in contrast to HERV-K sequences found in chimpanzees and other primates (Figure 4.1).

(ii) primers and probes for *HPRT* mRNA were designed to span an intron to exclude amplification of gDNA (Figure 4.2). Similarly, to amplify specifically spliced *env, rec* and *np9* mRNA, the primers or probe were designed to anneal across a splice junction (Figure 4.3). Figure 4.4 illustrates the splice donor (SD) sites used to generate *np9* and *rec* transcripts.

(iii) all amplicons were kept small (94 to 117 bp) to overcome the problem of RNA fragmentation in FFPE tissue.

(iv) optimised primer design (Apte & Daniel, 2003): the guanidine plus cytosine (GC) content was approximately 50%; the Tm (melting temperature) of the primers were 58-62°C; primer pairs with similar Tm values were designed (within 2°C of each other).

(v) optimised probe design (Apte & Daniel, 2003): the Tm of the probes were 8-10°C higher than the Tm of the primers; a guanidine residue was avoided at the 5' end of the probe, next to the reporter, since this can cause quenching so preventing fluorescence; runs of four or more of the same nucleotide were avoided, particularly of guanidine.

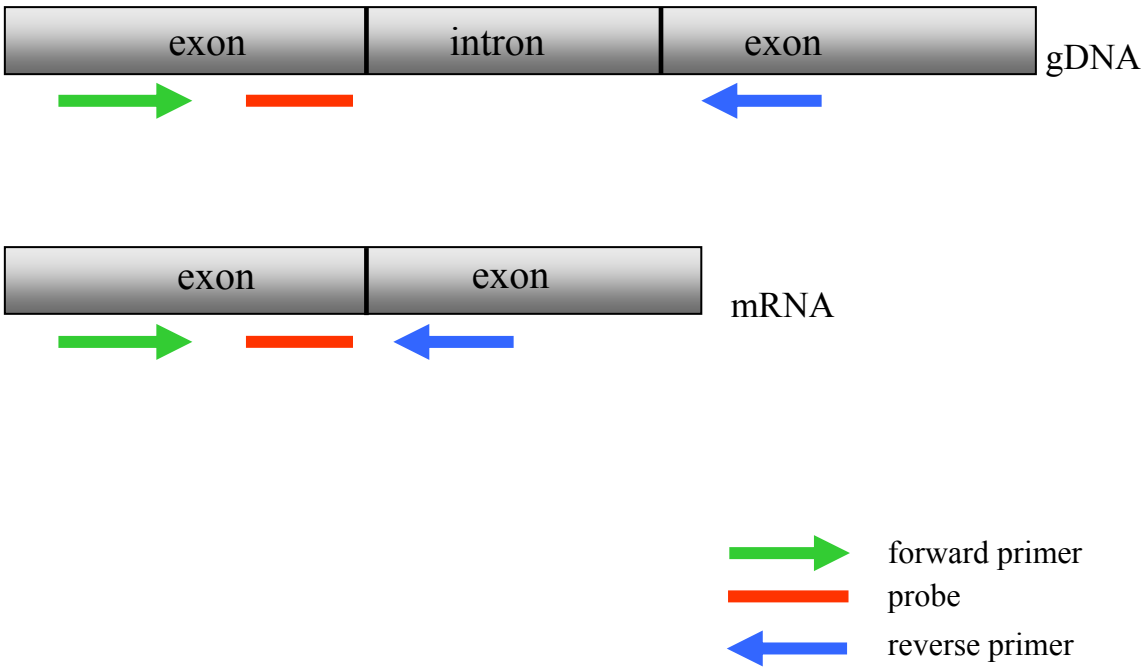
The primers and probes are shown in Table 4.1.

Figure 4.1: Example of the location of primers and probe in conserved regions of human specific HERV-K sequences

Legend:

Nucleotide sequence alignment of the *pol* forward primer (*pol* F), reverse primer (*pol* R) and probe (*pol* Pr) with the human specific HERV-K sequences K101-115, illustrating their locations in conserved regions. The nucleotide positions of the first base of the primers and probe with reference to HERV-K 108 (Gen Bank accession number AF164614) are shown. The dashes (-) indicate a six base insertion in HERV-K 107.

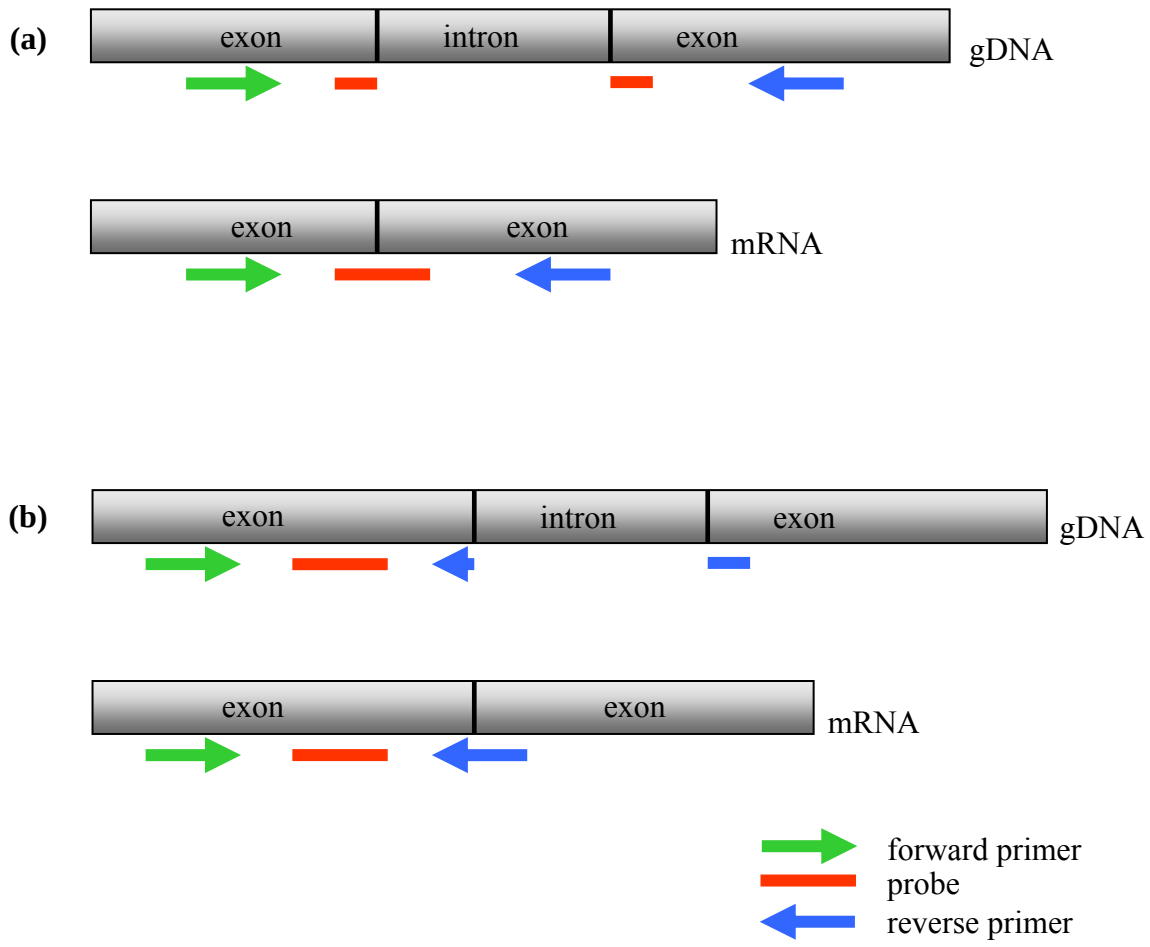
Figure 4.2: Diagram illustrating the locations of *HPRT* primers and probes



Legend:

To amplify *HPRT* mRNA, the primers and probe flank an intron. The product amplified from mRNA (no introns) is smaller than that amplified from gDNA (containing introns) and is preferentially amplified.

Figure 4.3: Diagram illustrating the locations of primers and probes used to amplify spliced HERV-K mRNA



Legend:

(a) To amplify *np9* and spliced *env* mRNA the probe anneals across a splice junction so that one half hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon. Thus the probe anneals to cDNA synthesised from spliced mRNA but not to gDNA, thereby eliminating amplification of any contaminating gDNA.

(b) Similarly, specifically to amplify *rec* mRNA the reverse primer anneals across a splice junction.

Figure 4.4: Splice donor (SD) sites used to generate *np9* and *rec* transcripts

Figure 4.4: Legend

Nucleotide sequence alignment of HERV-K101-115, illustrating the SD sites used to generate *np9* and *rec* transcripts. The SD sites are shown within the boxes and the nucleotide (nt) positions of the first base with reference to HERV-K 101 and 108 are indicated. The proviruses differ in a 292 bp deletion (indicated by dashes) that contains the SD site used to generate *rec* transcripts (SD2). Type 1 proviruses (labeled in purple) have an alternative upstream SD site (SD1) for *np9* messages.

Table 4.1: Primers and probes for the analysis of HERV-K mRNA expression by real-time qRT-PCR

Primer/probe	Location	Sequence	Amplicon size
HPRT F	*HPRT* (17895256-17895276)*	5'-TGACACTGGCAAAACAATGCA-3'	94bp
HPRT R	*HPRT* (17900128-17900148)	5'-AGCTTGCTGGTGAAAAGGACC-3'	
HPRT Pr	*HPRT* (17895284-17895308)	5'-CTTTCCTTGGTCAGGCAGTATAATC-3'	
pol F	HERV-K *pol* (5970 – 5992)†	5'-TTTGCTGTAATGGGAGTTCCAGA-3'	100bp
pol R	HERV-K *pol* (6046 – 6069)	5'-TCTTAAGTCAGTGGAAAATTTCAC-3'	
pol Pr	HERV-K *pol* (6014 – 6040)†	5'-ACCAGGATATTGTAGTAAAGCTTTCCA-3'	
unspliced *env* F	HERV-K *env* (7594 – 7616)†	5'-TGCGTAAAGCCCCCTTATATGCT-3'	117bp
unspliced *env* R	HERV-K *env* (7686 – 7710)†	5'-TTGCATTGATTCAACTTTTAATTGG-3'	
unspliced *env* Pr	HERV-K *env* (7640 – 7665)†	5'-AACCAGACTCCCAGACTATAACCTGT-3'	
spliced *env* F	HERV-K *gag* (1034 – 1053)†	5'-AAGTCGACGAGAGATCCCGA-3'	93bp
spliced *env* R	HERV-K *env* (6462 – 6483)†	5'-AGAGATGCAAAGAAAAGCACCT-3'	
spliced *env* Pr	HERV-K *env* (1064 – 1075 and 6433 – 6445)†	5'-AGTCAGCCTTACGACATTTGAAGTT-3'	
rec F	HERV-K *env* (6628 – 6651)†	5'-GCTACAAAATATCTAGAGAACACA-3'	96bp
rec R	HERV-K *env* (6701 – 6712 and 8412 – 8422)†‡	5'-TATCAATGGTG TCTGCAGGTGTA-3'	
rec Pr	HERV-K *env* (6667 – 6690)	5'-CCAGAGAGTATGCTGCTTGCAGCC-3'	
np9 F	HERV-K *env* (6450 – 6470)§	5'-ATGAACCCATCGGAGATGCAA-3'	92bp
np9 R	HERV-K *env* (8146 – 8165))§	5'-AGACAGCGACCATCGAGAAC-3'	
np9 Pr	HERV-K *env* (6482 – 6493+ 8118 – 8129)**	5'-TCCACGGAGATG TCTGCAGGTGTA	

Designation of the primers/probes, their location and nucleotide sequence are shown. F, forward primer; R, reverse primer; and Pr, probe. Primers and probes were designed by Dr S. Singh under the guidance of Dr S. Kaye.

* Nucleotide positions with reference to *HPRT1* (Gen Bank accession number NT011786.16)

† Nucleotide positions with reference to HERV-K 108 (Gen Bank accession number AF164614)

‡ Primer spans the SD and SA sites used to generate *rec* transcripts (originate from HERV-K Type 2 proviruses, for example HERV-K 108)

§ Nucleotide positions with reference to HERV-K 101 (Gen Bank accession number AF164609)

** Probe spans the SD and SA sites used to generate *np9* transcripts (originate from HERV-K Type 1 proviruses, for example HERV-K 101)

4.3 Real-time qRT-PCR analysis of HERV-K mRNA expression in human melanoma cell lines

In real-time PCR a threshold for detection of fluorescence above background is determined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold (Ct). The Ct value is inversely proportional to the amount of target nucleic acid sequence in the original sample. Thus the lower the Ct value, the greater the amount of target there is in the starting material. The correlation between fluorescence and amount of amplified product allows target molecules to be quantified (Arya et al., 2005; Farrell, 2005; Logan et al., 2009).

As an initial step toward establishing a quantitative gene expression methodology applicable to FFPE tissue, RNA extracted from human melanoma cell lines was examined by two-step real-time qRT-PCR (section 2.4.5).

4.3.1 Detection of HERV-K mRNA in melanoma cell lines

Expression of *pol,* unspliced *env,* spliced *env, rec* and *np9* mRNA (representing full-length and spliced HERV-K) in addition to *HPRT* mRNA was detected in A375 cells by real-time qRT-PCR as shown in Figure 4.5. In contrast to the conventional RT-PCR experiment described in section 3.2, spliced *env* mRNA was detected. This discrepancy can be explained by the superior sensitivity of real-time qRT-PCR (in which very low copy numbers of a target molecule can be detected) over conventional RT-PCR.

HERV-K mRNA expression was also investigated in the human melanoma cell line WM2664 and the embryonic kidney cell line 293T (included for comparison as a non-melanoma cell line). Full-length (*pol* and unspliced *env*) and *np9* mRNA was expressed in both cell lines but, in contrast to A375 cells, not spliced *env* or *rec* mRNA (Table 4.2).

4.3.2 Control of gDNA contamination

Reverse transcription was combined with a gDNA elimination step to remove residual gDNA from the RNA extracts (section 2.4.2). However, no currently available gDNA elimination procedures are 100% effective (Peters et al., 2004) hence the importance of performing parallel negative controls in which RT is omitted ("no-RT" controls) in order to exclude residual gDNA contamination. Although PCR cannot discriminate between cDNA targets synthesised by reverse transcription and gDNA contamination, the latter would result in higher Ct values for a given gene (Peters et al., 2004). Since the quantity of DNA doubles every cycle during the exponential phase of PCR, a difference of one between sample Cts means that the sample with the lower Ct value had double the target sequence of the other sample; a change in Ct of two represents a fourfold difference; a change in Ct of three means an eightfold difference, and so on.

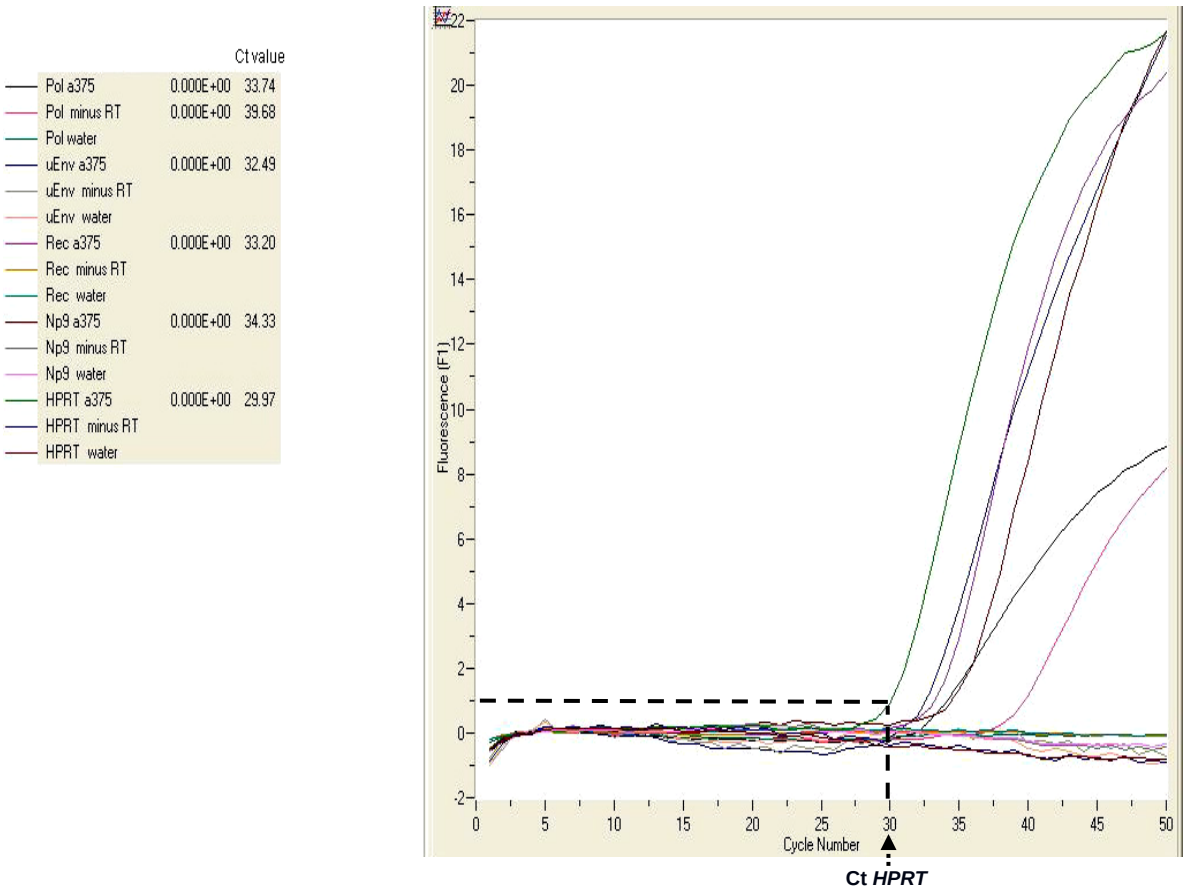
In this experiment, there was no amplification in the no-RT controls with the exception of a weak fluorescent signal with the *pol* primers (Figure 4.5). As the latter amplify full-length HERV-K mRNA, residual gDNA in the RNA extract can be amplified. However, the Ct was six cycles greater in the no-RT control thus indicating only a very small amount of gDNA contamination (approximately 1/64th the amount of cDNA).

For all experiments, significant residual gDNA contamination was deemed to be present if there was a Ct difference of ≤ 3 between the sample and its corresponding no-RT control.

4.3.3 Determination of intra and inter-assay variation

Assessment of reproducibility is essential to the development of a new assay. Intra- and inter-assay reproducibility was investigated by measuring *HPRT* gene expression in A375 cells. Intra-assay variation was determined by measuring the Ct value of a sample in triplicate in each PCR run. Inter-assay variation was investigated in four independent runs performed on two consecutive days (Table 4.3). There was no significant difference between inter- and intra-assay Ct values demonstrating that the assay has acceptable reproducibility (Kruskall-Wallace test, $p=0.144$).

Figure 4.5: Detection of HERV-K mRNA in A375 cells by real-time qRT-PCR



Legend:

RNA was isolated from A375 cells and HERV-K mRNA expression investigated by real-time qRT-PCR. Amplification plot (generated using LightCycler® software) depicting the detection of *pol*, unspliced *env* (labelled uEnv), *rec*, *np9* and *HPRT* mRNA is shown. The results for spliced *env* are not shown on this plot. Relative concentrations of amplicons generated during the exponential phase of the reaction are determined by plotting fluorescence against cycle number on a logarithmic scale. The Ct *HPRT* is indicated by the dashed lines and arrow. The Ct values for the genes are shown to the left of the amplification plot. Negative controls (water, no-RT) were performed to demonstrate the specificity of the PCR reaction. Water was included as a negative control to exclude environmental DNA contamination.

Table 4.2: Real-time qRT-PCR analysis of HERV-K mRNA expression in cell lines

	A375	A375 NO-RT	WM2664	WM2664 NO-RT	293T	293T NO-RT
Ct *HPRT*	32.30	>50	30.61	>50	29.93	>50
Ct *pol*	35.94	>50	34.21	>50	35.17	39.67
Ct unspliced *env*	33.55	>50	32.94	>50	34.02	39.78
Ct spliced *env*	36.89	>50	>50	>50	>50	>50
Ct *rec*	37.31	>50	>50	>50	>50	>50
Ct *np9*	35.89	>50	36.48	>50	37.33	>50

RNA was extracted from A375, WM2664 and 293T cells, cDNA synthesis was performed at the same time point and HERV-K mRNA expression investigated by real-time qRT-PCR in the same PCR run. Replicates were not performed and thus the raw Ct values are indicated. No-RT controls were performed to exclude any residual gDNA contamination. The Ct values for all genes investigated are shown. A Ct >50 indicates no detectable fluorescence, i.e. no amplification. The Ct value is inversely proportional to the amount of target mRNA in the original sample. Some gDNA contamination has resulted in amplification in the 293T cell line no-RT controls for full length HERV-K mRNA (*pol and* unspliced *env*). However, the Ct values are greater than four cycles higher than Ct values from amplified cDNA.

Table 4.3: Inter and intra-assay variation: RNA extract derived from A375 cells

Ct *HPRT*	Run 1	Run 2	Run 3	Run 4
Replicate 1	28.61	29.25	29.60	28.51
Replicate 2	27.69	28.25	29.70	28.20
Replicate 3	29.59	28.77	28.98	28.28
Mean	28.63	28.76	29.43	28.51
Standard deviation	0.95	0.50	0.39	0.16

RNA was extracted from A375 cells and real-time qRT-PCR analysis of *HPRT* mRNA expression performed. The Ct *HPRT* of the sample in triplicate in four independent runs was determined. The mean Ct and standard deviation for each run is indicated (Kruskall-Wallace test, p=0.144).

4.3.4 Relative quantification of HERV-K mRNA expression

(i) Normalisation of gene expression

Real-time qRT-PCR data should be normalised against a reference gene to correct for differences in the amount of starting material, RNA integrity and reverse transcription efficiency between samples (Antonov et al., 2005; Bustin, 2002; Bustin et al., 2005). In this work, HERV-K gene expression was normalised by quantification of the spliced mRNA of *HPRT* because (a) this gene has been shown to be the best reference to standardise gene expression measurements in human tumour tissue, including melanomas (de Kok et al., 2005) and (b) *HPRT* mRNA levels are very low, approximately one to ten molecules per cell (Steen et al., 1990) thus making it suitable as an endogenous control for the quantification of low copy or rare mRNAs (Specht et al., 2001).

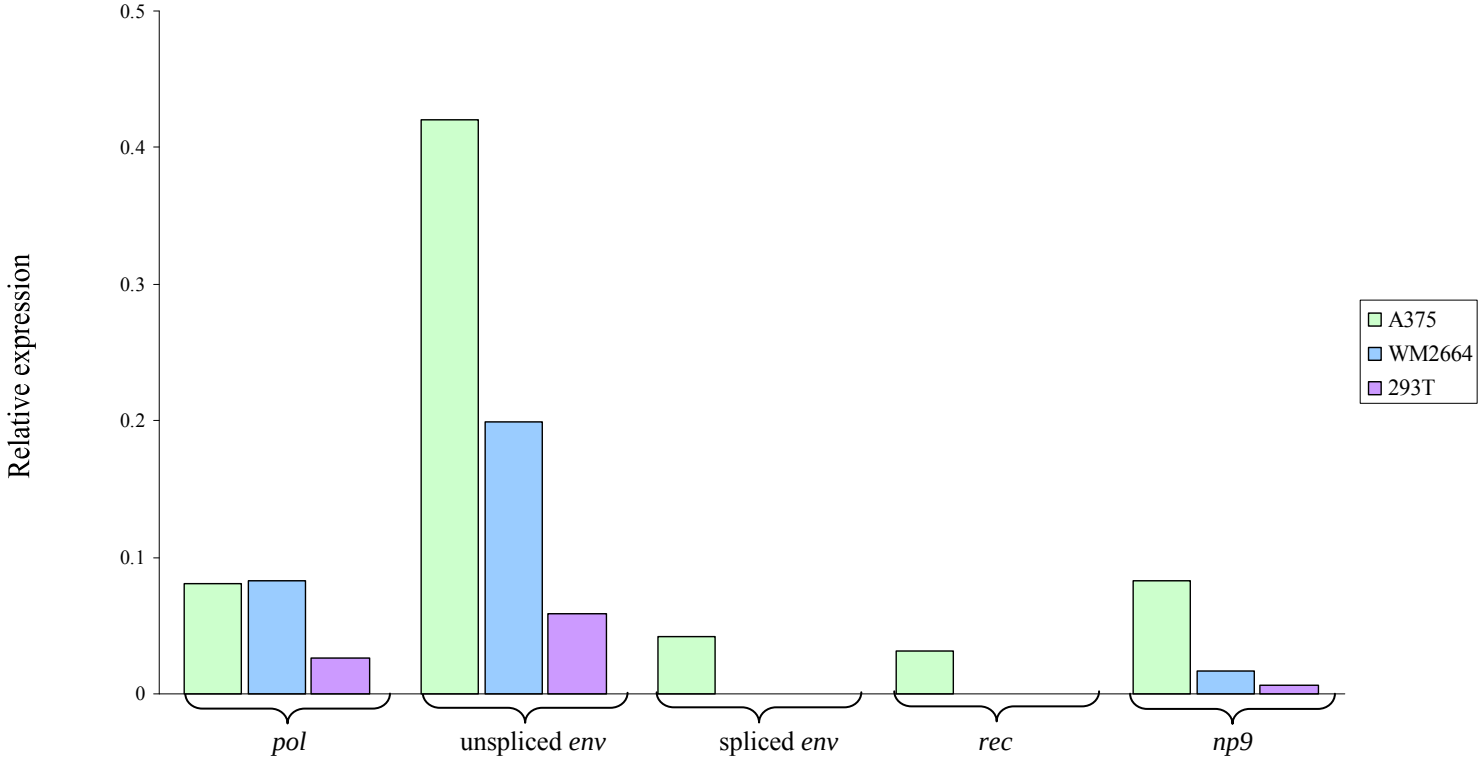
(ii) Calculation of relative gene expression

Relative target gene expression was calculated using the following formula (Livak & Schmittgen, 2001; Pfaffl, 2001):

$$2^{-\Delta Ct} \text{ where } \Delta Ct = Ct \textit{ target gene} - Ct \textit{ HPRT}.$$

Figure 4.6 illustrates the relative expression of HERV-K mRNA in the cell lines investigated. In the interests of practicalities and cost, replicate samples (to correct for intra-experiment variation) were not run, although experiments to investigate assay reproducibility showed no significant difference between inter- and intra-assay Ct values (Table 4.3). Full-length and *np9* transcripts were expressed in all three cell lines and at higher levels in the human melanoma cell lines. In all cell lines, the relative expression of *pol* was lower than that of unspliced *env*. Spliced *env* and *rec* transcripts were expressed in A375 cells only. The relative expression of all HERV-K genes was less than one, thus these genes were expressed at lower levels than *HPRT*.

Figure 4.6: Relative expression of HERV-K mRNA in cell lines



Legend:

RNA was extracted from the two human melanoma cell lines A375 and WM2664 and the embryonic kidney cell line 293T. The cDNA synthesis reaction was performed at the same time point for all three cell lines. HERV-K mRNA expression was investigated by real-time qRT-PCR in the same PCR run. Replicate samples were not run. The bars represent relative expression of the target gene (*pol*, unspliced *env*, spliced *env*, *rec*, *np9)* compared with the reference gene (*HPRT)* calculated as $2^{-\Delta Ct}$ where $-\Delta Ct = Ct_{target\ gene} - Ct_{HPRT}$.

4.4 RNA extraction from archival tissue

4.4.1 Determination of the suitability of FFPE-derived RNA extracts for investigation of HERV-K gene expression

It has been demonstrated that optical density at 260 nm (OD_{260}) readings less than 0.1 give an unreliable measure of RNA concentration (Farrell, 2005). Data from this study support this observation. All FFPE-derived RNA samples had an $OD_{260} < 0.1$, yet PCR-amplifiable RNA was acquired even from samples with an undetectable OD_{260}. In contrast, the Ct *HPRT* gives a more accurate indication of the amount of RNA in the starting material (the greater the amount of RNA in the extract, the lower the Ct value). Thus the Ct *HPRT* was used as an RNA quality control measure in this study.

An FFPE-derived RNA extract was deemed suitable for the analysis of HERV-K gene expression if *HPRT* mRNA was successfully amplified (Figure 4.7). The Ct *HPRT* was lower in the A375 sample than FFPE-derived samples. This is indicative of the greater amount of amplifiable RNA that can be extracted from cell lines compared with archival tissue.

4.4.2 Replicate end-point dilution PCR to determine sensitivity and cut-off values

It has previously been demonstrated that the input of a single copy of target DNA or cDNA will result in the generation of a specific PCR product (Saiki et al., 1988; Simmonds et al., 1990). To determine the Ct value associated with a single input copy and the level of expression of the housekeeping gene *HPRT*, dilutions of an RNA extract from an archival normal skin sample were amplified in quadruplicate.

At the end-point of the titration (often referred to as the stochastic or Poisson end-point) there is a finite probability of sampling one or zero copies of the target and hence only a proportion of reactions will generate a product (James & Patricia, 2003; Simmonds et al., 1990). At the end-point dilution the concentration of the DNA (cDNA) copies in the original sample is given as: *-Ln [F] x dilution factor/input volume*, where Ln=natural log,

F=frequency of negative reactions, input volume=volume of DNA added to the PCR reaction.

A dilution of 1 in 25 was chosen as the end-point (Table 4.4) and hence the number of copies of *HPRT* cDNA in the original sample was: -Ln [0.75] x 25/2 µl = 7 copies per 2 µl = 3.5 copies per µl. Therefore, assuming a reverse transcription efficiency of 25% (Pfaffl, 2001), the number of RNA copies in the original RNA extract was 3.5 copies per µl x 4 = 14 copies per µl.

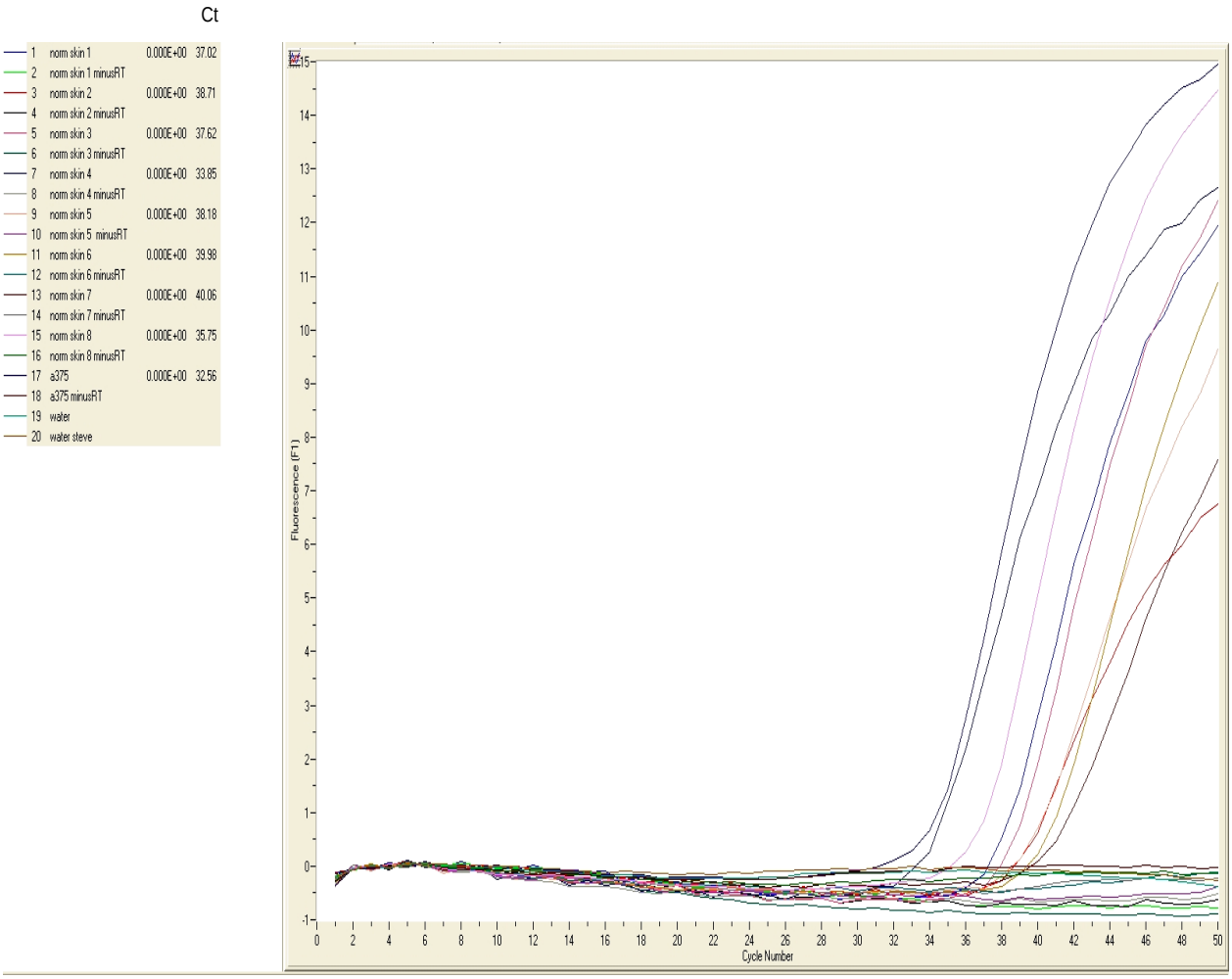
Based on these data a cut-off Ct value of 41 was determined, above which any PCR signal obtained was deemed to be non-specific.

Table 4.4: Replicate end-point dilution PCR

Dilution	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Neat	+ (36.61)	+ (37.21)	+ (36.86)	+ (36.20)
1 in 5	+ (38.62)	+ (38.16)	+ (39.91)	-
1 in 25	+ (39.72)	-	-	-
1 in 125	-	-	-	-
1 in 625	-	-	-	-
1 in 3125	-	-	-	-

Expression of *HPRT* mRNA was investigated in five- fold serial dilutions of cDNA derived from RNA extracted from a FFPE normal skin sample, four replicates per dilution. The Ct values are shown in brackets. +, *HPRT* mRNA detected; - , *HPRT* mRNA not detected (i.e. Ct >50).

Figure 4.7: Amplification of *HPRT* mRNA in archival tissue



Legend:

A real-time qRT-PCR amplification plot illustrating the detection of *HPRT* mRNA from eight archival tissue samples (normal skin 1-8). RNA was extracted from FFPE tissue blocks and real-time qRT-PCR for *HPRT* mRNA expression performed. RNA derived from A375 cells was included as a positive control. Negative controls (water, "minus RT") were performed to demonstrate the specificity of the PCR reaction. The Ct values for *HPRT* are shown to the left of the amplification plot.

Figure 4.8 summarises the approach used to determine the suitability of FFPE-derived RNA extracts for the investigation of HERV-K gene expression. RNA was extracted from 102 tissue blocks. Only 3% (3/102) of the blocks did not yield amplifiable *HPRT* mRNA, presumed due to degradation of RNA derived from those blocks.

4.4.3 The influence on RNA yield of incubation at 50°C in xylene

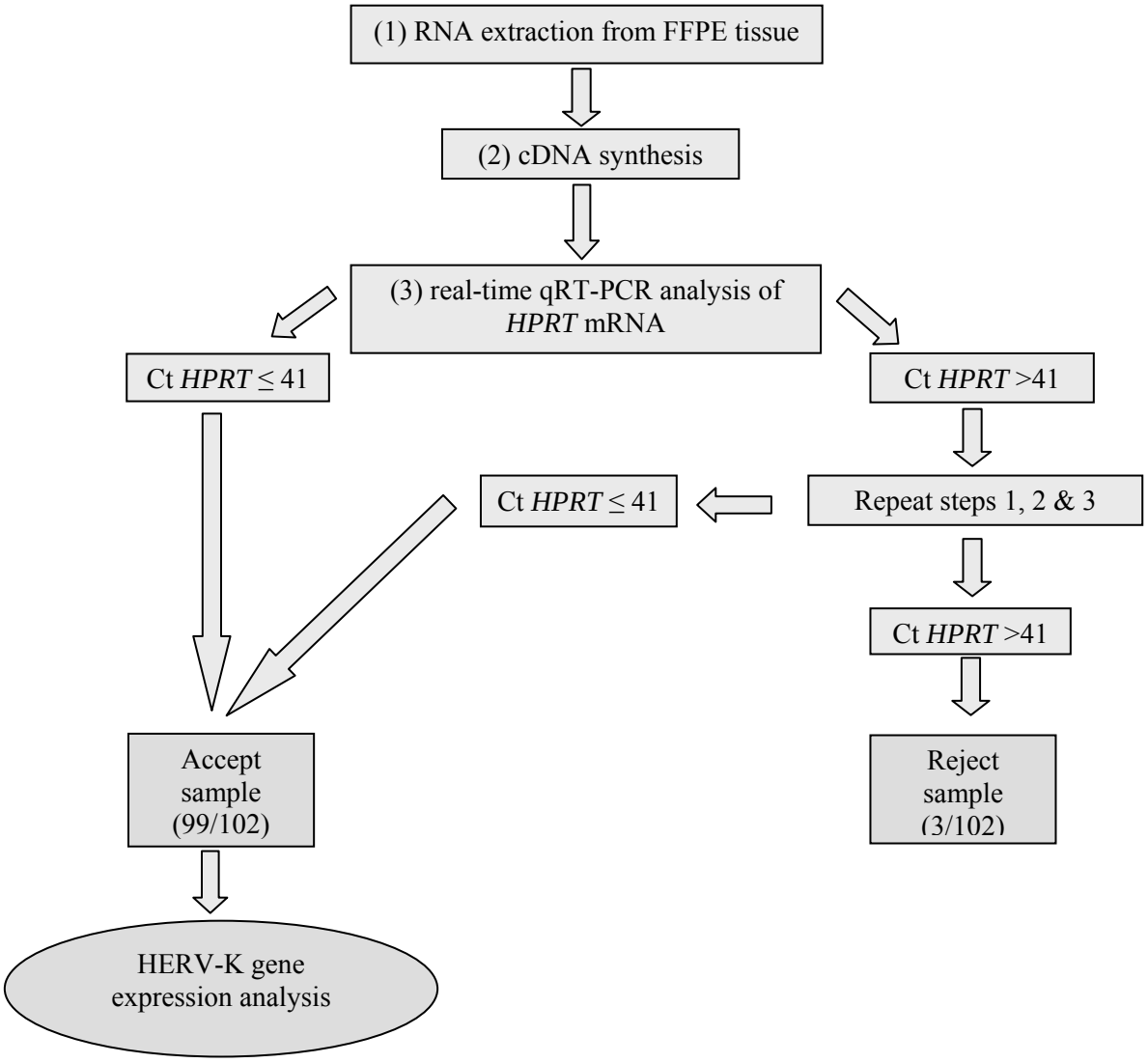
The effect of different fixation methods on RNA yields has previously been studied (Srinivasan et al., 2002; van Maldegem et al., 2008) and the use of archival tissue in this work did not allow the fixation or tissue processing protocol to be controlled. Incubation at higher temperatures during the FFPE-tissue deparaffinisation step has previously been reported to increase RNA yields (Chung et al., 2006). Thus the influence of incubation at 50°C in xylene during the pre-extraction procedure was investigated in more detail (Table 4.5). There was no significant correlation between incubation time with xylene and the Ct *HPRT* (Spearman's rho=0.234, p=0.705). Thus it was deemed unnecessary to incubate tissue sections in xylene.

Table 4.5: Incubation at 50°C in xylene during the pre-extraction procedure

Sample number	Incubation time	Ct *HPRT*
1	0 mins	37.78
2	5 mins	38.76
3	15 mins	39.30
4	20 mins	39.27
5	25 mins	37.90

Five FFPE samples, each comprising two 7 μm tissue sections of normal skin from the same block, were incubated in xylene for 0 minutes (no incubation) or at 50°C for 5, 15, 20 or 25 minutes. Extracted RNA was subjected to real-time qRT-PCR analysis of *HPRT* mRNA expression.

Figure 4.8: Algorithm to determine the suitability of FFPE tissue derived RNA extracts for HERV-K gene expression analysis



Legend:

RNA extracted from archival FFPE tissue was deemed suitable for the analysis of HERV-K gene expression if *HPRT* mRNA was successfully amplified (Ct *HPRT* ≤ 41). Ninety-nine of 102 (97%) FFPE-derived RNA extracts were suitable for analysis.

4.4.4 Assay reproducibility

Further to assay reproducibility experiments using A375-derived RNA extracts (section 4.3.3), reproducibility was also determined using RNA extracted from an archival normal skin specimen. Intra-assay variation was calculated from the Ct *HPRT* of the sample in triplicate in each run. Inter-assay variation was measured in two independent runs performed on two consecutive days. There was no significant difference between inter and intra-assay Ct values (Mann-Whitney test, p=0.1) confirming that the assay has acceptable reproducibility (Table 4.6).

Table 4.6: Inter and intra-assay variation: RNA extract derived from archival tissue

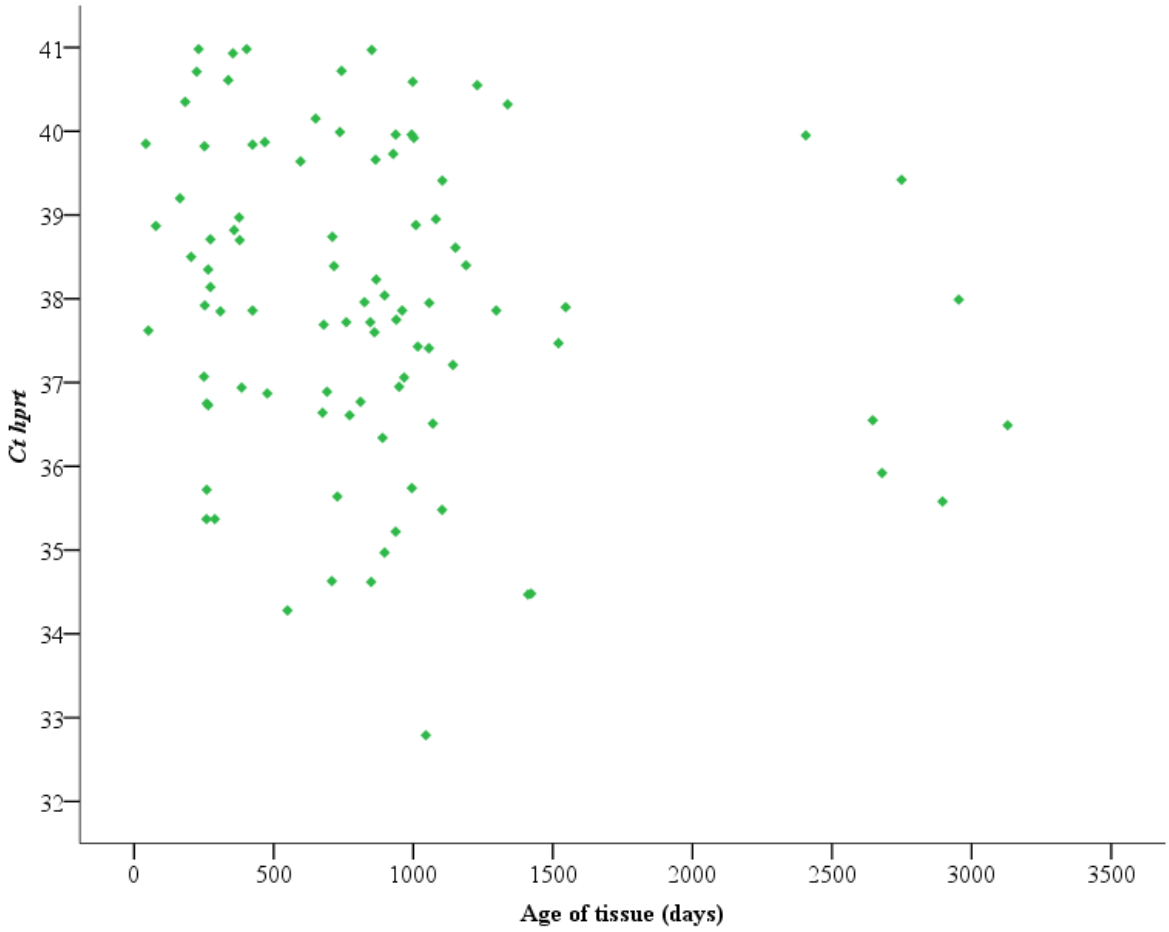
Ct *HPRT*	Run 1	Run 2
Replicate 1	33.60	33.99
Replicate 2	33.38	33.88
Replicate 3	33.53	33.77
Mean	33.50	33.88
Standard deviation	0.11	0.11

RNA was extracted from a FFPE benign naevus specimen and real-time qRT-PCR analysis of *HPRT* mRNA expression performed. The Ct *HPRT* of the sample in triplicate in two independent runs was determined. The mean Ct and standard deviation for each run is indicated (Mann-Whitney test, p=0.1).

4.4.5 The influence of age of tissue on RNA yield

It has previously been reported that the amount of amplifiable RNA in FFPE tissue decreases with length of storage of the tissue (Ribeiro-Silva et al., 2007). The FFPE tissue blocks in this study ranged in age from 42 to 3,129 days with a mean age of 866 days. No significant correlation (Spearman's rho=-0.2, p=0.06) was observed between the age of the tissue and the amount of PCR amplifiable RNA as determined by the Ct *HPRT* (Figure 4.9).

Figure 4.9: Influence of age of tissue on Ct *HPRT*



Legend:

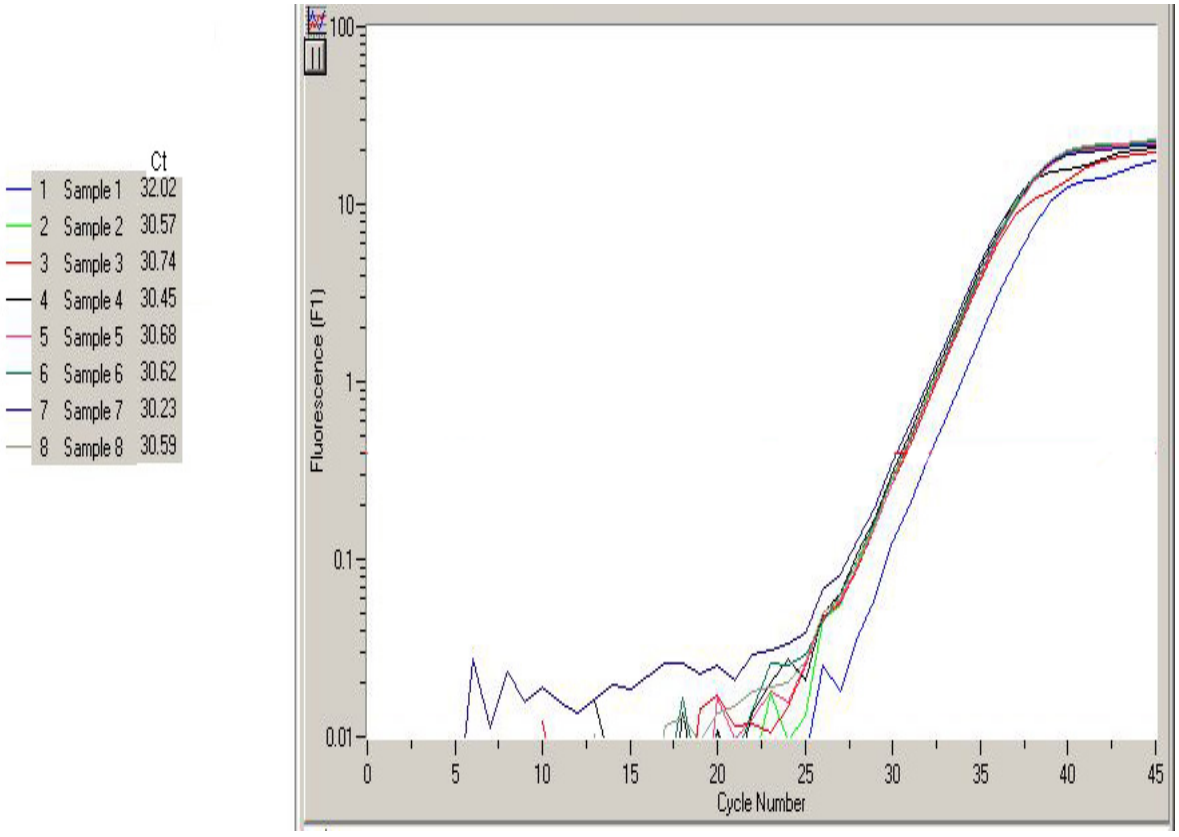
FFPE tissue blocks ranged in age from 42 to 3,129 days (n=94). In all cases, RNA was extracted from two tissue sections, 7 μm thick. The Ct *HPRT* (a measure of PCR amplifiable RNA) was determined for each sample and plotted against the age of the tissue block from which the RNA sample was derived. Spearman's rho=-0.2, p=0.06

4.5 Determination of PCR inhibition

Inhibitory elements in biological samples can reduce the sensitivity of real-time qRT-PCR. The inhibiting agents may be reagents used during nucleic acid extraction or co-purified components from the biological sample, such as bile salts, urea and haem (Wilson, 1997). Inhibitors can generate inaccurate quantitative results and give rise to false-negative results. It has previously been reported that melanin can co-purify with nucleic acids and inhibit PCR (Eckhart et al., 2000; Satyamoorthy et al., 2002).

To investigate if melanin or any other potentially inhibitory element present in the FFPE-derived RNA extracts were inhibitory to the PCR, serial dilutions of an RNA extract from an archival benign naevus specimen (a pigmented compound melanocytic naevus, three years old) were spiked into PCR reactions containing bacteriophage λ DNA and a primer pair directed against λ DNA (Aryee et al., 2005; Thornton & Passen, 2004). Amplification of λ DNA was not affected by the FFPE tissue-derived RNA extract, thus indicating no inhibition of PCR (Figure 4.10).

Figure 4.10: Determination of inhibition of real-time qRT-PCR



Legend:

RNA was extracted from a benign naevus archival specimen. Sample 1 (neat cDNA) and 10-fold serial dilutions (samples 2–8) were spiked into PCR reactions containing bacteriophage λ DNA and a primer pair directed against λ DNA. Amplification plot of bacteriophage λ DNA is shown. Ct values are indicated on the left of the plot.

4.6 Discussion

The main goal of this work was to develop a Taqman-based real-time qRT-PCR based methodology to study HERV-K gene expression in FFPE tissue because no methods specifically for the detection and quantification of HERV-K gene expression in archival tissue have previously been published.

The PCR primers were designed such that (a) they were located in the conserved regions of human-specific HERV-K sequences, (b) small amplicons (92-117 bp) were generated to circumvent the problem of RNA fragmentation and (c) amplification of gDNA was avoided where possible by amplification across splice junctions. Antonov et al. (2005) demonstrated that reliable gene expression measurements from degraded RNA by real-time qRT-PCR depend on short amplicons and correct normalisation. In this study, spliced mRNA of the housekeeping gene *HPRT* was used to normalise for the variability between samples.

HERV-K gene expression was initially investigated in the human melanoma cell lines WM2664 and A375, and the embryonic kidney cell line 293T using the novel real-time qRT-PCR assay that was developed. Full-length HERV-K mRNA and *np9* mRNA were expressed in all three cell lines investigated, but spliced *env* and *rec* mRNA were expressed in A375 cells only. Thus the suitability of RNA derived from these cells as a positive control in real-time qRT-PCR experiments to investigate HERV-K gene expression in archival tissue was confirmed. Expression of spliced *env* was not observed in A375 cells using a conventional RT-PCR methodology (section 3.2) but was detected using real-time qRT-PCR. This could be explained by the superior sensitivity of this method over conventional RT-PCR. Parallel experiments omitting RT showed that there was no significant gDNA contamination and the assay was shown to have acceptable reproducibility.

In this study, quantification of *HPRT* was used as an RNA quality control measure. Further quality control measures to check the quantity and quality of RNA could have been performed had the suitable instrumentation been available, for example the Agilent 2100

Bioanalyzer. The latter utilises a combination of microfluidics, capillary electrophoresis, and fluorimetry to determine RNA length, distribution and concentration. RNA extracted from archival FFPE tissue was deemed suitable for the analysis of HERV-K gene expression if *HPRT* mRNA was successfully amplified (Ct *HPRT* $\leq$ 41). Using this criteria, 99/102 FFPE-derived RNA samples were found to be suitable for the analysis of HERV-K mRNA expression by real-time qRT-PCR. Possible explanations for the high yields of amplifiable RNA obtained in this study are put forward in the General Discussion (Chapter 7).

In the real-time qRT-PCR assays, there was only one replicate of each sample per experiment. This was due to the high cost of Taqman probes and the difficulty in obtaining sufficient amounts of mRNA from precious archival primary melanoma tissue to study the expression of multiple genes (full-length and spliced HERV-K mRNAs in addition to *HPRT*). This practice is not uncommon, particularly when clinical samples are scarce, and is based on the assumption that the intra- and inter- experiment variation is of a small enough magnitude not to undermine the power of the assay to resolve interesting biological differences that may exist between samples. Furthermore, experiments to investigate assay reproducibility in this work showed no significant difference between inter- and intra-assay Ct values.

RNA yield, as determined by the Ct *HPRT*, was not affected by the age of the tissue although all of the tissue blocks in this work were less than eight years old. It has previously been demonstrated that less, and more fragmented, RNA is obtained from older tissue blocks. In one study RNA derived from breast cancer tissue archived for one year was less degraded than RNA archived for six and 17 years, indicating that fragmentation of RNA continues even after specimens are dehydrated and embedded in paraffin (Cronin et al., 2004). In another study, although the quality of RNA extracted from ten year old FFPE tissue was similar to RNA extracted from recently archived months old samples, the quantity, consistency and success rate of gene expression assays was higher in the months old group (Ribeiro-Silva et al., 2007). However, FFPE-derived RNA extracts were found

suitable for RT-PCR analysis if the final product of amplification was less than 151 bp, with a success rate of 100% for both ten year old and months old samples.

In this work, no PCR inhibition was observed but only RNA derived from one archival pigmented benign naevus was studied and samples with differing melanin contents were not compared. Melanin is known to inhibit PCR and the effect appears to be dose-dependent. Conway et al. (2009) visually scored the melanin content of melanoma tissue microarray cores in their study, and found that an increased melanin score was predictive of a reduced number of detected genes in an array-based assay. Potential mechanisms of PCR inhibition include binding of the inhibitor to the polymerase, interaction of the inhibitor with the DNA, and interaction with the polymerase during primer extension. In a recent study, melanin was found to inhibit the PCR reaction through sequence specific binding to DNA thus limiting the amount of available template (Opel et al., 2010). However, smaller amplicons appeared to be less inhibited presumably due to fewer binding sites. The addition of bovine serum albumin to the PCR reaction has been shown to reduce inhibition by melanin (Giambernardi et al., 1998; Opel et al., 2010).

In summary, a robust real-time qRT-PCR assay for the quantification of HERV-K gene expression in archival FFPE tissue samples has been developed. This work demonstrates that real-time qRT-PCR utilising short amplicons is a sensitive, accurate, and reproducible method for the analysis of gene expression in FFPE tissues. Archival tissue can be confidently accessed to study gene expression.

Chapter 5:
Expression of HERV-K mRNA in primary melanoma

5.0 Abstract

Introduction:

The expression of HERV-K in categorically defined clinicopathological subtypes of primary melanoma has not previously been investigated. The aims of this work were to quantify the expression of full-length (unspliced) and spliced HERV-K mRNA in archival primary melanoma and to correlate expression with subtype and Breslow thickness.

Methods:

Informed consent for the use of archival melanoma and control benign naevus FFPE specimens was obtained from patients. Control normal skin FFPE specimens were obtained from a tissue bank. Clinical and histopathological features of melanoma cases were obtained from patient records and histopathology reports. RNA was extracted from archival primary melanoma comprising MMIS (n=6), SSMM (n=12), NMM (n=7), ALMM (n=9) and LMM (n=5) sub-types, control normal skin (n=31) and benign naevus (n=16) tissues. To verify that RNA was derived from melanoma tissue, tissue sections adjacent to the sections from which RNA was extracted were stained with H&E. HERV-K mRNA expression was quantified using a novel real-time qRT-PCR.

Results:

Full-length HERV-K mRNA expression was observed at high levels in 100% tissues (*pol*: n=47 and unspliced *env*: n=40) but there were no statistically significant differences in relative expression between melanoma, benign naevus and normal skin tissues (*pol*: p=0.267 and unspliced *env*: p=0.823). There was no difference in expression between subtypes of melanoma (*pol,* p=0.046; unspliced *env,* p=0.213) or between ulcerated and non-ulcerated tumours (*pol,* p=0.1; unspliced *env,* p=0.296), nor was there any correlation with Breslow thickness (*pol*, p=0.111; unspliced *env*, p=0.421). The *np9* gene was expressed in 36% (14/39) melanoma, 25% (4/16) benign naevus and 29% (9/31) normal skin samples and there was no statistically significant difference in relative expression between the groups (p=0.964). However spliced *env* was expressed in 20.5% (8/39) melanoma, but only in 6% (1/16) benign naevus and 0% (0/31) normal skin tissues and this was statistically significant (p=0.003). Expression of *rec* was found in 23% (9/39) melanomas but not in benign naevi or normal skin and this was statistically significant (p=0.009). Expression of *rec* was not

detected in extra-lesional skin in *rec*-positive cases (n=5) but this was not statistically significant (p=0.06). The *rec*-positive melanomas were all histologically in VGP and thicker than *rec*-negative melanomas with a median Breslow thickness of 2.1mm versus 1.05mm, and this was statistically significant (p=0.009).

Conclusion: This is the first detailed study of HERV-K mRNA expression in primary melanoma. Full-length HERV-K was expressed at high levels in all tissues investigated. The expression of potentially oncogenic *rec* mRNA in some VGP primary melanoma, but not in benign naevus or normal skin tissues, has not previously been reported and merits further evaluation.

5.1 Introduction

Although expression of HERV-K mRNA has previously been demonstrated in human melanoma, studies thus far have focused on melanoma cell lines and metastasis tissue (section 1.4). This may partly be attributed to the limited availability of adequate numbers of primary melanoma tissue samples for research and corresponding clinical data. However the investigation of primary melanoma tissue, particularly comparative analyses involving normal and benign naevus tissues, is of paramount importance if molecular characteristics are to be correlated with clinical outcomes. Importantly, there has been a recent drive towards a molecular classification of primary melanoma because of molecular genetic studies demonstrating specific genotype-phenotype correlations (Fecher et al., 2007).

Two previous studies have examined HERV-K gene expression in primary melanoma (Muster et al., 2003; Schiavetti et al., 2002). However, only nine samples were studied and neither the characteristics of the patients nor the histopathological features were described. Furthermore, only *full-length* HERV-K transcripts were investigated in both studies. Muster et al. (2003) used fresh surgical touch preparations of primary melanoma tissue. Archival tissues were investigated in this work because of the lack of availability of fresh primary melanoma samples (Chapter 1, section 1.5). Thus the principal objective was to investigate full-length and spliced HERV-K gene expression in clearly defined clinicopathological subtypes of archival primary melanoma. The aims were to quantify the expression of full-length and spliced

HERV-K mRNA in primary melanoma, benign naevi and normal skin using real-time qRT-PCR (Chapter 4) and to correlate expression in primary melanoma with clinicopathological subtype and Breslow thickness.

5.2 Descriptive characteristics of study subjects

5.2.1 Clinical and histopathological characteristics

In total, 110 melanoma and 78 benign naevus subjects were recruited for potential study. It was not possible to investigate all recruited subjects due to time limitations. The details of the recruited melanoma subjects who were not included in this study are summarised in Appendix 4.

Thirty-nine archival primary melanoma (cases), 16 benign naevus (controls) and 31 normal skin samples (controls) were investigated. The clinical characteristics of the patients from whom the samples were derived and histopathological diagnoses are summarised in Table 5.1. The melanoma group was significantly older than the benign naevus and normal skin groups (Kruskall-Wallace test, p=0.025). The groups were similar in terms of gender (Pearson's chi-square test, p=0.5). It was intended to match cases and controls in terms of age and sex but this was not possible due to the small number of benign naevus and normal skin controls recruited. Normal skin samples (acquired from a tissue bank) were derived from the trunk or lower limb because they were acquired from skin removed during reduction mammoplasty, abdominoplasty or limb amputation.

Table 5.2 summarises the melanoma cases investigated. The cases were selected from the total of those recruited so that a range of subtypes, lesion sites and Breslow thicknesses were represented. The samples comprised six MMIS (15.4%), 14 SSMM (35.9%), eight NMM (20.5%), six ALMM (15.4%) and five LMM (12.8%). The median Breslow thickness of the samples was 1.2 mm (range 0 - 6.7 mm). Thirty-three of 39 (84.6%) melanomas were histologically in VGP and 11/39 (28.2%) had histological evidence of ulceration. Seven of 39 (17.9%) were associated with a dysplastic naevus histologically.

Table 5.1: Clinical and histopathological characteristics of study subjects

Characteristics	Melanoma	Benign naevus	Normal skin
Age of subjects: years			
Median	64	47.5	47
Range	24 – 88	22 – 79	19 – 84
Gender: no. subjects (%)			
Male	18 (46.2%)	7 (43.8%)	10 (32.3%)
Female	21 (53.8%)	9 (56.2%)	21 (67.7%)
Anatomical location: no. samples (%)			
Arm/shoulder	5 (12.8%)	3 (18.8%)	0
Leg	7 (17.9%)	1 (6.2%)	15 (48.4%)
Head/neck	9 (23.1%)	4 (25.0%)	0
Trunk	9 (23.1%)	8 (50%)	15 (48.4%)
Acral	5 (12.8%)	0	1 (3.2%)
Subungual	4 (10.3%)	0	0
Histological diagnosis: no. samples (%)			
Melanoma:			
MMIS	6 (15.4%)		
SSMM	14 (35.9%)		
NMM	8 (20.5%)		
ALMM	6 (15.4%)		
LMM	5 (12.8%)		
Benign naevus:			
junctional		3 (13.3%)	
intradermal		7 (46.7%)	
compound		6 (40%)	
Normal skin			31 (100%)
Total number of samples	**39**	**16**	**31**

MMIS, melanoma *in situ*; SSMM, superficial spreading melanoma; NMM, nodular melanoma; ALMM, acral lentiginous melanoma; LMM, lentigo maligna melanoma

Table 5.2: Summary of melanoma cases

Code	Sex	Age[i] (years)	Melanoma site	Subtype[ii]	Associated dysplastic naevus	Breslow (mm)	Growth phase[iii]	Ulceration	History other cancer[iv]	Status[v]
MM1	M	71	head/neck	LMM	no	0.9	VGP	no	no	Local recurrence (3y)
MM2	M	71	trunk	SSMM	no	0.5	VGP	no	no	Disease free (3y)
MM3	F	32	leg	NMM	no	1.1	VGP	no	no	Disease free (4y)
MM4	F	26	leg	SSMM	yes	0.4	VGP	no	no	Disease free (5y)
MM5	F	34	subungual	NMM	no	1.3	VGP	yes	no	Unknown
MM6	F	36	arm	SSMM	no	0.5	VGP	no	no	Disease free (3y)
MM8	F	45	leg	MMIS	no	0	IS	no	no	Disease free (2y)
MM9	F	24	leg	NMM	no	1.2	VGP	no	no	Disease free (2y)
MM10	M	77	leg	SSMM	no	2.3	VGP	yes	no	Disease free (4y)
MM11	M	52	trunk	SSMM	no	1	VGP	no	no	Unknown
MM12	F	48	trunk	SSMM	yes	0.7	VGP	no	no	Disease free (3y)
MM13	M	39	trunk	SSMM	no	1.2	VGP	no	Hodgkin's (B)	Disease free (4y)
MM14	F	56	head/neck	NMM	no	3	VGP	yes	no	Unknown
MM15	M	37	trunk	SSMM	no	1.6	VGP	no	no	Disease free (2y)
MM16	M	51	trunk	MMIS	no	0	IS	no	no	Disease free (2y)
MM17	F	54	head/neck	SSMM	no	1.2	VGP	no	no	Disease free (5y)
MM18	F	83	acral	SSMM	yes	1.5	VGP	no	breast (B)	Local recurrence (2y), disease free (4y)
MM19	M	71	leg	SSMM	yes	1.6	VGP	no	no	Disease free (4y)
MM20	M	72	acral	ALMM	no	6.7	VGP	yes	no	Unknown
MM21	M	68	arm	NMM	no	2.5	VGP	no	no	Nodal disease (1y), Deceased

Code	Sex	Age[i] (years)	Melanoma site	Subtype [ii]	Associated dysplastic naevus	Breslow (mm)	Growth phase[iii]	Ulceration	History of other cancers[iv]	Status[v]
MM22	F	88	head/neck	LMM	no	2.3	VGP	no	no	Unknown
MM23	M	58	arm	SSMM	yes	1.8	VGP	no	no	Disease free (2y)
MM24	F	80	arm	MMIS	no	0	IS	no	MMIS (A) bowel (B)	Disease free (3y)
MM25	F	88	head/neck	LMM	no	0.5	VGP	no	no	Unknown
MM26	M	70	head/neck	LMM	no	0.5	VGP	no	MM (B)	Disease free (4y)
MM27	F	79	acral	SSMM	yes	1.4	VGP	no	no	Disease free (4y)
MM28	M	85	trunk	NMM	no	6.3	VGP	yes	no	Unknown
MM29	F	69	subungual	ALMM	no	6	VGP	yes	no	Nodal disease (1y), Disease free (4y)
MM30	F	84	head/neck	LMM	no	0.3	VGP	no	no	Unknown
MM31	F	83	head/neck	MMIS	no	0	IS	no	no	Disease free (5y)
MM32	M	63	subungual	ALMM	no	2.2	VGP	no	prostate (A)	Disease free (5y)
MM33	F	44	trunk	MMIS	no	0	IS	no	no	Disease free (5y)
MM34	M	81	acral	ALMM	no	1.4	VGP	yes	no	Unknown
MM35	M	66	head/neck	MMIS	no	0	IS	no	no	Local recurrence (3y)
MM36	F	64	leg	SSMM	no	1.7	VGP	yes	no	Disease free (4y)
MM37	F	59	arm	NMM	no	1.3	VGP	yes	no	Unknown
MM38	M	84	subungual	ALMM	no	0.7	VGP	no	no	Unknown
MM39	F	62	acral	ALMM	no	3.6	VGP	yes	no	Disease free (5y)
MM40	M	55	trunk	NMM	yes	2.1	VGP	yes	no	Unknown

[i] Age at diagnosis

[ii] LMM, lentigo maligna melanoma; SSMM, superficial spreading melanoma; NMM, nodular melanoma; ALMM, acral lentiginous melanoma; MMIS, melanoma *in situ*

[iii] VGP, vertical growth phase; IS, *in situ*

[iv] History of cancers including other melanomas. B, occurring before and; A, occurring after development of melanoma investigated in this study.

[v] Disease status at number of years (y) post excision of primary melanoma. Unknown – patient's care transferred to another Trust or patient lost to follow-up.

5.2.2 Verification of melanoma diagnosis in samples

Tissue sections taken from either side of the two sections from which RNA was extracted showed the presence of representative melanoma tissue in all cases, thus confirming that RNA had been extracted from tumour tissue. Figure 5.1 shows a selection of the H&E stained tissue sections. The histological subtype, Breslow thickness and tumour growth phase in these sections were found to be in concordance with the original histopathology sections and reports with the exception of two cases. When the original and newly sectioned H&E stained slides were reviewed, case MM31, originally reported as a SSMM, was rediagnosed as a MMIS (lentigo maligna type). Case MM39, originally reported as 2.8 mm Breslow thickness, was restaged to be 3.6 mm thick.

Figure 5.1 : Histopathological verification of primary melanoma cases

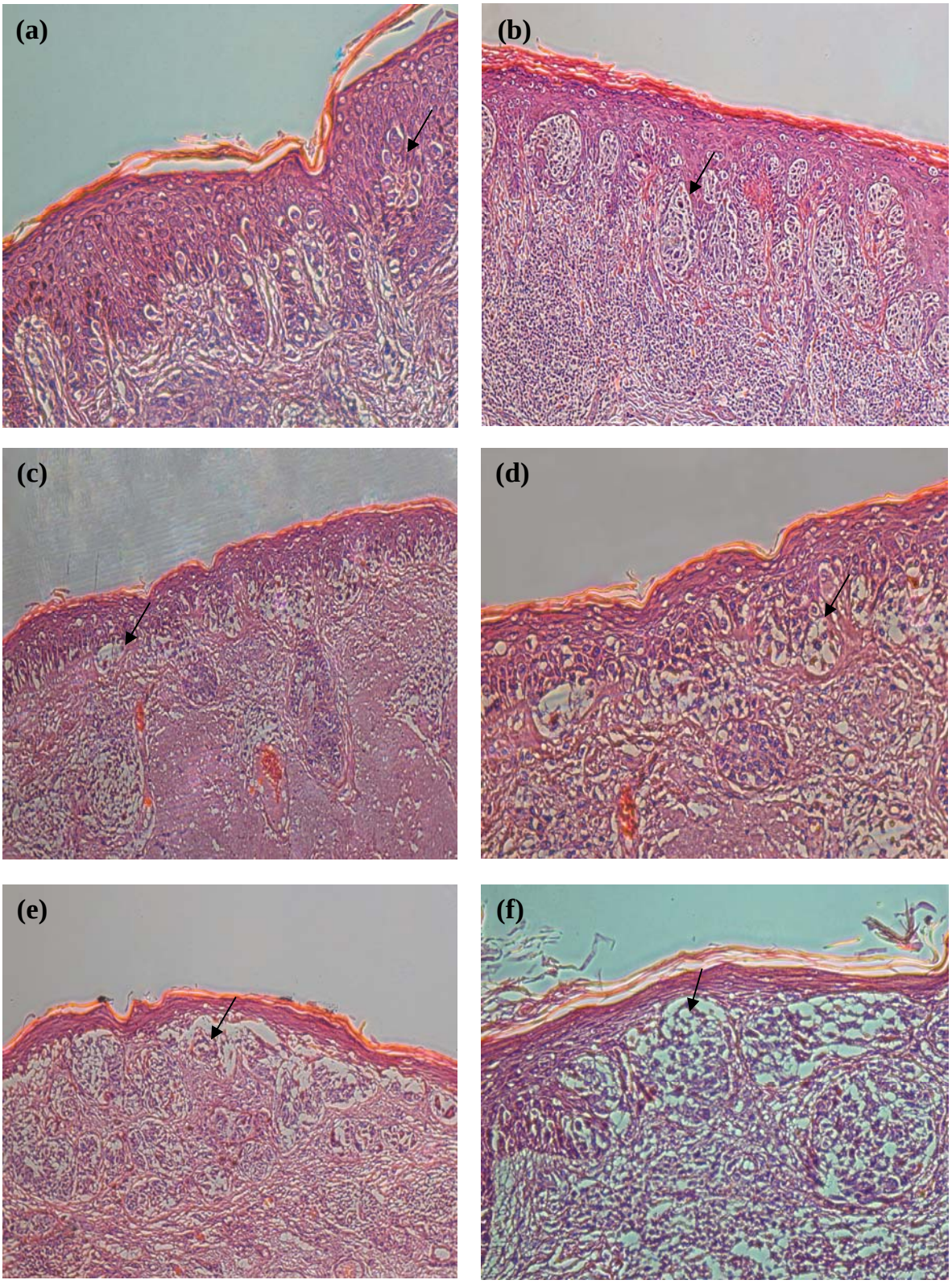


Figure 5.1: Legend

To verify that RNA had been extracted from tumour tissue and to confirm the histological subtype of melanoma and growth phase, sections were taken from either side of the two sections from which RNA was extracted and stained with H&E according to routine laboratory processing. A selection of the H&E stained tissue sections is shown. The histopathological diagnoses with the original magnifications in parentheses are as follows: (a) MMIS (x 20), case MM8; (b) SSMM (x 10), case MM10; (c) LMM (x 10), case MM1; (d) LMM (x 20), case MM1; (e) SSMM (x 10), case MM2; (f) SSMM (x20), case MM2. The arrows indicate examples of nests of atypical melanocytes. Dermal invasion (VGP) is evident in images (b) to (f).

5.3 Full-length HERV-K mRNA expression

5.3.1 Expression of *pol* mRNA

Full-length HERV-K mRNA expression was initially evaluated by quantification of the *pol* gene in 21 melanoma, 16 benign naevus and 14 normal skin samples. Expression of *gag* was not investigated in clinical tissues because *gag* and *pol* expression are analogous (both are a measure of full-length HERV-K expression so it was unnecessary to expend time and reagents to investigate expression of both). Two melanoma and two normal skin samples were excluded from the analysis because of evidence of significant residual gDNA contamination (defined as a Ct difference of ≤3 between the sample and its corresponding no-RT control).

The *pol* gene was highly expressed in 100% tissues, and at levels greater than the housekeeping gene *HPRT* (Figure 5.2). There was no significant difference in expression between the melanoma (median relative expression=7.8, n=19), benign naevus (median relative expression=10.7, n=16) and normal skin (median relative expression=11.4, n=12) groups (Kruskall-Wallace test, p=0.267). Extremely high expression levels, approximately 20 to120-fold greater than *HPRT*, were observed in two melanoma and two normal skin samples (extreme and outlying sample values shown in Figure 5.2).

5.3.2 Expression of unspliced *env* mRNA

Subsequently, unspliced *env* mRNA expression (also a measure of full-length HERV-K mRNA expression) was quantified in 18 melanoma, 16 benign naevus and eight normal skin samples. One melanoma and one normal skin sample were excluded from the analysis because of significant residual gDNA contamination.

High levels of unspliced *env* expression were observed in 100% tissues (Figure 5.2) and, analogous to *pol*, there were no statistically significant differences in expression between melanoma (median relative expression=17.1, n=17), benign naevus (median relative expression=17.4, n=16) and normal skin (median relative expression=19.8, n=7) samples (Kruskall-Wallace test, p=0.823). Extremely high expression levels, approximately 70 to

140-fold greater than *HPRT*, were observed in one melanoma, one benign naevus and one normal skin sample.

Data for both *pol* and unspliced *env* expression in the same sample were available for 15 melanoma, 16 benign naevus and 7 normal skin samples. Unspliced *env* mRNA was expressed at significantly higher levels (about 10-fold greater) than *pol* in melanoma samples (Wilcoxon matched pairs test, p=0.001), but there were no statistically significant differences between the expression of unspliced *env* and *pol* in benign naevus (p=0.105) and normal skin samples (p=0.469).

5.3.3 Expression of full-length HERV-K mRNA in lesional versus extra-lesional skin

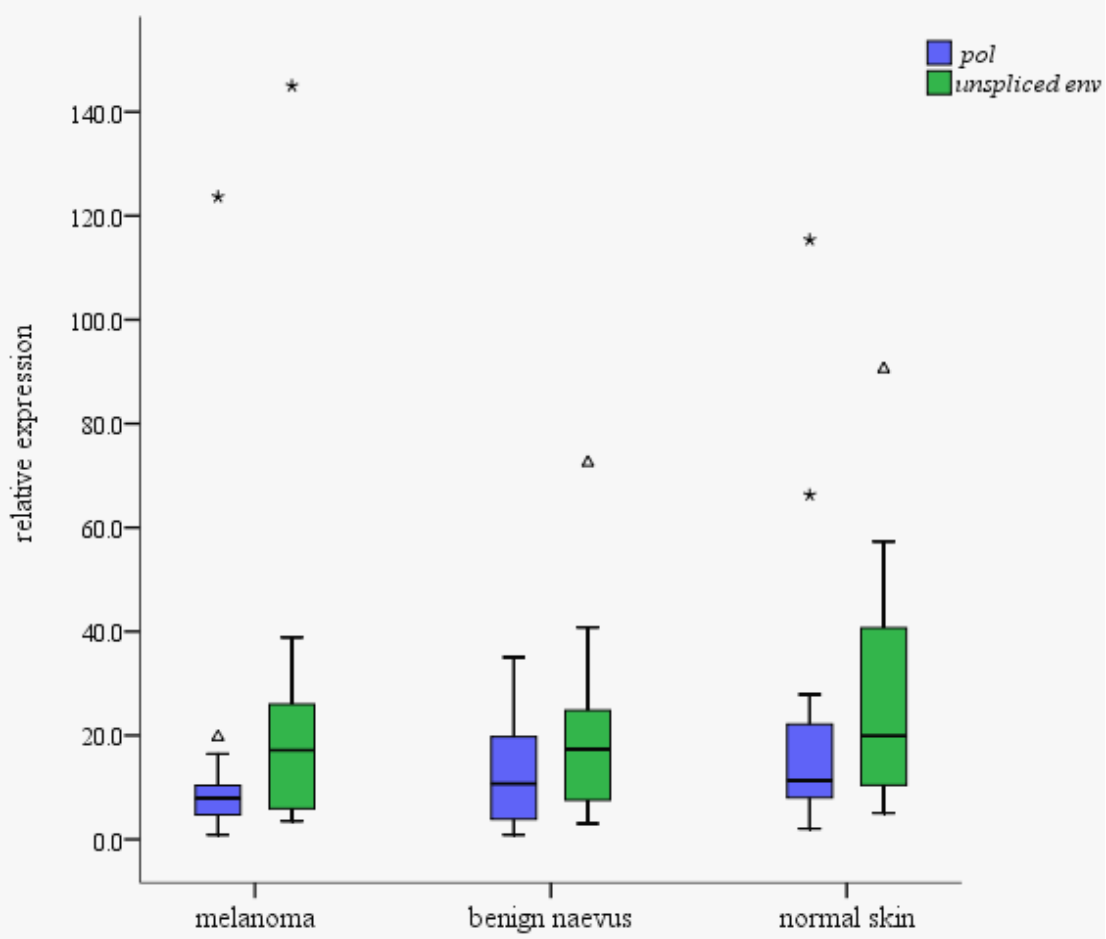
Eight matched extra-lesional (un-involved normal skin) samples were investigated from among the melanoma (n=5) and benign naevus (n=3) samples. Both *pol* and unspliced *env* were expressed in extra-lesional skin but there were no statistically significant differences in the level of expression between lesional and extra-lesional skin (Wilcoxon matched pairs test: *pol*, p=0.156; unspliced *env,* p=0.383).

5.3.4 Expression of full-length HERV-K mRNA: clinical and histopathological correlates

A statistically significant correlation was not found between full-length HERV-K mRNA expression and age of the patient (Spearman's rho: *pol* p=0.560; unspliced *env,* p= 0.1) and there were no statistically significant differences in expression between males and females (Mann-Whitney test: *pol,* p=0.418; unspliced *env*, p=0.766) or lesion sites (Kruskall-Wallace test: *pol,* p=0.930; unspliced *env,* p=0.555).

Regarding the melanoma group, there were no statistically significant differences in full-length HERV-K mRNA expression between the subtypes (Kruskall-Wallace test: *pol,* p=0.05; unspliced *env,* p=0.213), nor between ulcerated and non-ulcerated tumours (Mann-Whitney test: *pol,* p=0.1; unspliced *env,* p=0.296). There were no statistically significant correlations between the level of expression and Breslow thickness of the tumour (*pol*, n=19, Spearman's rho, p=0.111; unspliced *env*, n=17, Spearman's rho, p=0.421).

Figure 5.2: Full-length HERV-K mRNA expression in melanoma, benign naevus and normal skin samples



Legend:

RNA was extracted from archival melanoma, benign naevus and normal skin tissue samples and full-length HERV-K mRNA expression quantified by real-time qRT-PCR. Relative gene expression was calculated as $2^{-\Delta Ct}$ where ΔCt = Ct *target gene* - Ct *HPRT*. The box plots show the relative expression of full-length (*pol* and unspliced *env*) HERV-K mRNA. The box plot markings represent median, 25–75th percentile and the range of values. Extreme sample values are denoted by asterisks and outlying sample values by triangles.

TT5.4 Spliced HERV-K mRNA expression

Spliced HERV-K mRNA (spliced *env*, *rec* and *np9*) expression was subsequently studied in the tissues already investigated for full-length mRNA expression and in additional melanoma and normal skin samples (Table 5.3).

5.4.1 Expression of spliced *env* mRNA

Spliced *env* mRNA was expressed in 8/39 (20.5%) melanoma but only 1/16 (6.3%) benign naevus (at very low levels) and 0/31 normal skin samples and this was statistically significant (Pearson's chi-square test, p=0.003). The median relative expression in the melanoma samples was approximately three-fold higher than *HPRT* mRNA (median relative expression=3.4).

5.4.2 Expression of *rec* mRNA

Expression of *rec* mRNA was found in 9/39 (23.1%) melanoma but not in benign naevus or normal skin samples and this was statistically significant (Pearson's chi-square test, p=0.009). Extra-lesional skin in *rec*-positive melanoma samples (n=5) did not show expression of *rec*. This was not statistically significant (McNemar's test, p=0.06), although the number of samples analysed was small. Expression levels of *rec* were lower than that of *HPRT* (median relative expression=0.3) with the exception of samples MM23 and MM32 (three to four-fold higher) and MM40 (45-fold higher).

Co-expression of spliced *env* and *rec* mRNA was observed in seven samples. There was a strong positive correlation between spliced *env* and *rec* expression (Spearman's rho=1, p=0.001).

The *rec*-positive cases are summarised in Table 5.4. The tumours were all in VGP and were statistically significantly thicker than *rec*-negative tumours with a median Breslow thickness of 2.1 mm compared with 1.05 mm (Mann-Whitney test, p=0.009). Five of 11 *rec*-positive tumours were ulcerated compared with 6/28 *rec*-negative tumours but this was not statistically significant (Pearson's chi-square test, p=0.082).

Table 5.3: Spliced HERV-K mRNA expression in primary melanoma, benign naevus and normal skin samples

	Melanoma	Benign naevus	Normal skin	p value*
Total no. of samples	39	16	31	
Spliced *env* mRNA (% samples expressed)	**8 (20.5%)**†	1 (6.3%)	0	p=0.003
Median relative expression‡ spliced *env* mRNA	3.4 (0.003- 47.8)	0.1	N/A	
***rec* mRNA** (% samples expressed)	**9 (23.1%)**†	0	0	p=0.009
Median relative expression‡ *rec* mRNA	0.3 (0.07 – 44.6)	N/A	N/A	
***np9* mRNA** (% samples expressed)	14 (35.9%)	4 (25.0%)	9 (29.0%)	p=0.964
Median relative expression‡ *np9* mRNA	0.5 (0.06 – 2.0)	0.4 (0.2– 1.4)	0.3 (0.09 – 2.1)	

RNA was extracted from archival melanoma, benign naevus and normal skin tissue samples and spliced (*env*, *rec* and *np9*) HERV-K mRNA expression quantified by real-time qRT-PCR. The median relative expression (and range) is shown.

* Pearson's chi-square test
† Co-expression of spliced *env* and *rec* mRNA was observed in seven samples
‡ Relative gene expression was calculated as $2^{-\Delta Ct}$ where ΔCt = Ct *target gene* - Ct *HPRT*

Table 5.4: *rec*-positive melanoma cases

Code	Sex	Age[i] (years)	Melanoma site	Subtype[ii]	Associated dysplastic naevus	Breslow (mm)	Growth phase[iii]	Ulceration	History other cancer[iv]	Status[v]	Relative expression *rec*
MM5	F	34	subungual	NMM	no	1.3	VGP	yes	no	Unknown	0.61
MM10	M	77	leg	SSMM	no	2.3	VGP	yes	no	Disease free (4y)	0.07
MM19	M	71	leg	SSMM	yes	1.6	VGP	no	no	Disease free (4y)	0.14
MM20	M	72	acral	ALMM	no	6.7	VGP	yes	no	Unknown	0.11[vi]
MM23	M	58	arm	SSMM	yes	1.8	VGP	no	no	Disease free (2y)	44.63[vi]
MM25	F	88	head/neck	LMM	no	0.5	VGP	no	no	Unknown	0.26[vi]
MM29	F	69	subungual	ALMM	no	6	VGP	yes	no	Nodal disease (1y) Disease free (4y)	0.11[vi]
MM32	M	63	subungual	ALMM	no	2.2	VGP	no	prostate (A)	Disease free (5y)	3.78[vi]
MM40	M	55	trunk	NMM	yes	2.1	VGP	yes	no	Unknown	3.05

[i] Age at diagnosis
[ii] LMM, lentigo maligna melanoma; SSMM, superficial spreading melanoma; NMM, nodular melanoma; ALMM, acral lentiginous melanoma
[iii] VGP, vertical growth phase
[iv] History of cancers including other melanomas. (A), occurring after development of the melanoma investigated in this study.
[v] Disease status at number of years (y) post- excision of primary melanoma.
[vi] Co-expression of spliced *env*, *rec* and *np9* detected in sample

5.4.3 Expression of *np9* mRNA

Expression of *np9* mRNA was detected in 14/39 (35.9%) melanoma, 4/16 (25.0%) benign naevus and 9/31 (29.0%) normal skin samples (Table 5.3). There were no statistically significant differences between melanoma (median relative expression=0.5), benign naevus (median relative expression=0.4) and normal skin (median relative expression=0.3) samples (Pearson's chi-square test, p=0.964). Median expression levels in all three groups were lower than those of *HPRT* mRNA (i.e. <1) and full-length HERV-K transcripts.

Co-expression of spliced *env*, *rec* and *np9* mRNA was observed in five melanoma samples, including 3/6 (50%) ALMM investigated (Table 5.4).

5.5 Discussion

In this work, HERV-K gene expression was investigated in clearly defined clinicopathological subtypes of archival primary melanoma using a novel real-time qRT-PCR assay. The expression of full-length and spliced HERV-K mRNA in primary melanoma, normal skin and benign naevus samples was compared.

(i) Full-length HERV-K mRNA expression

Full-length HERV-K mRNA expression was observed at high levels in all tissue types and there were no statistically significant differences in relative expression between melanoma, normal skin and benign naevi.

No statistically significant differences in expression were observed between males and females and there was no correlation with age of the patient. Some studies have suggested that gender might influence HERV-K activity, for example expression of HERV-K *gag* was found to be significantly higher in females with rheumatoid arthritis compared with males (Freimanis, 2010) and it has been postulated that HERV-K activity is influenced by sex hormones such as oestrodiol (Nelson et al., 2004; Wang-Johanning et al., 2003).

It could be surmised that HERV-K expression was not influenced by the of degree sun exposure because there were no statistically significant differences in expression between lesion sites. However, histological evidence of chronic sun-exposure was not sought in this study. Curtin et al. have previously defined the presence of solar elastosis of the dermis surrounding the melanomas as indicative of chronic sun-induced damage. In nearly all cases, melanomas associated with chronic sun-induced damage occurred on the face and those that were not associated with chronic sun-induced damage occurred on the trunk, arms and legs (Curtin et al., 2005).

In contrast to the findings in this investigation, increased transcriptional activity of full-length HERV-K mRNA has been reported in patients with diseases, such as breast cancer and rheumatoid arthritis, compared with healthy controls. For example, the expression of HERV-K *env* transcripts was significantly higher in most breast cancer tissues than in normal breast tissues (Wang-Johanning et al., 2003). However, Haupt et al. (2011) recently compared HERV-K transcription in renal cell cancer with normal kidney tissue and found that there was no specific up or down regulation of HERV-K in the cancer tissue, analogous to the findings in this work. This suggests that there are tumour type-specific differences in the transcriptional regulation of HERV-K.

There was no difference in the level of expression of full-length HERV-K mRNA between subtypes of melanoma or between ulcerated and non-ulcerated tumours, and there was no correlation between expression levels and Breslow thickness. Although unspliced *env* mRNA was expressed at statistically significantly higher levels than *pol* in melanoma samples ($p=0.001$) there were no statistically significant differences in unspliced *env* expression between melanomas, benign naevi and normal skin. Thus the importance of this observation is difficult to interpret.

The finding of ubiquitous and similar levels of expression of full-length HERV-K mRNA in melanoma, benign naevi and normal skin is in contrast to previously published work on HERV-K expression in melanoma. Muster et al. (2003) observed high copy numbers of the HERV-K108 *pol* sequence in the tumour cells of primary melanomas (n=9), lymph node

and cutaneous metastases by *in situ* hybridization (ISH). However, benign naevus tissue samples from healthy individuals were negative. There are several possible explanations for this discrepancy. Firstly, real-time Taqman-based qRT-PCR is much more sensitive and specific than ISH. The former can detect even a few copies of a transcript and is a true objective quantitative method, particularly when relative quantification is used, whereas ISH is a semi-quantitative test. Secondly, Muster et al. used a probe specific for HERV-K108 *pol* in contrast to this work where the primers were designed to amplify all human-specific HERV-K HML-2 *pol* transcripts. Finally, these workers did not describe the clinical or histopathological characteristics of the study subjects and thus discrepancies due to differences in phenotypes cannot be excluded.

A number of recent studies have, however, demonstrated full-length HERV-K mRNA expression in many normal human tissues analogous to the findings in this work (Flockerzi et al., 2008; Muradrasoli et al., 2006; Seifarth et al., 2005). Furthermore, a defective HERV-K HML-6 provirus (designated the *HERV-K-MEL* gene) was found to be expressed in a proportion of tissues, namely 6/9 primary melanomas samples, 11/13 benign naevi and 3/5 normal skin samples (Schiavetti et al., 2002). The potential roles that HERV-K proviruses might play in normal skin physiology are discussed in Chapter 7.

(ii) Spliced HERV-K mRNA expression

The expression of *np9* mRNA is unlikely to be important in melanoma because it was expressed at similar and low levels in a proportion of all tissue types. Buscher et al. (2006) observed *np9* expression in 29% of metastatic melanoma samples and 21% of melanoma cell lines using RT-PCR but these workers did not examine normal tissue. The potential significance of *np9* expression in skin is discussed in Chapter 7.

In contrast, expression of *rec* mRNA was observed in 9/39 primary melanoma but not in benign naevus or normal skin samples and this was statistically significant ($p=0.009$). Buscher et al. reported the expression of *rec* in 45% of metastatic melanoma samples in one study and in 39% of samples in a subsequent study (Buscher et al., 2006; Buscher et al., 2005). Thus, this is the first study to demonstrate the selective expression of *rec* in primary

melanoma. Moreover, the *rec*-positive melanomas were all in VGP and were statistically significantly thicker than *rec*-negative tumours with a median Breslow thickness of 2.1 mm versus 1.05 mm ($p = 0.009$). The biological significance of these findings is discussed in Chapter 7.

Co-expression of spliced *env* and *rec* mRNA was observed in seven samples and there was a strong positive correlation between expression of these genes ($p=0.001$). This is not an unexpected finding since *rec* mRNA is derived from spliced *env* mRNA by a second splicing event (Figure 1.8). In two melanoma samples, *rec* was expressed in the absence of spliced *env* possibly because *env* transcripts were efficiently spliced down to *rec* in these samples. Co-expression of spliced *env*, *rec* and *np9* mRNA was observed in 3/6 (50%) ALMM investigated in this work but the significance of this finding is unclear because of the small numbers of each melanoma subtype studied. However, this suggests that within melanoma tissue, there are populations of cells that have complex splicing patterns resulting in expression of both types 1 and 2 HERV-K transcripts (Figure 1.8). Buscher et al (2006) also observed co-expression of spliced *env* and *rec* mRNA in the metastatic melanoma samples they studied by RT-PCR. Analogous to this study, Wang-Johanning et al showed that HERV-K spliced *env* is selectively expressed in breast cancer but not normal breast tissue (Wang-Johanning et al., 2003).

(iii) Limitations

In this study, sections were processed whole for RNA extraction and were not microdissected. This did not allow confirmation of the tumour content, so there was a possibility of sampling normal tissue. In addition to neoplastic cells, tumours are composed of a variable component of non-neoplastic stromal and inflammatory cells. The effects of tumour heterogeneity can be limited by analyzing several different areas of a tumour and by selecting tissue samples under microscopic control. VGP melanomas, especially thicker ones, are likely to be much larger and more cellular than naevi thus making comparisons between them potentially unreliable. The use of laser microdissection might have addressed this issue although this approach would not only have been

technically difficult with FFPE tissue, but also extremely time consuming. Alternative approaches are discussed in Chapter 7.

(iv) Summary

This is the first detailed study of HERV-K mRNA expression in primary melanoma. In summary, full-length HERV-K transcriptional activity was not increased in melanoma compared with benign naevi and normal skin controls. A proportion of all tissue types expressed *np9*. However expression of potentially oncogenic *rec* mRNA was observed in some VGP primary melanoma, but not in benign naevus or normal skin tissues, and *rec*-positive melanomas were significantly thicker than *rec*-negative tumours. It was not possible to determine whether *rec* expression was associated with an adverse disease outcome (relapse) because of the small number of samples studied and insufficient follow-up data. The biological significance of full-length and spliced HERV-K expression is discussed in Chapter 7.

Chapter 6:
Characterisation of expressed HERV-K sequences

6.0 Abstract

Introduction:

Expressed HERV-K sequences in primary melanoma have not previously been characterised. The principal aim of this investigation was to characterise expressed HERV-K sequences derived from the A375 human melanoma cell line, primary melanoma and normal skin tissues to ascertain if expression of particular sequences is associated with malignancy.

Methods:

Expressed HERV-K unspliced *env* sequences derived from A375 cells, primary melanoma (n=4) and normal skin samples (n=4) were cloned. Individual clones were sequenced and aligned with published HERV-K sequences.

Results:

Fourteen distinct HERV-K *env* sequences were expressed in A375 cells, with 96 to 100% homologies to published human-specific HERV-K *env* sequences. Eight HERV-K loci, comprising four type 1 and four type 2 proviruses, were expressed in both melanoma and normal skin tissues. Expression of more than one locus was found in three of four normal skin and two of four melanoma samples. None of the loci or provirus types was expressed at a higher frequency in melanoma. The four base deletion previously observed in A375 derived sequences was seen in a number of sequences derived from both melanoma and normal skin samples and was thus not unique to melanoma. Homologies to published human genome HERV-K sequences ranged from 99 to 100%. A number of nucleotide substitutions were found in both melanoma and normal skin derived sequences but these were deemed to be of uncertain biological significance.

Conclusions:

Multiple HERV-K loci were found to be transcribed in melanoma and normal skin, even in samples derived from the same individual. Expression of a particular HERV-K locus, provirus type or mutation was not associated with melanoma in this study although the number of samples analysed was small. Expression of novel HERV-K elements (additional to those identified as present within the human genome) was not found.

6.1 Introduction

A limited number of RVLP-associated sequences derived from melanoma cell line supernatants have been described (Buscher et al., 2005; Hirschl et al., 2007; Muster et al., 2003) but expressed HERV-K sequences in primary melanoma have not previously been characterised. In this work, full-length HERV-K mRNA expression was observed at high levels in all melanoma, benign naevus and normal skin samples investigated (section 5.3). Expression of *rec* mRNA was found in melanoma tissue only (9/39 samples). Sequencing of a variable region of *env* mRNA derived from A375 cells suggested expression of a mixture of mRNA species with or without a four base deletion (section 3.4). Together, these findings prompted further investigation of expressed HERV-K sequences in A375 cells and melanoma tissues.

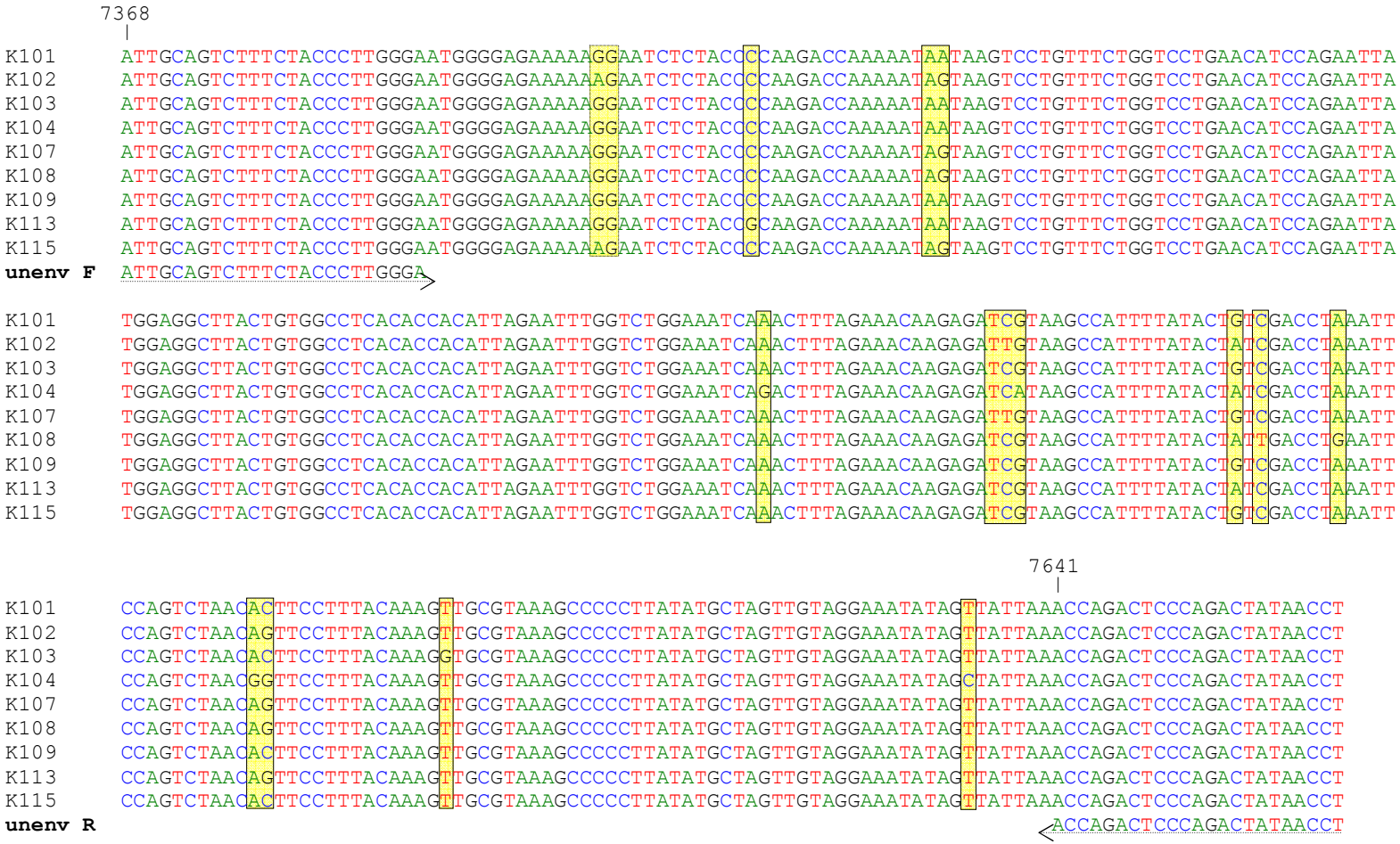
The aims were to: investigate expressed HERV-K sequences derived from A375 cells in further detail; characterise and compare expressed HERV-K sequences in melanoma and normal skin samples to ascertain if expression of particular sequences is associated with malignancy; determine if the four base deletion in expressed *env* is characteristic of melanoma.

6.2 Expressed HERV-K sequences in A375 cells

Expressed HERV-K *env* sequences from A375 cells were cloned for sequence analysis (section 2.6). The PCR primers were designed to allow typing of expressed HERV-K *env* sequences (Figure 6.1). The *pol* gene is highly conserved between HERV-K subtypes 101-115, whereas *env* contains the most variable region and was therefore selected for sub-typing.

Twenty-three clones were sequenced and aligned with published human-specific HERV-K *env* sequences (HERV-K 101-115) using Clustal W (www.ebi.ac.uk). An alignment of the sequences with HERV-K 108 *env* sequence is illustrated in Figure 6.2. Eleven sequences were represented by a single clone, one sequence was represented by four clones and one sequence by seven clones. Sequence homologies to HERV-K 101-115 ranged from 96 to 100% (Table 6.1). Only one sequence (clone 2) showed 100% homology with a published HERV-K sequence (HERV-K 108). Three of the 14 sequences had the four base deletion that was observed in expressed *env* sequences in preliminary experiments (section 3.4).

Figure 6.1: Location of primers used to amplify *env*



Legend: Figure 6.1

To investigate HERV-K *env* expression, a 296 bp region was amplified by RT-PCR and cloned into the vector pCR4-TOPO. Nucleotide sequence alignment of forward (F) and reverse (R) primers with human-specific HERV-K *env* sequences is shown. The primers, designed to allow typing of expressed *env* sequences, were located in conserved regions (nucleotide positions with reference to HERV-K 108 indicated), did not span a splice junction and encompassed the most variable region of the HERV-K genome. Regions of sequence variation are highlighted.

Figure 6.2: Nucleotide sequence alignment of expressed *env* sequences derived from the melanoma cell line A375 with HERV-K 108.

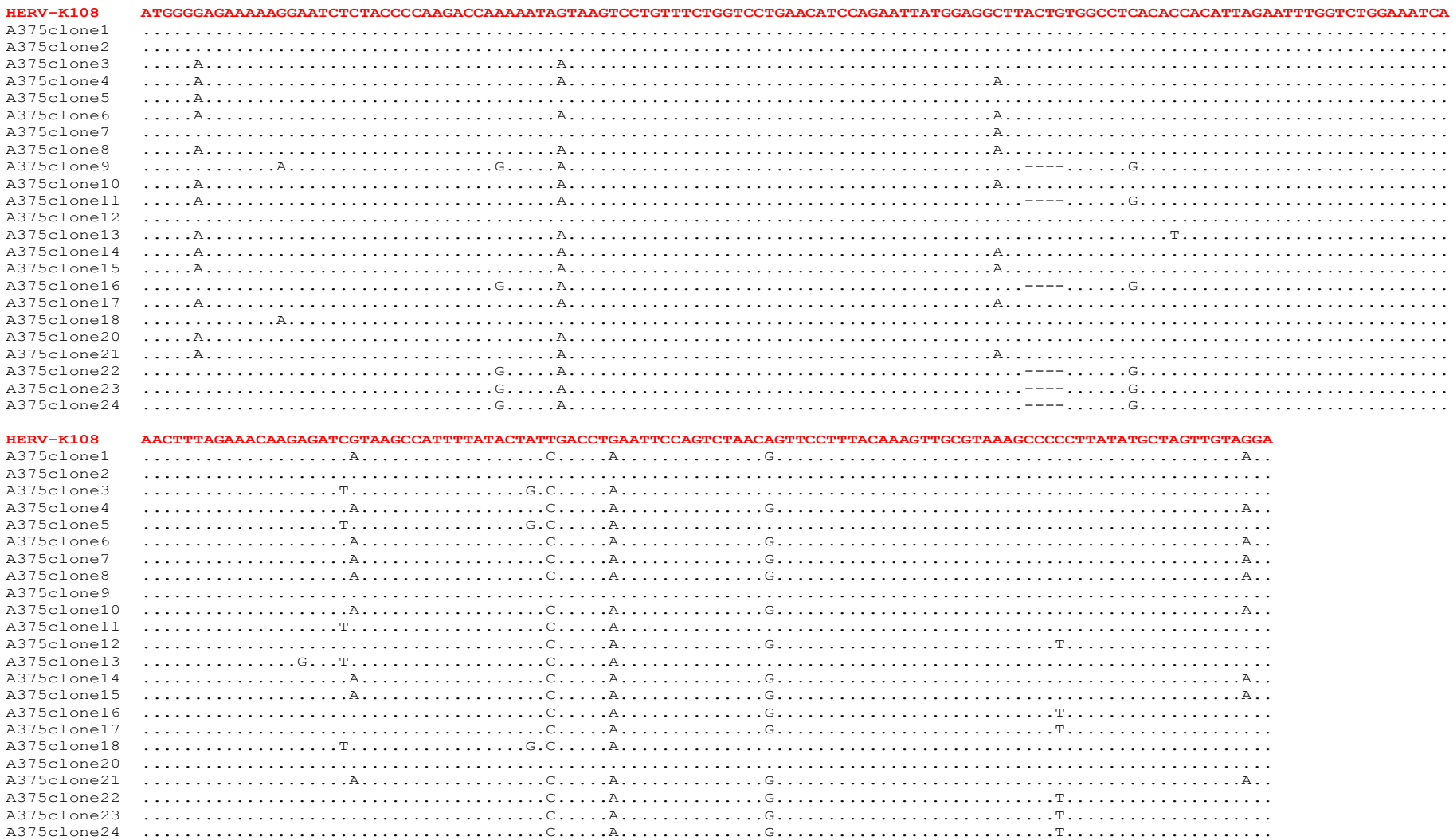


Figure 6.2 Legend:

Expressed HERV-K *env* sequences from A375 cells were cloned for sequence analysis. Twenty-three clones were sequenced and aligned with the HERV-K108 sequence using Clustal W (clone 19 was discarded as the sequence was unreadable). Eleven sequences were represented by a single clone, one sequence was represented by four clones and one sequence by seven clones. Dot (.) indicates identical nucleotide. Hyphens (-) indicate the four base deleted region. Only clone 2 showed 100% homology with HERV-K 108.

Table 6.1: Nucleotide sequence homologies of expressed *env* sequences derived from A375 cells with HERV-K 101-115

A375 clone number and percentage homology with HERV-K														
	C1	**C2**	**C 3**	**C 4**	**C 5**	**C7**	**C 9**	**C 11**	**C 12**	**C 13**	**C 16**	**C 17**	**C 18**	**C 20**
HERV-K 101	97%	97%	98%	97%	98%	97%	97%	97%	97%	97%	97%	97%	98%	97%
HERV-K 102	97%	98%	98%	96%	98%	97%	97%	98%	98%	97%	97%	97%	99%	97%
HERV-K 103	97%	97%	98%	96%	97%	96%	96%	97%	97%	97%	97%	97%	97%	97%
HERV-K 104	98%	97%	97%	97%	96%	97%	96%	97%	97%	96%	97%	97%	96%	97%
HERV-K 107	97%	98%	99%	96%	99%	97%	96%	98%	98%	97%	97%	97%	99%	97%
HERV-K 108	97%	100%	97%	96%	97%	97%	98%	97%	98%	97%	97%	97%	97%	99%
HERV-K 109	97%	97%	98%	97%	98%	97%	97%	97%	97%	97%	97%	97%	98%	97%
HERV-K 113	97%	98%	98%	97%	97%	97%	97%	98%	98%	97%	97%	97%	97%	98%
HERV-K 115	97%	97%	97%	96%	98%	97%	97%	97%	97%	96%	96%	96%	99%	97%
4 base deletion	No	No	No	No	No	No	Yes	Yes	No	No	Yes	No	No	No

Expressed HERV-K *env* sequences from A375 cells were cloned and the sequences aligned with human specific HERV-K *env* sequences (101-115) using Clustal-W. There were 14 distinct sequences (C1-C20) with only one (C2) showing 100% homology with a published HERV-K sequence. Three sequences (C9, C11 and C16) had the four base deletion.

6.3 Expressed HERV-K sequences in melanoma and normal skin tissues

6.3.1 Selection of samples for cloning of HERV-K *env*

Expression of unspliced *env* was found in all tissues analysed by real-time qRT-PCR (section 5.3.2). Twenty cDNA samples derived from melanoma and normal skin samples were analysed by conventional RT-PCR (Figure 6.3) and nine of these (four melanoma and five normal skin samples) were deemed suitable for cloning (296 bp product detected). Thus, expressed HERV-K *env* sequences from four melanoma (Table 5.2: MM13; MM17; MM18; and MM20) and four normal skin samples were cloned for sequence analysis. Control experiments excluded the presence of contaminating gDNA in all samples studied.

6.3.2 Sequence analysis of HERV-K *env* clones

(i) Transcriptionally active HERV-K loci

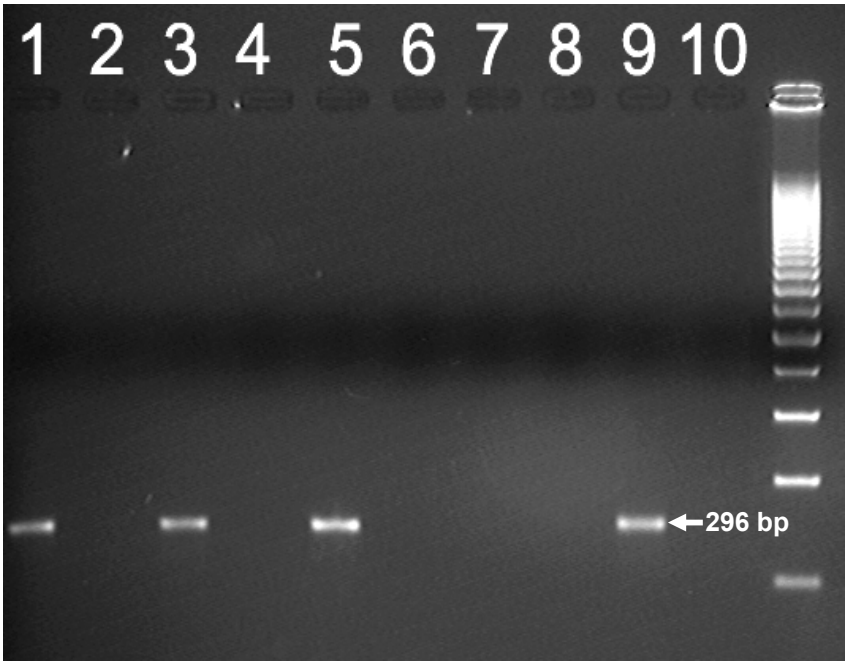
Expression of eight HERV-K loci, comprising both type 1 and type 2 proviruses, was observed (Table 6.2). Expression of more than one locus was found in three of four normal skin and two of four melanoma samples. A separate analysis was performed for each locus to test, as a comparison of proportions, the number of melanoma versus the number of normal skin samples in which the locus was expressed. The comparison of proportions was not statistically significant (Fisher's exact test), for any of the eight loci (p values shown in Table 6.2). Thus none of the HERV-K loci were more likely to be expressed in melanoma.

The four base deletion was seen in a number of sequences derived from both melanoma and normal skin samples and was thus not unique to melanoma (Appendices 5 and 6).

(ii) Nucleotide differences

Homologies to human genome HERV-K sequences ranged from 99 to 100% (Table 6.3). Random substitutions were considered to represent the error rate of the experiment (mutations generated during the reverse transcription, amplification and cloning processes) and substitutions found in most or all clones within a sample were considered to represent inter-patient variability (Table 6.4).

Figure 6.3: RT-PCR analysis of full-length HERV-K *env* mRNA expression in archival tissue samples



Legend:

To identify cDNA samples that were suitable for cloning of HERV-K *env*, the PCR product from amplification of a 296 bp variable region of *env* was analysed by agarose gel electrophoresis. Lanes 1 and 3, samples derived from archival melanoma; lane 5, sample derived from archival normal skin; lane 7, sample derived from archival normal skin, not amplified; lane 9, A375 melanoma cell line sample (positive control). Parallel experiments omitting RT (negative controls, lanes 2, 4, 6 and 10) showed that gDNA was successfully eliminated. A 200 bp MW ladder is adjacent to lane 10.

Table 6.2: Expressed HERV-K *env* sequences in melanoma and normal skin

Expressed HERV- K chromosome/start base (type)	Melanoma sample				Normal skin sample				Proportion of samples in which locus expressed		Fisher's exact p value
	1	2	3	4	1	2	3	4	Melanoma	Normal skin	
1/153864913 (type 1)	y	y	-	-	y	-	-	-	2/4	1/4	1.0
3/102900472 (type 1)	y	y	-	-	y	y	y	y	2/4	4/4	0.43
5/156019127 (type 1)	y	-	-	-	-	-	-	-	1/4	0/4	1.0
16/34089573 (type 1)	y	-	-	-	-	-	y	-	1/4	1/4	1.0
10/6907979 (type 2)	-	-	-	-	-	-	y	-	0/4	1/4	1.0
3/127099218 (type 2)	-	-	y	-	-	-	-	-	1/4	0/4	1.0
5/30524341 (type 2)	-	-	-	-	-	-	y	y	0/4	2/4	0.43
7/4590415 (type 2)	-	-	-	y	y	-	-	-	1/4	1/4	1.0

Expressed HERV-K *env* sequences from melanoma (n=4) and normal skin (n=4) samples were cloned and the sequences aligned with human genome HERV-K sequences by BLASTn searching. Fisher's exact test was performed to determine whether there were any significant differences in the HERV-K loci expressed between melanoma and normal skin tissues. Expressed HERV-K loci for each sample are shown (y=expressed).

Table 6.3: Nucleotide sequence homologies of expressed *env* sequences in melanoma and normal skin samples with human genome HERV-K sequences

	Percentage Homology to published HERV-K							
Expressed HERV- K locus (chromosome/start base)	**Melanoma sample number**				**Normal skin sample number**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1/153864913	100%	99.6%	-	-	99.6%	-	-	-
3/102900472	100%	100%	-	-	99.6%	98.8%	99.6%	99.6%
5/156019127	100%	-	-	-	-	-	-	-
16/34089573	99.6%	-	-	-	-	-	-	-
10/6907979	-	-	-	-	-	-	-	-
3/127099218	-	-	100%	-	-	-	-	-
5/30524341	-	-	-	-	-	-	99.6%	99.6%
7/4590415	-	-	-	99.6%	100%	-	-	-

Nucleotide sequence homologies with published human genome HERV-K sequences for each transcriptionally active HERV-K locus are shown.

Table 6.4: Nucleotide substitutions observed in expressed HERV-K *env* sequences in melanoma and normal skin

Nucleotide substitution (amino acid change)								
Expressed HERV- K (chromosome/start base)	**Melanoma sample number**				**Normal skin sample number**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1/153864913	-	6a to g (silent)	-	-	14a to g (E to G)	-	-	-
3/102900472	-	-	-	-	212t to c (F to S)	209t to c (silent) 210t to c (P to F) 212t to c (F to S)	212t to c (F to S)	90t to c (silent)
5/156019127	-	-	-	-	-	-	-	-
16/34089573	215t to c (F to S)	-	-	-	-	-	-	-
10/6907979	-	-	-	-	-	-	-	-
3/127099218	-	-	-	-	-	-	-	-
5/30524341	-	-	-	-	-	-	147g to a (silent)	147g to a (silent)
7/4590415	-	-	-	25t to c (S to P)	-	-	-	-

Nucleotide substitutions for each transcriptionally active HERV-K locus with resultant amino acid changes in parentheses. Silent mutations are mutations that do not result in a change to the amino acid sequence.

6.4 Discussion

Expressed HERV-K sequences in primary melanoma have not previously been characterised. The primary objective of this investigation was to ascertain whether expression of a particular HERV-K sequence (or sequences) is associated with melanoma. Expressed *env* sequences from four melanoma and four normal skin samples were cloned to identify distinct transcriptionally active HERV-K (HML-2) proviral loci.

Expression of eight HERV-K loci was observed in both primary melanoma and normal skin. Furthermore, multiple loci were transcriptionally active in samples derived from the same individual. About 30 to 50 HERV-K loci are estimated to be present in the human genome (Mayer et al., 1999; Tonjes et al., 1999). Expression of multiple HERV-K sequences, some with random nucleotide substitutions, has previously been described in normal tissues including human placenta, foetal lung and adult testis (Sugimoto et al., 2001). Wang Johanning et al. investigated the expression of HERV-K sequences in breast cancer. Sequence analysis of *env* cDNA clones derived from four breast tumour samples revealed that the clones had >97% identity with HERV-K102, similar to the 99-100% homologies observed in this work (Wang-Johanning et al., 2001). These workers also found that independent isolates from the same breast tumour sample showed nucleotide sequence differences. Flockerzi et al. identified 23 different HERV-K (HML-2) proviruses as transcriptionally active in a number of normal tissues and several proviruses were transcribed in every tissue investigated (Flockerzi et al., 2008)

In this work, random substitutions were considered to represent the error rate of the experiment (because one HERV-K DNA sequence cannot give rise to differing RNA sequences) and substitutions found in most or all clones within a sample were considered to represent inter-patient variability. Previous studies have identified single nucleotide polymorphisms within HERV-K (HML-2) coding regions (de Parseval et al., 2005; Mayer et al., 2005).

Expression of a specific HERV-K locus, provirus type (type 1 versus type 2) or mutation was not associated with melanoma in this study. However, the number of

samples analysed was small. This was due to limitations in extracting RNA of sufficient integrity (296 bp in length) for cloning experiments. It is possible that HERV-K *env* loci that are transcribed at very low rates could be missed in the cloning procedure. Furthermore, different splicing efficiencies for HERV-K transcripts may also influence cloning of corresponding proviral transcripts if, for example, full-length *env* transcripts are efficiently spliced down to spliced *env*, *rec* or *np9* mRNA. At present there are no data in the literature regarding the splicing efficiencies of transcripts from different HERV-K loci.

Sequencing of unspliced *env* mRNA derived from A375 cells in preliminary experiments (section 3.4) suggested expression of a mixture of mRNA species with or without a four base deletion as previously reported by Sugimoto et al. (2001). This finding was confirmed by cloning expressed HERV-K *env* sequences derived from A375 cells. However, the four base deletion was also seen in a number of sequences derived from both melanoma and normal skin samples and was thus not unique to melanoma.

In summary, consistent with the real-time qRT-PCR data showing that expression of full-length HERV-K transcripts is a ubiquitous phenomenon (Chapter 5), it has been demonstrated that at least eight HERV-K *env* loci are transcribed in melanoma and normal skin. Furthermore multiple loci were expressed even in samples derived from the same individual. However, expression of a particular HERV-K locus, provirus type or mutation was not associated with melanoma and expression of novel HERV-K elements (additional to those identified as present within the human genome) was not found, although the number of samples analysed was small.

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Chapter 7:
General Discussion

7.0 Introduction

Substantial aspects of the pathogenesis of melanoma remain unresolved, although genetic and environmental factors are thought to contribute. It is likely that, in addition to the pigmentation phenotype, there are other as yet undetermined genetic elements that contribute to the UVR-induced transformation of melanocytes. During the past decade our understanding of the molecular aberrations that underlie melanomagenesis has greatly increased. Current evidence suggests that melanoma, like other cancers, is not a single disease but a heterogeneous group of disorders that arise from complex molecular changes. Novel insights into melanoma are urgently needed. A more comprehensive understanding of molecular changes is essential for better diagnosis, accurate assessment of prognosis and the development of new effective treatments. Ultimately, it is hoped that a more detailed molecular understanding of melanoma will result in personalised predictors of outcome and the development of individually tailored treatment strategies. Molecular research in melanoma has previously been impeded by the lack of availability of fresh or cryopreserved tissue. However, considerable advances have been made in techniques to extract nucleic acids from FFPE tissue for use in downstream applications such as real-time qRT-PCR.

HERVs, remnants of ancestral exogenous retroviral infections that are fixed in the germ-line DNA, have been implicated in melanomagenesis. Members of the HERV-K provirus family are the most likely to be pathogenic and encode genes for two potentially oncogenic proteins, Rec and Np9. Human-specific HERV-K full-length proviruses were specifically investigated in this work because, as evolutionary recent integrations with nearly intact ORFs, they are more likely to be transcriptionally active and thus potentially pathogenic. When this work was conceived there were only a handful of studies in the literature regarding HERV expression in human melanoma, largely based on melanoma cell lines and metastasis tissue. Therefore, the aim was to carry out a detailed investigation of HERV-K gene expression in archival primary melanoma with the hope that the findings will illuminate the molecular pathogenesis of melanoma and contribute to its emerging molecular classification. Downstream benefits are potential novel prognostic markers and therapeutic targets. It was hypothesised that HERV-K gene expression is associated with primary

melanoma and varies according to the clinicopathological subtype and Breslow thickness. Additionally, it was postulated that primary melanoma is associated with the expression of novel HERV-K elements additional to those identified as present within the human genome.

In the first part of this work, HERV-K mRNA expression was studied in two human melanoma cell lines, principally to corroborate previously published data and to establish positive controls for the investigation of expression in clinical tissue (Chapter 3). Secondly a novel real-time qRT-PCR methodology to study HERV-K gene expression in FFPE tissue was developed (Chapter 4). Thirdly, the expression of full-length and spliced HERV-K mRNA in primary melanoma, benign naevi and normal skin was quantified using this assay (Chapter 5). Finally, expressed HERV-K sequences in melanoma and normal skin were characterised (Chapter 6).

7.1 Summary of findings and their implications

The main findings of this work and their implications are as follows:

(i) Human melanoma cell lines differ in their expression of spliced HERV-K mRNA and production of putative viral particles

Expression of full-length mRNA derived from type 1 and type 2 HERV-K proviruses was detected in both human melanoma cell lines investigated but spliced *env*, *rec* and *np9* mRNA expression was observed in A375 cells only. These cells also produced putative viral particles that contained sequences with homology to the *env* region of HERV-K. Given the expression of all HERV-K genes in A375 cells, RNA derived from these cells was identified as a suitable positive control for experiments to investigate HERV-K gene expression in archival clinical tissue.

These findings are concordant with previously published work (section1.4.2). Not all human melanoma cell lines express spliced HERV-K mRNA or release RVLPs. The biological significance and consequences of HERV-K RNA packaging into RVLP are unclear. HERV-K, which is the only HERV family capable of producing RVLP, has never been shown to be infectious (replication competent) and is only known to be transmitted via the germ line. It has however been hypothesised that recombination

between different HERV-K genomes might give rise to novel infectious viral genomes able to re-infect human cells, including germ cells (Dewannieux et al., 2006).

(ii) HERV-K expression can be reliably detected in FFPE tissue using real-time qRT-PCR

In this study, a high proportion (97%) of FFPE-derived RNA samples was found to be suitable for the analysis of HERV-K mRNA expression using a Taqman based real-time qRT-PCR assay. All tissue blocks emanated from the same pathology laboratory and were thus subject to similar tissue handling and processing procedures. Other studies have used FFPE tissues derived from different pathology departments. A major challenge is the lack of strict standardisation of tissue handling and processing, which results in significant diversity in the quality and quantity of RNA extracted from FFPE tissues obtained from different hospitals.

One explanation for the good results seen this work is that RNA extraction was commenced on-site in the pathology department within minutes of tissue sectioning, thus overcoming the detrimental effects of exposure to air and other environmental factors that can degrade RNA. Furthermore, the tissue blocks were all less than eight years old. Another explanation is the superior performance of the Qiagen kit FFPE RNA isolation kit used in this study over other commercially available kits. A recent study comparing five different kits showed greater total RNA concentrations and RNA purity with the Qiagen kit (Boeckx et al., 2011).

The findings of this study have wider implications for translational research in melanoma (and medicine generally) given the paucity of fresh or cryopreserved primary melanoma tissue for research. This work has demonstrated that real-time qRT-PCR utilising short amplicons is a robust method for the analysis of gene expression in samples where the amount of RNA available for analysis is limited. As the quantification of a target mRNA is measured relative to that of a reference housekeeping gene mRNA, accuracy and specificity is maintained despite any RNA degradation. The assay had good reproducibility and no PCR inhibition was observed.

In this work, the RNA yield was not found to be affected by age of the FFPE tissue contrary to some published studies, although the tissue samples used in this study were all less than eight years old. Old samples are particularly valuable for research because (a) they are inevitably derived from cases where there is longer clinical follow up and (b) some diseases are very rare so archival samples are particularly precious. One group recently reported successful gene expression profiling using tissue blocks stored for over 24 years (Hoshida et al., 2008).

This work has highlighted the vast potential of the histopathology archive as a source of material for gene expression studies in primary melanoma (and other diseases). The development of improved techniques to analyse FFPE tumours would allow studies using large numbers of samples stored from melanoma cohorts or clinical trials with long follow-up data. Since the start of this work, a high-throughput bead array termed DASL (cDNA-mediated annealing, selection, extension and ligation assay, Illumina) has been specifically developed for gene expression studies in FFPE tissue (Bibikova et al., 2008). DASL has been used to study gene expression in archival primary melanoma tissue (Conway et al., 2009; Mitra et al 2010).

Improved guidance regarding the optimal preparation of specimens for the recovery of nucleic acids should be provided to clinicians, pathology laboratories and individuals involved in the collection of tissue in clinical trials. To this end a better appreciation that tissue might have wider usefulness than solely for routine histopathology, but also for molecular assays, is urgently required.

(iii) Full-length HERV-K mRNA is ubiquitously expressed, at high levels, in melanomas, benign naevi and normal skin and is not associated with melanoma

To explore a possible relationship between HERV-K and melanoma, the transcriptional activities of HERV-K genes were compared in primary melanomas, benign naevi and normal skin. The finding of ubiquitously high and similar levels of expression of full-length HERV-K mRNA in all groups is in contrast to previously published data as discussed in Chapter 5, section 5.5. No differences were identified in a number of paired samples of melanoma tissue and extra-lesional skin from the same case. Furthermore, there was no difference in expression levels between

subtypes of melanoma or between ulcerated and non-ulcerated tumours, and there was no correlation between expression and Breslow thickness.

These data indicate that melanoma is not related to increased expression of HERV-K mRNA *per se*. There is little published data regarding the transcriptional activity of HERV-K in normal tissue. To this end, the identification of a transcriptional "core" profile that is characteristic for a certain tissue and detectable in all individuals, would be of importance to determine the significance of alterations in transcription in disease.

HERV expression is regulated by transcription factors (TFs) and by CpG-dinucleotide methylation of the LTRs (Lavie et al., 2005). High levels of HERV-K mRNA expression in the skin could be the result of up-regulation of HERV-K activating TFs and LTR de-methylation. Analogous to the findings in this work, Seifarth et al (2005) found high transcriptional activities of nearly all HERV families, including HERV-K, in normal skin (although only one sample was examined) as well as reproductive organ tissues (uterus, cervix, and testes) and placental tissue. In contrast, less HERV activity was observed in rectum, stomach heart, and skeletal muscle. These workers postulated that HERV activity might be a reflection of the high level of transcriptional and proliferative activity in tissues such as the skin, placenta and uterus.

Although HERV-K is expressed in many normal tissues, the high transcriptional activity of full-length HERV-K implies that these proviruses might play a role in skin physiology. It has been postulated that HERVs have been retained and have persisted during evolution because they performed and continue to perform useful biological functions (Ryan, 2004; Ryan, 2009). Perhaps a high level of HERV-K expression contributes to the skin's defence system against exogenous infectious agents. It has been suggested that HERVs may confer resistance to viral infections by a number of mechanisms including retroviral receptor blockade (by HERV products), interference of replication by antisense mRNA or by priming an immune response to an undesirable viral agent (Buzdin, 2007; Nelson et al., 2003). Exposure of the skin to environmental agents that potentially up-regulate HERV-K expression, such as UVR and hormones, might provide an alternative explanation for the high levels of HERV-K mRNA observed (Nelson et al., 2004). For example, UVR induces the expression

of HERV mRNA transcripts in primary epidermal keratinocytes (Hohenadl et al., 1999). Further studies to elucidate the role of HERV-K in the skin are required.

(iv) Spliced *np9* mRNA is expressed in a proportion of all tissue types and is thus unlikely to be involved in melanomagenesis

The expression of *rec* and *np9* mRNA has not previously been studied in *primary* melanoma. A proportion of all tissue types expressed *np9* at low levels and there were no significant differences in relative expression between the groups. Np9 has been postulated to be oncogenic because of the observation of *np9* transcripts in certain tumours and transformed cell lines. Armbruester et al. (2002) observed expression of *np9* in 52% of mammary carcinoma biopsies, 37% of germ cell tumour biopsies and 33% of leukaemia blood lymphocytes that they studied. However, no direct association of the protein/gene with the induction of tumours has been found and there is little data regarding expression in normal tissues compared with tumours.

Based on the findings in this work, *np9* is unlikely to be important in melanomagenesis and indeed might even play a role in normal skin biology. It could be postulated that the expression of type 1 (*np9*-encoding) HERV-K proviruses is switched on during melanocyte proliferation. The Np9 protein interacts with the Ligand of Numb-protein X (LNX), an ubiquitin ligase, and thus might modulate melanonocyte proliferation through the LNX/Numb/Notch pathway (Armbruester et al., 2004). Notch signalling controls a number of cellular processes, including proliferation, differentiation and survival/apoptosis, and is thought to regulate keratinocyte stem cells and keratinocyte differentiation (Okuyama et al., 2007; Pinnix & Herlyn, 2007). Further studies on the function of Np9 are required to more clearly elucidate the roles of this protein.

(v) Potentially oncogenic *rec* mRNA is differentially expressed in some VGP primary melanomas and thus might play a role in melanoma progression

Expression of *rec* was found to be confined to melanomas: the gene was expressed in 9/39 melanomas but not in benign naevi or normal skin and this was statistically significant ($p=0.009$). Interestingly, the *rec*-positive melanomas were histologically in VGP and statistically significantly thicker than *rec*-negative tumours (median Breslow 2.1 mm compared with 1.05 mm, $p=0.009$). Thus it could be postulated that

rec expression is associated with melanoma progression. Furthermore, data published by Serafino et al. implicate the activation of HERV-K expression as an important contributory element to morphological and functional cell changes during melanoma progression (Serafino et al., 2009).

Several molecular events occur during the transition from RGP to VGP to metastatic melanoma but the mechanisms underlying this progression are not well characterised (Zaidi et al., 2008). Rec physically and functionally interacts with the PLZF tumour suppressor; a transcriptional repressor implicated in carcinogenesis, and thus might be oncogenic by inhibiting PLZF (Denne et al., 2007). PLZF silencing could represent one of the events that occur during melanoma progression.

PLZF has been shown to be expressed in melanocytes but not in melanoma cells, and melanoma growth is suppressed by enforced expression of PLZF (Fellicetti et al. 2004). In their study, Fellicetti et al. demonstrated that melanoma cell lines retrovirally transduced with *PLZF* induced a more differentiated, less malignant phenotype. These workers observed that several genes involved in melanoma progression are modulated, either directly or indirectly, by PLZF. Gene expression profiling of PLZF-negative versus PLZF-positive melanomas showed that PLZF down-regulates tumour-promoting genes, such as integrin avb3 and matrix metalloproteinase 9, and induces genes that promote melanoma cell differentiation, such as *c-KIT* and the downstream *MITF*, *TYR*, and *TRP-1*.

PLZF also targets the *c-myc* proto-oncogene, thus inhibition of PLZF by Rec could result in the up-regulation of *c-myc* with downstream effects on *c-myc* regulated genes. C-MYC is an oncogenic TF that is frequently up-regulated in human malignancies. Although its role in melanomagenesis has not been elucidated, one of the major functions of C-MYC over-expression in melanoma progression is thought to be the suppression of $BRAF^{V600E}$ or $NRAS^{Q61}$-dependent senescence (Zhuang et al. 2008).

Therefore, based on these data, it is possible that Rec is involved in melanoma progression through its inhibitory action on PLZF.

(vi) Multiple HERV-K loci are transcribed in melanoma and normal skin but expression of a particular HERV-K locus is not associated with melanoma

Multiple HERV-K loci were found to be transcribed in melanoma and normal skin, even in samples derived from the same individual. Expression of a particular HERV-K locus, provirus type or mutation was not significantly associated with melanoma and expression of novel HERV-K elements (additional to those identified as present within the human genome) was not found, although the number of samples analysed was small.

This is the first study to characterise expressed HERV-K sequences in melanoma. Epigenetic differences in HERV methylation status or chromatin structure and in the availability of cellular TFs may account for the transcriptional activation of specific HERV-K loci in the skin (Lavie et al., 2005; Okahara et al., 2004; Schon et al., 2001).

Tissue-specific expression patterns of HERV-K loci have previously been demonstrated (Flockerzi et al., 2008; Muradrasoli et al., 2006; Seifarth et al., 2005) but further studies are required. There are limited data regarding factors that control locus-specific transcription of HERVs that are selectively expressed in different tissues.

Mammalian cells limit the functions of retroviral LTRs primarily through DNA methylation. Genome-wide CpG methylation is established at implantation in vertebrate embryos and favours transcriptional silencing. As development proceeds, discrete regions within the genome are de-methylated in a cell type–specific manner, generating patterns of gene expression specific to a particular lineage. Inappropriate gene expression might occur if CpG methylation is reversed on silenced HERV LTRs, which can then act as promoters for cellular genes.

Interestingly, Lamprecht et al. (2010) recently demonstrated that aberrant expression of the proto-oncogene *CSF1R* (encoding colony-stimulating factor-1 receptor) in human lymphomas is driven by activation of a nearby endogenous LTR that is normally silenced thus permitting the *CSF1R* signals that are needed for tumour cell survival. This is the first example of ERV–driven activation of a proto-oncogene in human malignancy. These data strongly suggest that loss of epigenetic control and

the subsequent transcriptional activation of ERV elements have an important role in the malignant transformation of human cells.

7.2 Study limitations

The limitations of this study are related to the small number of samples investigated and to sampling of melanoma tissue. As discussed in section 5.5, the tumour content in the sections from which RNA was extracted was not determined and there was a possibility of sampling normal tissue. An approach similar to that used by Conway et al. (2009), which is more practicable than laser microdissection, could be employed. In their study, an H&E-stained slide was first examined to identify the deepest part of the tumour having a diameter of >0.8 mm, containing the lowest admixture of inflammatory or stromal cells. A tissue microarray needle was then used to take a core from the deepest part of the tumour for RNA extraction. Tissue microarray cores were embedded horizontally in wax blocks, sections obtained throughout the whole core and H&E slides prepared to determine the percentage tumour content of the core by light microscopy. The tumour content of tissue cores sampled in this way was estimated to be at least 70% tumour cells. However, one limitation of this technique is the inability to sample very small tumours.

In this work, it was not determined whether *rec* was transcribed specifically in melanoma cells. This could be elucidated using ISH. Wang-Johanning et al. (2001) used ISH to demonstrate the expression of HERV-K *env* transcripts in breast tumour cells but not in normal or uninvolved breast epithelial cells. Immunohistochemistry could be used to determine the localisation of Rec in melanomas but there are no commercially available antibodies at present. Busher et al. (2005 & 2006) generated rabbit anti-serum against a recombinant Rec protein and goat antiserum against TM Env in their laboratory and used these to study the in-situ expression of Env, Rec and Np9 in metastatic melanoma samples by immunohistochemistry. Interestingly, these workers demonstrated the expression of Env and Rec in melanoma cells but not in surrounding lymphoid tissue in some metastatic melanoma samples.

7.3 Conclusions

This is the first detailed study of HERV-K mRNA expression in primary melanoma. Although full-length HERV-K mRNA appears to be ubiquitously expressed at high levels in skin and a definitive role for HERV-K in melanomagenesis has not been demonstrated, the differential expression of potentially oncogenic *rec* in some VGP primary melanoma merits further evaluation. It is possible that *rec* expression is an epiphenomenon rather than a causative event or step involved in tumour progression. The potential impact of *rec* expression on melanomagenesis and its role in molecular pathways known to be involved in melanoma require further investigation. This work suggests that *rec* expression is associated with melanoma progression but further large scale studies are needed. Furthermore, this work has demonstrated that gene expression can be reliably detected in FFPE tissue.

Thus far, a clear association between UVR-induced DNA damage and melanoma initiation has not been demonstrated. It is therefore plausible to suggest that other mechanisms exist by which UVR mediates melanocyte transformation. One possible mechanism that warrants consideration is the transcriptional activation of HERV-K in melanocytes. Furthermore polymorphic HERV-K elements might represent novel genetic risk factors for melanoma in susceptible populations, but this remains to be investigated.

7.4 Future work

Following on from this work, a number of further investigations are proposed to address the following questions:

(i) Is *rec* expression a prognostic biomarker?

Investigation of *rec* expression in further melanoma and control samples (with associated long term follow-up data) would be required to ascertain if *rec* expression is a prognostic biomarker (and potential therapeutic target).

(ii) Are HERV-K proteins expressed in melanoma?

Work to study HERV-K protein expression by immunohistochemistry is currently in progress.

(iii) Are insertionally polymorphic members of the HERV-K family associated with melanoma?

To investigate this, DNA would be isolated from melanoma patients and healthy controls. The presence or absence of the polymorphic HERV-K proviruses 113 and 115 would be determined by conventional PCR with primers spanning the pre-integration sites and sites within the proviral sequences as previously described by Moyes et al. (2005).

(iv) Is photo-activation of HERV-K associated with melanoma?

The effects of UVR exposure on expression of HERV-K genes could be studied by real-time qRT-PCR in melanoma cell lines.

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Zhuang, D., Mannava, S., Grachtchouk, V., Tang, W.H., Patil, S., Wawrzyniak, J.A., et al. (2008) C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. *Oncogene,* **27,** 6623-6634.

Appendices

Appendix 1: Ethics approval

The Royal Marsden Local Research Ethics Committee
St George's University of London
South London REC Office (1)
Room 1.13 & 1.14
1st Floor - Jenner Wing
Blackshaw Road
Tooting, London
SW17 0RE

Telephone: 020 8725 0262
Facsimile: 020 8725 1897

18 August 2006

Professor C. Bunker
Consultant Dermatologist
Chelsea & Westminster Hospital
369 Fulham Road
London
SW10 9NH

Dear Professor Bunker,

Full title of study: **THE ROLE OF HUMAN ENDOGENOUS RETROVIRUS K (HERV-K HML2) IN THE PATHOGENESIS OF MELANOMA**

REC reference number: **06/Q0801/55**

Thank you for your letter of 26 July 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered by the members of the Sub-Committee of the REC.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA. There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.1	07 June 2006
Investigator CV	1	
Protocol	1	01 June 2006

Covering Letter	1	01 June 2006
Statistician Comments	1	
Letter of invitation to participant	1	01 June 2006
Participant Information Sheet – Archival Control Tissue Samples	2	26 July 2006
Participant Information Sheet – Archival Melanoma Samples	2	26 July 2006
Participant Information Sheet – Control Subjects (Mouth Swabs)	2	26 July 2006
Participant Consent Form - Archival Control Tissue Samples	2	26 July 2006
Participant Consent Form - Archival Melanoma Samples	2	26 July 2006
Participant Consent Form - Control Subjects (Mouth Swabs)	2	
Response to Request for Further Information	1	26 July 2006
Letter from Funder	1	29 September 2005

Research governance approval

You should arrange for the R&D department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0801/55	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Sheree Manson

Mrs Sheree Manson
Committee Co-ordinator

Email: sheree.manson@stgeorges.nhs.uk

Enclosures: *Standard approval conditions*

Copy to: Chelsea and Westminster Healthcare NHS Trust
Research and Development
369 Fulham Road, London
SW10 9NH

Appendix 2: Study invitation letter, patient information and consent forms

1st September 2007

Dear Sir/Madam

You are being invited to take part in a research study on melanoma. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you would like to take part, please sign and return the consent form and the attached short questionnaire in the stamped envelope provided. If you would like to meet with us and discuss the study before making a decision please contact us on the number or email below.

Thank you for taking the time to read this information.

Yours sincerely

Professor Christopher Bunker
Dr Sarita Singh

Telephone: 0208 7468169
Email: saritasingh@doctors.org.uk

Participant Information Sheet- Archival Melanoma Samples

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- *Part 1 tells you the purpose of this study and what will happen to you if you take part.*
- *Part 2 gives you more detailed information about the conduct of the study.*

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1

Study title

The role of an internal virus called "human endogenous retrovirus" in the development of melanoma (The role of Human Endogenous Retrovirus K in the pathogenesis of melanoma).

What is the purpose of the study?

In the UK each year about 8,000 people are diagnosed with melanoma, which is a type of skin cancer. Melanoma cancer rates are set to treble over the next thirty years. Understanding how cancers form and grow out of control is an important part of finding new and better cancer treatments.

Scientists now know that the ultraviolet light from the sun can damage your genetic code (DNA). The damaged DNA can cause normal skin cells to become cancerous. However some people are more at risk than others. Everybody's DNA contains genes that code for internal viruses. These viruses are not infectious, that is they cannot be passed on from person to person. These genes are normally switched off and are considered to be "junk" bits of DNA. However recent studies have shown that in some people with melanoma, these viral genes have been switched on. We would like to explore the possibility that this virus might be involved in the causation of melanoma. One hypothesis might be that the development of melanoma is triggered by ultraviolet light "switching on" production of the virus in pigment producing cells in the skin. This may have a knock-on effect on other genes which normally protect the cells from becoming cancerous. Our research will involve looking for the presence of specific "markers" produced by the HERV-K retrovirus which may play a role in the development of melanoma.

Why have I been chosen?

We are asking people who have had a melanoma surgically removed in the past to take part. At the time of your surgery, when tissue was removed, it was sent to the pathology department for routine testing and diagnosis. After that process was completed, the remaining tissue sample was stored in the pathology department. We are asking for your permission to use the remainder of your melanoma tissue sample for research. Since this tissue was removed at the time of surgery the permission to use this tissue will not lead to any additional procedures.

Do I have to take part?
No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?
If you choose to be in this study, we will obtain some of your left over tissue which has been stored in the pathology department. We will carry out tests to determine whether "internal virus" (human endogenous retrovirus) is present in the genetic code (DNA) of your melanoma cells. We will not be taking any new samples from you. In order to understand the significance of the research done on your tissue, we may need to know some things about you, for example your gender, your age, your past medical history and your family history. This information will be obtained from your medical records with your consent.

What do I have to do?
Nothing else is required. We will not be taking any new samples from you. We will compare the results with tests on people who have not had melanoma ("control" subjects).

What are the possible disadvantages and risks of taking part?
There are no medical risks to you for donating tissue. However, while every effort is made to protect your identity, there is a small risk of loss of privacy.

What are the possible benefits of taking part?
The kind of information we will look for in this study will not tell you anything specific about your personal health. This study will not help you but the information we get might help improve the treatment of people with melanoma in the future. You will not be paid for taking part in this study.

What if there is a problem?
Any complaint about the way you have been dealt with during the study will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact Details
For any further information of if you have any questions please contact Dr Sarita Singh at:

Department of Dermatology
Chelsea and Westminster Hospital
369, Fulham Road
London SW10 9NH
Telephone: 0208 7468169
saritasingh@doctors.org.uk

This completes Part 1 of the Information Sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

What will happen if I don't want to carry on with the study?
Only stored tissue samples provided for this research **that can still be identified as yours** will be destroyed if you do not wish to carry on with the study.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Contact email as above). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Will my taking part in this study be kept confidential?
Yes. To protect your information, we will not keep your name and address with the sample, only a code number. Files that link your name to the code number will be kept in a locked cabinet and only the study staff will be allowed to look at them. Although no one can absolutely guarantee confidentiality, using a code number greatly reduces the chance that someone other than the study staff will ever be able to link your name to your sample or to your test results.

What will happen to my tissue sample after the study has been completed?
After our study is over, we would like to keep any left over sample for future research. We don't have specific research plans at this time but we would like to use the samples for studies on melanoma. We will store the sample under a code number and we will keep the file that links the code number to your name private. A Research and Ethics Committee will review and approve all future projects.

You can choose not to have your sample stored for future research and still be part of this study. You will have the chance to state your choice about this at the end of this form.

Will any genetic tests be done?
Yes. The genetic testing performed on your tissue sample will be looking for the presence of internal virus genes, and depending on how the study is progressing we may go on to look for other genes that have been implicated in melanoma development. We will not be looking for genes associated with any other diseases, that is we will not be carrying out "genetic profiling".

What will happen to the results of the research study?
The studies we do on the samples we collect are to add to our knowledge about melanoma. We are gathering this knowledge by studying groups of people, and the study is not meant to test your personal medical status. Results from research using your tissue may not be ready for many years and will not affect your care right now, but the results of the research may be helpful to people like you in the future. For these reasons, we will not give you or your GP the results of our research on your sample and the results will not appear on your medical records. However, we will share what we learn with other health professionals through medical publications. You may contact the researchers if you wish to have a copy of any such publications.

Who is organising and funding the research?
Chelsea and Westminster Healthcare NHS Trust Charity.

Who has reviewed the study?
The Royal Marsden Research Ethics Committee.

Thank you for taking the time to read this information.

CONSENT FORM

PLEASE DETATCH AND RETURN IN PRE- PAID ENVELOPE

Your Name:
Your Date of Birth:

Title of Project: The role of Human Endogenous Retrovirus K in the pathogenesis of melanoma

Name of Researcher: Dr Sarita Singh

Please initial box

1. I confirm that I have read and understand the information sheet dated 26th July 2006 (version 2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I have read the part of this form about storing my tissue for future research. My choice about having my sample stored and used for future melanoma research is:

I refuse to have my tissue stored or used for any kind of future research on melanoma. ☐

It is OK to store my tissue with a code number and to use it for any kind of future research on melanoma. ☐

5. I understand that my individual results from the study will not be given to me. ☐

6. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix 3: Patient questionnaire

The role of HERV K in the pathogenesis of melanoma
Patient Questionnaire

1. What is your hair colour?
   - ☐ Black/dark brown
   - ☐ Brown
   - ☐ Blonde/fair
   - ☐ Red

2. What is your eye colour?
   - ☐ Brown
   - ☐ Green or hazel
   - ☐ Blue or grey

3. How many moles do you have in total?
   - ☐ 0–20
   - ☐ 21–100
   - ☐ More than 100

4. How would you describe your skin colour?
   - ☐ Very fair
   - ☐ Fair
   - ☐ Medium
   - ☐ Olive
   - ☐ Dark brown
   - ☐ Black

5. How does your skin react when spending time in the sun/sunbathing?
   - ☐ Always burn, never tan
   - ☐ Always burn, occasionally tan
   - ☐ Occasionally burn, always tan
   - ☐ Rarely burn, always tan
   - ☐ Never burn, always tan

6. Have you had any episodes of severe sunburn after the age of 19?
   - ☐ Never
   - ☐ 1–5 times
   - ☐ More than 5 times

7. Have you had any episodes of severe sunburn before the age of 19?

Never ☐
1–5 times ☐
More than 5 times ☐

8. Have you ever used or do you use sun beds?

Never ☐
1–10 times ☐
10–100 times ☐
More than 100 times ☐

9. When you spend time out in the sunshine do you use sunscreen?

Never ☐
Occasionally ☐
Always ☐

10. How many sunny vacations have you had to date?

None ☐
1–10 ☐
11–20 ☐
More than 20 ☐

10. Do you have a family history of melanoma?

Yes ☐
No ☐

11. What is/was your occupation? ……

Thank you for taking the time to answer these questions

Appendix 4: Melanoma cases recruited but not included in the study

Sex	Age (years)	Melanoma site	Histological subtype	Breslow (mm)	Ulceration
M	63	head/neck	NMM	9.5	yes
M	76	trunk	SSMM	0.8	no
F	50	trunk	SSMM	0.7	no
F	32	not stated	SSMM	0.2	no
M	79	head/neck	LMM	2.6	no
M	81	head/neck	SSMM	1.0	no
M	81	head/neck	SSMM	1.3	no
F	85	head/neck	LMM	1.4	no
M	43	leg	SSMM	0.4	no
M	72	head/neck	SSMM	1.5	yes
M	51	trunk	MMIS	0.0	no
F	79	leg	LMM	0.0	no
F	79	leg	SSMM	0.6	no
F	51	arm	SSMM	0.4	no
F	35	other	SSMM	0.9	no
M	77	head/neck	LMM	0.8	no
F	60	trunk	other variant	0.2	no
F	88	head/neck	LMM	not stated	no
M	79	head/neck	LMM	1.0	no
M	60	leg	SSMM	0.7	no
M	65	leg	SSMM	0.8	no
F	56	other	MMIS	0.0	no
F	55	head/neck	LMM	1.0	no
F	63	arm	SSMM	1.1	no
M	69	trunk	SSMM	1.3	no
M	53	trunk	SSMM	0.2	no
F	75	not stated	SSMM	1.8	no
F	32	arm	MMIS	0.0	no
M	71	trunk	SSMM	0.4	no
F	51	leg	SSMM	0.8	no
F	84	head/neck	SSMM	not stated	no
M	66	trunk	SSMM	0.4	no

Sex	Age (years)	Melanoma site	Histological subtype	Breslow (mm)	Ulceration
M	69	arm	LMM	0.6	no
M	63	leg	SSMM	0.8	no
F	52	leg	SSMM	0.7	no
F	53	acral	MMIS	0.0	no
F	44	trunk	SSMM	0.8	no
M	52	trunk	SSMM	not stated	no
M	36	leg	SSMM	0.5	no
F	46	leg	SSMM	0.5	no
F	66	head/neck	other variant	1.2	no
F	88	head/neck	LMM	0.5	no
F	58	leg	SSMM	0.5	no
M	61	trunk	SSMM	1.0	yes
M	61	trunk	MMIS	0.0	no
F	67	leg	SSMM	1.1	no
M	38	head/neck	MMIS	0.0	no
F	74	subungual	ALMM	4.3	yes
M	32	trunk	SSMM	0.3	no
M	70	trunk	SSMM	0.8	no
M	76	head/neck	LMM	1.0	no
F	71	leg	SSMM	0.8	no
M	66	trunk	SSMM	1.0	no
M	46	leg	SSMM	0.8	no
M	31	arm	MMIS	0.0	no
F	76	leg	SSMM	0.9	no
F	30	trunk	MMIS	0.0	no
M	76	head/neck	NMM	4.0	no
M	77	arm	NMM	6.0	yes
F	46	leg	other variant	0.6	no
M	30	arm	SSMM	0.9	no
F	52	leg	SSMM	0.7	no
F	60	head/neck	LMM	0.6	no
F	55	arm	SSMM	0.6	no
F	35	head/neck	NMM	1.0	no
F	66	head/neck	LMM	0.1	no
F	66	head/neck	LMM	0.3	no

Sex	Age (years)	Melanoma site	Histological subtype	Breslow (mm)	Ulceration
M	42	not stated	not stated	not stated	not stated
F	56	arm	SSMM	1.2	yes
F	66	leg	SSMM	1.4	no
M	48	arm	SSMM	0.4	no
F	33	arm	other variant	1.2	yes
F	50	trunk	SSMM	1.0	no
F	61	leg	SSMM	1.6	no
F	89	leg	NMM	0.6	no
M	95	leg	SSMM	0.6	no
F	82	arm	SSMM	1.6	no
M	79	arm	SSMM	1.2	no

LMM, lentigo maligna melanoma; SSMM, superficial spreading melanoma; NMM, nodular melanoma; ALMM, acral lentiginous melanoma; MMIS, melanoma in situ; not stated, information not stated on histopathology report

Appendix 5: HERV-K *env* clones - melanoma samples

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
1	1	3	102900472	100	yes	0		
	2	5	156019127	100	no	0		
	3	5	156019127	98.8	yes	3	34	g -> a
							40	a -> g
							91	g -> c
	4	5	156019127	100	no	0		
	5	5	156019127	100	no	0		
	6	3	102900472	100	yes	0		
	7	3	102900472	100	yes	0		
	8	3	102900472	99.2	yes	2	34	a -> g
							40	g -> a
	9	3	102900472	100	yes	0		
	10	3	102900472	100	yes	0		
	11	5	156019127	100	no	0		
	12	3	102900472	100	yes	0		
	13	1	153864913	99.2	no	2	14	a -> g
							213	t -> c
	14	16	34089573	99.2	no	2	41	g -> a
							215	t -> c
	15	1	153864913	99.2	no	2	186	g -> a
							215	t -> c
	16	3	102900472	100	yes	0		
	17	3	102900472	99.6	yes	1	34	a -> g
	18	5	156019127	100	no	0		
	19	16	34089573	98.8	no	3	34	g -> a
							145	t -> c
							215	t -> c
	21	5	156019127	100	no	0		
	23	5	156019127	99.6	no	2	14	a -> g
							164	_ -> g

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
2	1	3	102900472	100	yes	0		
	2	3	102900472	100	yes	0		
	3	3	102900472	100	yes	0		
	4	1	153864913	98.8	no	3	6	a -> g
							186	g -> a
							215	t -> c
	5	3	102900472	99.2	yes	2	34	a -> g
							40	g -> a
	6	3	102900472	100	yes	0		
	7	3	102900472	100	yes	0		
	8	3	102900472	100	yes	0		
	9	3	102900472	99.6	yes	1	63	g -> a
	10	3	102900472	98.8	yes	3	141	t -> c
							182	a -> g
							211	c -> t
	11	3	102900472	100	yes	0		
	12	3	102900472	100	yes	0		
	13	3	102900472	100	yes	0		
	14	3	102900472	100	yes	0		
	15	3	102900472	98.8	yes	3	27	t -> c
							98	g -> a
							205	g -> a
	16	3	102900472	100	yes	0		
	17	3	102900472	100	yes	0		
	18	3	102900472	100	yes	0		
	19	3	102900472	100	yes	0		
	21	3	102900472	100	yes	0		
	22	3	102900472	99.2	yes	2	34	a -> g
							40	g -> a
	23	3	102900472	100	yes	0		
	24	1	153864913	99.2	no	1	6	a -> g

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
3	1	3	127099218	100	no	0		
	2	3	127099218	100	no	0		
	3	3	127099218	100	no	0		
	4	3	127099218	99.6	no	1	80	g - > a
	5	3	127099218	100	no	0		
	6	3	127099218	100	no	0		
	7	3	127099218	100	no	0		
	8	3	127099218	100	no	0		
	9	3	127099218	100	no	0		
	10	3	127099218	99.6	no	1	4	g -> a
	11	3	127099218	100	no	0		
	12	3	127099218	100	no	0		
	13	3	127099218	100	no	0		
	14	3	127099218	100	no	0		
	15	3	127099218	100	no	0		
	16	3	127099218	100	no	0		
	17	3	127099218	100	no	0		
	18	3	127099218	100	no	0		
	19	3	127099218	100	no	0		
	20	3	127099218	99.6	no	1	218	c -> t
	21	3	127099218	100	no	0		
	22	3	127099218	100	no	0		
	23	3	127099218	100	no	0		
	24	3	127099218	100	no	0		

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
4	1	7	4590415	99.2	no	2	25	t -> c
	2	7	4590415	99.6	no	1	25	t -> c
	3	7	4590415	99.6	no	1	25	t -> c
	4	7	4590415	99.6	no	1	25	t -> c
	5	7	4590415	99.6	no	1	25	t -> c
	6	7	4590415	99.6	no	1	25	t -> c
	7	7	4590415	99.6	no	1	25	t -> c
	8	7	4590415	99.6	no	1	25	t -> c
	9	7	4590415	99.6	no	1	25	t -> c
	10	7	4590415	99.6	no	1	25	t -> c
	11	7	4590415	99.6	no	1	25	t -> c
	12	7	4590415	99.6	no	1	25	t -> c
	13	7	4590415	99.2	no	2	25	t -> c
							150	g -> a
	14	7	4590415	99.6	no	1	25	t -> c
	15	7	4590415	99.6	no	1	25	t -> c
	16	7	4590415	99.6	no	1	25	t -> c
	17	7	4590415	99.6	no	1	25	t -> c
	18	7	4590415	99.2	no	2	25	t -> c
							160	c -> t
	19	7	4590415	99.6	no	1	25	t -> c
	20	7	4590415	99.6	no	1	25	t -> c
	21	7	4590415	99.6	no	1	25	t -> c
	22	7	4590415	99.6	no	1	25	t -> c
	23	7	4590415	99.6	no	1	25	t -> c

Appendix 6: HERV-K *env* clones - normal skin samples

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
1	1	7	4590415	100	no	0		
	2	1	153864913	99.6	no	1	14	a -> g
	3	7	4590415	100	no	0		
	4	7	4590415	99.6	no	3	14	a -> g
							144	_ -> c
							235	_ -> t
	5	1	153864913	99.6	no	1	14	a -> g
	6	7	4590415	100	no	0		
	7	1	153864913	99.6	no	1	14	a -> g
	8	3	102900472	98.8	yes	3	162	t -> c
							168	g -> a
							212	t -> c
	9	7	4590415	99.2	no	2	34	g -> a
							40	a -> g
	10	1	153864913	100	no	0		
	11	7	4590415	99.2	no	2	16	a -> g
							145	t -> c
	12	7	4590415	99.2	no	2	215	t -> c
							216	t -> c
	13	7	4590415	99.2	no	2	20	g -> t
							45	c -> t
	14	7	4590415	100	no	0		
	15	3	102900472	99.6	yes	1	212	t -> c
	16	1	153864913	100	no	0		
	17	7	4590415	100	no	0		
	18	7	4590415	100	no	0		
	20	1	153864913	99.6	no	1	14	a -> g
	21	1	153864913	99.6	no	1	14	a -> g
	22	1	153864913	99.6	no	1	14	a -> g
	23	7	4590415	100	no	0		
	24	1	153864913	99.6	no	1	14	a -> g

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
2	1	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	2	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	3	3	102900472	98.4	yes	4	153	g -> t
							209	t -> c
							210	t -> c
							212	t -> c
	4	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	5	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	6	3	102900472	98.4	yes	4	64	g -> a
							209	t -> c
							210	t -> c
							212	t -> c
	7	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	8	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	9	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	10	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	11	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c

							212	t -> c
	12	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	13	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	14	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	15	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	16	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	17	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	18	3	102900472	98.4	yes	4	7	g -> a
							209	t -> c
							210	t -> c
							212	t -> c
	19	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	20	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	21	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	22	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c
	23	3	102900472	98.4	yes	4	209	t -> c

							210	t -> c
							211	c -> t
							212	t -> c
	24	3	102900472	98.8	yes	3	209	t -> c
							210	t -> c
							212	t -> c

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
3	1	5	30524341	99.6	no	1	147	g -> a
	2	16	34089573	99.2	no	2	215	t -> c
							216	t -> c
	3	3	102900472	99.2	yes	2	35	a -> g
							212	t -> c
	4	5	30524341	99.2	no	2	35	g -> a
							147	g -> a
	5	3	102900472	99.2	yes	2	211	c -> t
							239	c -> t
	6	5	30524341	99.6	no	1	147	g -> a
	7	3	102900472	98.8	yes	3	123	g -> a
							211	c -> t
							239	c -> t
	8	16	34089573	99.2	yes	2	92	g -> c
							139	c -> t
	9	3	102900472	99.6	yes	1	212	t -> c
	10	3	102900472	98.8	yes	3	123	g -> a
							211	c -> t
							239	c -> t
	11	3	102900472	98.8	yes	1	212	t -> c
	12	16	34089573	99.6	no	1	127	g -> a
	13	5	30524341	99.2	no	2	35	g -> a
							147	g -> a
	14	3	102900472	99.2	yes	2	211	c -> t
							239	c -> t
	15	16	34089573	98.8	no	3	127	g -> a
							215	t -> c
							216	t -> c
	16	16	34089573	98.8	no	3	127	g -> a
							215	t -> c
							216	t -> c
	17	16	34089573	99.2	no	2	127	g -> a
							212	a -> c
	18	3	102900472	99.2	yes	2	212	c -> t

							239	c -> t
	19	5	30524341	99.6	no	1	147	g -> a
	20	5	30524341	99.6	no	1	147	g -> a
	21	10	6907979	100	no	0		
	22	3	102900472	99.6	yes	1	212	t -> c
	23	3	102900472	99.6	yes	1	212	t -> c
	24	3	102900472	99.6	yes	1	212	t -> c

Sample	Clone	Chromosome	Start base	Identity (%)	4bp deletion	Nucleotide differences	Position	Mutation
4	1	3	102900472	99.2	yes	2	90	t -> c
							149	t -> c
	2	3	102900472	99.6	yes	1	90	t -> c
	3	3	102900472	99.2	yes	2	90	t -> c
							101	c -> t
	4	3	102900472	99.6	yes	1	90	t -> c
	5	3	102900472	99.6	yes	1	90	t -> c
	6	3	102900472	99.6	yes	1	90	t -> c
	7	3	102900472	99.6	yes	1	90	t -> c
	8	5	30524341	99.6	no	1	147	g -> a
	10	3	102900472	99.6	yes	1	90	t -> c
	11	3	102900472	98.8	yes	3	93	g -> a
							212	t -> c
							239	c -> t
	13	3	102900472	99.6	yes	1	90	t -> c
	14	3	102900472	99.6	yes	1	90	t -> c
	15	3	102900472	99.6	yes	1	90	t -> c
	16	3	102900472	99.6	yes	1	90	t -> c
	17	3	102900472	99.6	yes	1	90	t -> c
	19	3	102900472	99.6	yes	1	90	t -> c
	20	3	102900472	99.6	yes	1	90	t -> c
	21	3	102900472	99.6	yes	1	90	t -> c
	22	3	102900472	99.6	yes	1	90	t -> c
	23	3	102900472	99.2	yes	2	13	g -> a
							90	t -> c

Publications

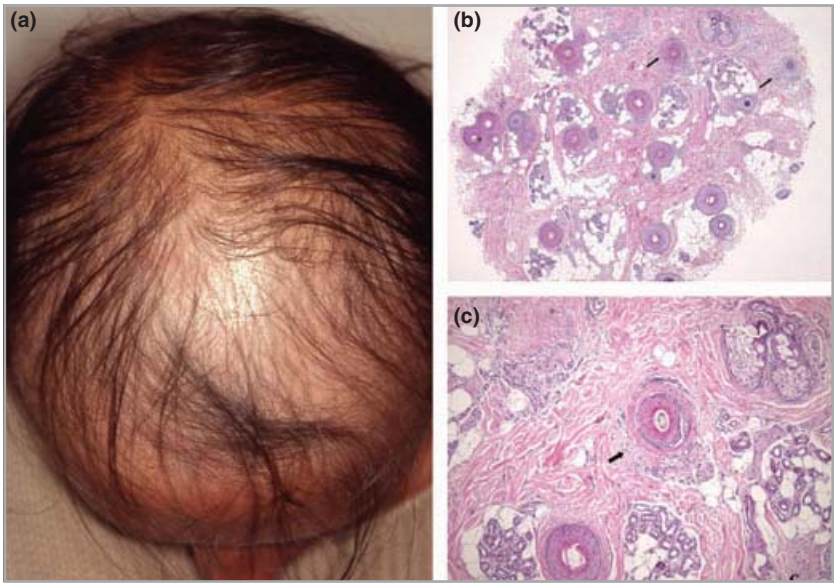

Fig 2. Patient 2. (a) Severe scalp alopecia mimicking diffuse alopecia areata. (b) Transverse section at the dermis showing a discrete peribulbar lymphocytic infiltrate (black arrows) with absence of fibrosis. We observe some vellus hairs and terminal anagen follicles of intermediate diameter (haematoxylin and eosin; original magnification × 10). (c) Closer view of the peribulbar lymphocytic infiltrate (black arrow) (haematoxylin and eosin; original magnification × 100).

To our knowledge, these are the first cases of irreversible alopecia linked with the use of taxanes. A hair loss of such severity due to the sole use of letrozole has not been described and does not seem plausible. A possible explanation of the irreversibility could be the permanent damage of the stem cells situated in the hair bulge or the matrix keratinocytes along with an idiopathic sensitivity of the hair follicle to these agents.[9] Explaining and preventing such complications is very important as the possibility of developing irreversible alopecia plays a major role in a patient's decision regarding the proposed chemotherapy regimen.

Sabouraud Centre for Scalp and Hair Disorders, 2 place du Dr Alfred Fournier, 75010 Paris, France
**Department of Dermatology 1, Saint Louis Hospital, Paris, France*
E-mail: chrisprevezas@yahoo.com

C. Prevezas
B. Matard
L. Pinquier*
P. Reygagne

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Key words: *alopecia, breast cancer, chemotherapy, taxanes*

Conflicts of interest: none declared.

Revisiting the past: utilizing archival tissue for the detection and quantification of gene expression

DOI: 10.1111/j.1365-2133.2009.09039.x

Sir, A significant challenge in dermatology research is the lack of availability of fresh or snap-frozen tissue, particularly from primary melanoma. However, histopathology archives, which are historical collections of formalin-fixed, paraffin-embedded tissue specimens representing an immense range of dermatological disease, are an excellent resource for retrospective studies. In the past decade, vast strides have been made in techniques to extract nucleic acids of sufficient quality and quantity from formalin-fixed, paraffin-embedded tissue for use in downstream applications, such as the polymerase chain reaction (PCR). DNA extracted from archival tissue is now frequently used in molecular dermatology research. For example, Curtin *et al.*[1] identified distinct sets of genetic alterations in melanoma by array-based comparative genomic hybridization of DNA extracted from archival melanoma specimens. Data generated from such studies can result in the identification of novel biomarkers for disease

classification, diagnosis and prognosis and illuminate potential therapeutic targets.

Determination of gene expression by real-time quantitative reverse transcriptase PCR is a powerful approach in the comparative analysis of normal and diseased tissues. However, the reliable quantification of gene expression in formalin-fixed, paraffin-embedded tissue has been limited by the quality of mRNA that can be extracted from such samples.[2] Extensive degradation of mRNA can occur before completion of the formalin fixation process. Furthermore the latter causes covalent modification of the mRNA by the addition of monomethylol groups to the bases and strand breakage, resulting in low yields. In addition, formalin fixation causes cross-linkage between nucleic acids and proteins, making subsequent mRNA extraction, reverse transcription and quantification difficult.[3] In recent years, many of these problems have been overcome and amplifiable mRNA can now be extracted successfully from archival specimens.[2,4–6] Yet, despite these advances, histopathology archives remain an under-used resource for gene expression analysis in dermatology research. Many still regard the extraction of useable mRNA from formalin-fixed, paraffin-embedded tissue as impossible, but our own experience belies this view.

We have developed a sensitive and reproducible method for analysing the expression of human endogenous retrovirus K (HERV-K) mRNA in archival skin tissue.[7] RNA was extracted from 1 to 5-year-old formalin-fixed, paraffin-embedded tissues (two sections, 7 μm thick) using the RNeasy formalin-fixed, paraffin-embedded tissue kit, including on-column DNase digestion (Qiagen, Crawley, U.K.). Reverse transcription was preceded by a genomic DNA (gDNA) elimination step to remove residual gDNA, and cDNA synthesis was primed with a mix of oligo-dT and random primers (Quantitect kit; Qiagen). A TaqMan®-based real-time quantitative PCR method for analysing the expression of HERV-K mRNA in archival tissue was developed, using primers spanning an amplicon range of 94–117 base pairs. The small target size minimized the effects of RNA fragmentation. RNA quality and normalization of gene expression were controlled by quantification of the spliced mRNA of the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*). The *HPRT* gene is ubiquitously and consistently expressed in all human cells, and has been shown to be the best reference to standardize gene expression measurements in cancer research.[8] Relative target gene expression was calculated using the formula $2^{-\Delta Ct}$ where ΔCt (cycle threshold) = Ct target gene − Ct *HPRT*.[9] Using this assay, expression of *HPRT* and full-length HERV-K mRNA was detected in 100% of the archival tissues analysed, which comprised eight benign naevi, eight primary melanoma and eight normal skin formalin-fixed, paraffin-embedded tissue samples ($n = 24$). Specificity of the PCR products was confirmed by sequencing. Good intra- and inter-assay reproducibility was observed and parallel experiments omitting reverse transcriptase showed no gDNA contamination.

Our results confirm that real-time quantitative PCR utilizing short amplicons is a sensitive, accurate and reproducible method for the retrospective analysis of gene expression in archival skin tissue. The potential of the histopathology archive as a source of material for gene expression studies can now be explored further.

Ethical approval was obtained for this work from the Royal Marsden Research Ethics Committee.

Acknowledgment

This work was supported by the Spyros Retsas Melanoma Fund, Chelsea and Westminster Healthcare Charities, Westminster Medical School Research Trust and the Skin Treatment and Research Trust (START).

Dermatology, Chelsea and Westminster Hospital, Imperial College School of Medicine, 369 Fulham Road, London SW10 9NH, U.K. — S. Singh†
†Infectious Diseases, Imperial College School of Medicine, London, U.K. — S. Kaye†
‡Histopathology, Charing Cross Hospital, Imperial College School of Medicine, London, U.K. — N.D. Francis‡, D. Peston‡, M.O. McClure†
§Cancer Medicine, Royal Marsden Hospital, London, U.K. — M.E. Gore§, C.B. Bunker*
E-mail: saritasingh@doctors.org.uk

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Key words: *gene expression, formalin-fixed, paraffin-embedded tissue*

Conflicts of interest: none declared.

REVIEW ARTICLE

BJD British Journal of Dermatology

The role of human endogenous retroviruses in melanoma

S. Singh,*† S. Kaye,† M.E. Gore,‡ M.O. McClure† and C.B. Bunker*

*Department of Dermatology, Chelsea and Westminster Hospital, Imperial College Faculty of Medicine, London, U.K.
†Department of Infectious Diseases, Imperial College Faculty of Medicine, London, U.K.
‡Department of Cancer Medicine, The Royal Marsden Hospital, London, U.K.

Summary

Sequencing of the human genome has established that our DNA harbours many endogenous retrovirus (ERV) sequences, remnants of ancestral exogenous retroviral infections fixed in the germline DNA. In recent years, human ERVs (HERVs) have been implicated in melanomagenesis. Retrovirus-like particles and the expression of HERV mRNA and proteins have been demonstrated in melanoma tissue. In addition, antibodies to HERV proteins have been observed in patients with melanoma. *In vitro* and mouse models have provided fascinating insights into the potential mechanisms of HERVs in melanomagenesis. This review considers the evidence associating HERVs with melanoma.

Correspondence
Sarita Singh.
E-mail: saritasingh@doctors.org.uk

Accepted for publication
8 July 2009

Key words
human endogenous retrovirus, melanoma

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2009.09415.x

In 1911, Peyton Rous demonstrated that sarcoma could be transmitted in chickens through a cell-free filtrate.[1] This discovery gave rise to the theory that cancer could be caused by a virus. The transforming agent in the filtrate was subsequently confirmed to be a virus, designated Rous sarcoma virus, and in 1966 Rous was awarded the Nobel Prize for Medicine for his ground-breaking work. It is now established that viruses are the aetiological agents in approximately 15% of cancers, for example, Epstein–Barr virus in nasopharyngeal carcinoma and Burkitt's lymphoma, hepatitis B and C viruses in hepatocellular carcinoma and human herpesvirus 8 in Kaposi sarcoma.[2–5] In the skin, human papillomavirus has been implicated in nonmelanoma skin cancer[6,7] and a novel human polyomavirus, the Merkel cell polyomavirus, has recently been reported in Merkel cell carcinoma.[8]

Retroviruses are well-recognized tumour-inducing agents in animals, hence their initial designation as 'RNA tumour viruses'.[9] Regarding human retroviruses, human T-cell lymphotropic virus (HTLV) type 1 has been shown to cause adult T-cell leukaemia and lymphoma.[10] Our genome harbours many endogenous retrovirus (ERV) sequences, remnants of ancestral exogenous retroviral infections fixed in the germline DNA. In recent years, it has been proposed that human ERVs (HERVs) play a role in the aetiology of certain cancers, including melanoma. In this review, we consolidate the evidence associating HERVs with melanomagenesis.

The origin and classification of human endogenous retroviruses

It is estimated that ERV sequences comprise about 8% of our DNA.[11,12] HERVs integrated into the genome following ancient germline infections by exogenous retroviruses and are thus transmitted vertically as proviruses, rather than by the horizontal transmission characteristic of an infectious virus.[13] They originated in our ancestors at least 25 million years ago, as most of them are also present in the genomes of apes and Old World monkeys. It is unclear whether they were retained because they performed or perform a useful biological function and thus persisted during evolution. For example, the HERV-W envelope gene on chromosome 7 encodes the protein syncytin, thought to play a key role in placenta formation.[14,15]

Over 20 HERV families have been identified.[16,17] They are classified according to the single letter amino acid code for the tRNA specificity of the primer binding site used to initiate reverse transcription.[11,18] Therefore, if it were an infectious virus, HERV-K would use lysine and HERV-W would use a tryptophan tRNA. HERVs have a similar genomic structure to exogenous retroviruses, such as the human immunodeficiency virus (HIV) type 1, but are not infectious (Fig. 1).

HERVs are composed of *gag*, *pol* and *env* genes flanked by two long terminal repeat (LTR) regions. The latter contain sequences that regulate retroviral gene expression. The *gag* and *env* genes encode retroviral capsid and envelope proteins,

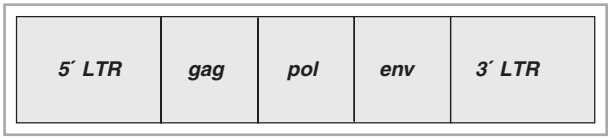

Fig 1. Genomic structure of a human endogenous retrovirus. LTR, long terminal repeat.

respectively. The *pol* gene encodes enzymes necessary for viral replication, namely the reverse transcriptase (RT), integrase and protease.

Human endogenous retrovirus-K

Most HERV sequences are defective due to mutations, deletions or termination signals within coding sequences.[18] Most copies exist as solitary LTRs. Only members of the HERV-K (HML-2) provirus family have open reading frames (ORFs) for all viral genes, and are therefore the most likely to be biologically active and potentially pathogenic.[19,20] Two forms of HERV-K exist: type 2 proviruses are complete, whereas type 1 proviruses have a 292-bp deletion at the boundary of the *pol* and *env* genes. In addition to the genes encoding structural proteins and retrovirus-specific enzymes, there are genes for two accessory proteins, Rec (formerly known as cORF) and Np9. Type 1 proviruses encode Np9, of unknown function but expressed in a number of tumours and transformed cell lines.[21] Type 2 proviruses encode Rec, a nuclear export factor and functional homologue of the HIV and HTLV encoded accessory proteins Rev and Rex, respectively.[22,23]

Genome-wide screening has revealed high levels of insertional polymorphism in the HERV-K (HML-2) family.[16] Insertionally polymorphic HERVs are proviruses that are present only in a proportion of the human population, i.e. some individuals have the insertion (provirus) while other individuals have the empty, preinsertion site (no provirus). HERV-K113 and HERV-K115, both full-length insertionally polymorphic proviruses, are recent additions to the human genome, approximately < 200 000 and 1·2 million years old, respectively.[24] They have ORFs for all their genes, apart from a frameshift mutation in the *gag* gene of HERV-K115. Hence HERV-K113 can potentially encode all elements necessary for a functional retrovirus. To date, 10–13 further insertional polymorphisms of HERV-K (HML-2) have been reported.[16,25–27]

Human endogenous retroviruses and disease

HERVs have been implicated in the pathogenesis of human diseases including multiple sclerosis, type 1 diabetes, rheumatoid arthritis, schizophrenia and certain cancers.[28,29] Regarding skin pathology, there is evidence to suggest that HERVs are associated with systemic lupus erythematosus,[30,31] psoriasis,[30–35] CD30-positive lymphoproliferative disorders[36] and melanoma.[37–54] Putative pathogenic mechanisms for HERVs include disrupting host genes at their integration site, suppressing or stimulating immune responses or pathogenesis induced by expression of accessory proteins such as Rec and Np9.[27,29] However, evidence that these mechanisms occur *in vivo* is lacking. It has been suggested that insertionally polymorphic HERVs are more likely to cause disease.[27] Their recent insertion might have disrupted host genes and, as evolutionarily recent integrations, they are more likely to have retained functional coding sequences that might modify cellular proliferation or immune responses. However, thus far, there have been no significant associations of polymorphic HERVs with disease.[55–57]

A clear-cut role for HERVs in carcinogenesis has not been established, but there is mounting evidence that HERV-K may be involved. HERV-K is transcriptionally active in several cancers, including testicular germ cell tumours (TGCTs),[11,29,58–61] and breast[62–64] and ovarian cancer.[65,66] It has been postulated that HERVs could be involved in carcinogenesis as a result of expression of mRNA, functional proteins or virus-like/retrovirus-like particles (VLPs/RVLPs). It is quite possible that the expression of HERV-K is a bystander effect in carcinogenesis. However, the HERV-K Rec and Np9 proteins bind the promyelocytic leukaemia zinc finger (PLZF) protein which plays a role in spermatogenesis.[67,68] As PLZF can act as a transcriptional repressor, impairment of its function might promote cell proliferation through the activation of proto-oncogenes, such as c-*myc*. Interestingly, mice transgenic for *rec* show neoplastic changes in the testis typical of early seminoma.[69]

Human endogenous retroviruses and melanoma

Evidence that a retrovirus might be involved in melanomagenesis has been accumulating since the 1970s (summarized in Table 1).

Retrovirus-like particles in melanoma

Following the discovery of VLPs in hamster melanoma,[70] evidence was sought for a viral aetiology of human melanoma. RVLPs were first detected in human melanoma by electron microscopy (EM) and biochemically in the 1970s.[37–43] Initially, VLPs resembling C-type virions, and RT activity, were found in lymph node metastases of human melanoma,[37] and subsequently more detailed studies followed. In particular, the biochemical features for oncogenic RNA viruses were sought, namely a single-stranded high molecular weight (70S) RNA, and RNA-dependent DNA polymerase activity.[39,41] Balda *et al.*[41] prepared virus particle-enriched fractions from primary and metastatic melanoma tissue and examined them by 'the simultaneous detection test', popular at that time, for the presence of high-molecular-weight RNA associated with an RT. The authors determined the density of the particles which had a positive simultaneous detection test and found that most of the positive particles showed density characteristics of RNA tumour viruses (retroviruses).

Table 1 Summary of evidence associating human endogenous retroviruses (HERVs) with melanoma

1970s	RVLPs observed in human melanoma tissue and melanoma cell lines by EM and biochemically[37–43]
2002	A HERV-K antigen, presented in association with HLA-A2, on the autologous tumour cells of a patient with melanoma shown to be targeted by CTLs[44]
2003	RVLPs in supernatants of human melanoma cell lines shown to have RT activity, package sequences with homology to HERV-K108, and to contain Gag and Env proteins.[45] Expression of the HERV-K108 *pol* gene and of Gag, Rec and Env proteins demonstrated in primary and metastatic melanoma tissue[45]
2005	Expression of full-length HERV-K mRNA and spliced *env* and *rec* mRNA demonstrated in metastatic melanoma tissue and cell lines. HERV-K TM protein and HERV-K Gag expression detected in primary and metastatic melanoma samples by immunohistochemistry.[46] RVLPs in melanoma cell line supernatants shown to be defective and noninfectious[46]
	ERV expression shown to be required for murine melanoma tumour growth *in vivo* by RNAi experiments. Proposed that ERVs promote melanomagenesis by a regulatory T cell-mediated subversion of immunosurveillance[48]
	Inhibition of endogenous RT by RT inhibitors and RNAi shown to antagonize melanoma growth *in vivo* in mouse experiments[49]
2006	Expression of spliced *env*, *rec* and *Np9* mRNA demonstrated in metastatic melanoma tissue and melanoma cell lines by RT-PCR. TM and Rec, but not Np9, protein expression demonstrated in metastatic melanoma tissue by immunohistochemistry[50]
	Antibodies directed against HERV-K TM and Env proteins reported retrospectively in sera from patients with melanoma.[50,51] However, HERV-K Rec and Np9-specific antibodies not detected[50]
	Characterization of the MelARV proviruses present in the murine primary B16 melanoma cell line and a metastatic subline revealed mobility of the element with new proviral insertions targeting critical genes and altering their transcriptional profile, namely *itga2* and *dok5*, that are likely to be involved in cell mobility and metastatic spread[52]
2007	RVLP-derived sequences found to be defective and to vary between different melanoma cell lines. Particles from the same cell line contained heterologous sequences[53]
2008	Anti-HERV-K Gag and Env antibodies detected retrospectively in 16% of sera from patients with melanoma but not in sera from healthy controls.[54] Anti-HERV-K reactivity elevated in patients with acrolentiginous, mucosal and uveal melanoma compared with patients with lentigo maligna, and nodular and superficial spreading melanoma. Serological HERV-K reactivity shown to be an independent marker of reduced survival probability

RVLPs, retrovirus-like particles; EM, electron microscopy; CTLs, cytolytic T lymphocytes; RT, reverse transcriptase; TM, transmembrane envelope; ERV, endogenous retrovirus; RNAi, RNA interference; PCR, polymerase chain reaction; MelARV, melanoma-associated retrovirus.

More recently, RVLPs have been demonstrated in melanoma cell line supernatants by EM.[45,46] They were shown to have RT activity, package sequences with homology to HERV-K108, and to contain Gag and Env proteins.[45] No RT activity was detected in supernatants from cultured human neonatal melanocytes (NEHM), indicating that the production of particles containing RT is exclusive to melanoma. However, these RVLPs were found to be defective and noninfectious.[46] Another study, which aimed to characterize particle-derived sequences, found them to be defective and to vary between different melanoma cell lines: even particles from the same cell line contained heterologous sequences.[53]

Human endogenous retrovirus-K mRNA and protein expression in melanoma

In 2003, expression of the *pol* gene and of Gag, Rec and Env proteins was demonstrated in primary and metastatic melanoma tissue.[45] Surgical touch preparations of primary melanomas, lymph node metastases and cutaneous metastases, studied by *in situ* hybridization, showed high copy numbers of the HERV-K108 *pol* sequence in tumour cells. Lymph node and benign naevus tissue samples from healthy individuals were negative. Using immunofluorescence, retroviral proteins were found to be expressed in melanoma cell lines and all primary melanomas ($n = 9$), lymph node and cutaneous metastases but in only one of 25 naevi and none of the benign lymph nodes or cultured NEHM.

Büscher *et al.*[46] investigated the expression of HERV-K mRNA in metastatic melanoma tissue and melanoma cell lines by RT-polymerase chain reaction (PCR). Expression of full-length mRNA derived from type 1 and 2 HERV-K proviruses was detected in all tissues and cell lines investigated. Expression of spliced *env* and *rec* was found in 45% of melanoma metastasis samples, and in 44% of melanoma cell lines. In cultured NEHM, spliced *rec* was detected in the absence of spliced *env*. Expression of retroviral proteins was studied in a melanoma cell line by immunofluorescence and Western blot analyses, and in metastatic and six primary melanoma samples by immunohistochemistry. Expression of HERV-K transmembrane envelope (TM) protein was observed in the cell line. However, in contrast to the findings of Muster *et al.*,[45] HERV-K TM protein expression was detected in approximately 50% and HERV-K Gag in approximately 70–80% of primary and metastatic melanoma samples.

The expression of TM, Rec and Np9 mRNA and proteins has been studied in further detail.[50] As discussed, these proteins are of particular interest because Rec and Np9 are poten-

tially oncogenic and TM is putatively immunosuppressive.[29] Env-specific, Rec-specific and Np9-specific antisera were generated, characterized and used to study protein expression in metastatic melanomas and melanoma cell lines. Analogous to previous findings, spliced *env* and *rec* mRNA was detected by RT-PCR in 39% of metastatic melanoma samples and in 40% of melanoma cell lines, and *np*9 mRNA was detected in 29% and 21%, respectively. Immunohistochemical analyses showed 38% of the metastatic melanoma samples to be positive for TM protein, 14% for Rec but none for Np9.

Immunological evidence

Nearly three decades after the early EM studies, interest in the potential association of retroviruses with human melanoma was reignited when a HERV-K antigen, presented in association with the HLA class I molecule HLA-A2 on the autologous tumour cells of a patient with melanoma, was shown to be targeted by cytolytic T lymphocytes (CTLs).[44] The antigenic peptide, comprising nine amino acids, was found to be encoded by a short ORF in the *env* region of a defective HERV-K (HML-6) provirus. The gene encoding the antigen, designated *HERV-K-MEL*, was expressed in most samples of cutaneous and ocular melanoma tested, but also in a majority of benign naevi and in some normal skin samples. The authors suggested that *HERV-K-MEL* is a source of antigens that are targeted by CTLs in patients with melanoma and could therefore be used for vaccination. Intriguingly, a multicentre case–control study revealed a reduced risk of melanoma associated with bacille Calmette-Guérin and/or vaccinia vaccination in early childhood and/or with infectious diseases later in life.[71] Consequently, Krone *et al.*[47] postulated that these vaccinations or infections, shown to have analogues of the *HERV-K-MEL* epitope, generate populations of cross-reactive T cells that participate in immunosurveillance and repair or kill melanocytes expressing this antigen.

Conversely, it has been proposed that HERVs promote melanomagenesis by a regulatory T cell-mediated subversion of immunosurveillance.[48] In general, cancers may arise from a population of transformed cells that have developed the ability to escape immunosurveillance.[72,73] Using RNA interference (RNAi), Mangeney *et al.*[48] demonstrated that knocking down the melanoma-associated retrovirus (MelARV), an ERV that is spontaneously induced in B16 murine melanoma,[74] resulted in the rejection of tumour cells in immunocompetent mice. Re-expression of the sole MelARV *env* gene in the knocked-down cells partially ablated tumour rejection, thus indicating that the *env* gene contributes to tumour immune escape. The authors hypothesized that ERV expression by the B16 melanoma is directly responsible for regulatory T-cell induction, thus resulting in tumour progression. Supporting this, adoptive transfer of regulatory T cells restored tumour progression in the knocked-down cells, suggesting that they are key to the ERV-mediated effect. Interestingly, several of the fully coding HERV *env* genes are immunosuppressive *in vivo* in a murine model and, the authors have postulated, might promote tumour escape in humans.[75]

Regarding human melanoma, there is evidence that the expression of HERV-K proteins may induce humoral immune responses. Antibodies directed against retroviral proteins have previously been reported in patients with TGCTs and may be of diagnostic and prognostic value in these patients.[61,76,77] Büscher *et al.*[46] detected antibodies specific for HERV-K TM protein in 22% of sera from 60 patients with metastatic melanoma by Western blot analyses. Sera from 20 normal blood donors and patients with alopecia were negative. However, HERV-K Rec- and Np9-specific antibodies were not detected in the sera of patients with metastatic melanoma nor in sera from control normal blood donors and patients with alopecia.[50] Humer *et al.*[51] confirmed that some patients with melanoma have antibodies against the Env protein of HERV-K. Following prediction analyses of potential epitopes on HERV-K, they identified an immunodominant epitope on the Env protein that is recognized by antibodies from sera of patients with melanoma. The prevalence of antibodies was significantly higher in sera from 81 patients with melanoma with American Joint Committee on Cancer (AJCC) stage I–IV disease, compared with 95 control sera from healthy individuals.

In a recent study to investigate the prognostic relevance of serological anti-HERV-K reactivity in melanoma, anti-HERV-K Gag and Env antibodies were detected retrospectively in 51 of 312 randomly selected and blinded sera from patients with melanoma and in none of 70 sera from healthy controls.[54] Anti-HERV-K reactivity was elevated in patients with acrolentiginous, mucosal and uveal melanoma (melanoma subtypes that occur at sun-protected sites) compared with patients with lentigo maligna, and nodular and superficial spreading melanoma. Patients with anti-HERV-K antibodies had a significantly decreased disease-specific overall survival. Serological HERV-K reactivity was shown to be an independent marker of reduced survival probability in patients with melanoma with AJCC stages I–III.

Other studies

Sciamanna *et al.*[49] have investigated whether high levels of endogenous RT activity are directly linked to cell transformation. They inhibited RT activity in melanoma cell lines using two methods: pharmacological inhibition by two RT inhibitors (nevirapine and efavirenz) and downregulation of expression of RT-encoding LINE-1 (long interspersed elements) by RNAi. Both treatments reduced proliferation, induced morphological differentiation and reprogrammed gene expression. These features were reversible upon discontinuation of the anti-RT treatment, suggesting that RT contributes to an epigenetic level of control. Additionally, inhibition of RT activity *in vivo* antagonized tumour growth in mouse experiments. In further experiments, HERV-K interfered melanoma cells were found, *in vivo*, to have reduced tumorigenic potential after inoculation in nude mice.[78] Based on previous studies, the authors suggested that HERV-K expression may favour tumour progression by reducing immunosurveillance in mice.

Characterization of the MelARV proviruses present in the primary B16 melanoma cell line and a subline selected *in vivo* for increased metastatic activity revealed mobility of the element with new proviral insertions targeting critical genes and changing their transcriptional profile.[52] The genes specifically targeted by new proviral insertions in the metastatic tumour cells, namely integrin α2 (*itga2*) and docking protein 5 (*dok5*), are likely to be involved in cell mobility and metastatic spread. Integrins are cell membrane proteins that play a vital role in cell adhesion and signal transduction. Fascinatingly, the development of the vertical growth phase in human melanoma is associated with loss of expression of cell adhesion and extracellular matrix molecules including integrin α2.[79] Thus MelARV can act both at the genetic level, inducing mutations by insertion (insertional mutagenesis) and at the epigenetic level, promoting immunosuppression of the host. The authors proposed that these properties may also be relevant to human melanoma although there is no firm evidence to support this.

Conclusion

Although there is theoretical and laboratory evidence to implicate HERV-K in melanomagenesis, an aetiopathogenic role has not yet been proven. It is possible that expression of HERV-K mRNA and proteins, and formation of RVLPs, are epiphenomena associated with tumorigenesis. Moreover, HERV-K transcripts have been reported in normal skin, benign naevi and NEHM.[44,46] Work so far has focused on melanoma cell lines and metastasis tissue, and further comparative investigations of HERV-K expression in primary melanoma and normal skin are required. In addition, *prospective* studies investigating humoral anti-HERV-K immune responses are required to support the current evidence that serological HERV-K reactivity provides prognostic information additional to that of established melanoma markers.[54]

Given their ubiquity in the population, it is difficult to assign a pathological role to the HERVs investigated so far. Perhaps the contribution of individual proviral loci rather than the HERV-K family as a whole should be examined. In this respect, insertionally polymorphic HERVs merit further investigation as they may represent novel genetic risk factors for melanoma. Importantly, there has been a recent drive towards a molecular classification of melanoma as a result of molecular genetic studies demonstrating specific genotype–phenotype correlations.[80] Curtin *et al.*,[81] for example, demonstrated a significant correlation of ultraviolet radiation exposure and melanoma site to *BRAF/NRAS* gene mutations and additional genetic events using array-based comparative genomic hybridization. Investigation of the association of insertionally polymorphic HERVs with melanoma, and correlation of HERV expression in primary melanoma with clinicopathological subtype, site and Breslow thickness may add to this molecular classification.

In vitro and mouse models have provided fascinating insights into the potential mechanisms of HERVs in melanomagenesis. However, oncogenic viruses cause cancers by inducing genetic mutations directly or by expressing oncoproteins[82] and there is little direct evidence that HERVs act through these mechanisms. Further functional analyses of the HERV-K accessory proteins Rec and Np9 are required to ascertain whether they are important in the causation of melanoma.

Key points

1 ERV sequences comprise about 8% of the human genome.
2 Expression of HERV-K mRNA and proteins has been observed in human melanoma cell lines and tissue.
3 Antibodies directed against HERV-K proteins have been reported in patients with melanoma and may be of diagnostic and prognostic value.
4 Although there is theoretical and laboratory evidence to implicate HERV-K in melanomagenesis, an aetiopathogenic role has not yet been proven and further studies are required.

Acknowledgments

The following funding sources are acknowledged: Spyros Retsas Melanoma Fund, Chelsea and Westminster Healthcare Charities, Westminster Medical School Research Trust and Skin Treatment and Research Trust (START).

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Comprehensive characterization of skin microbiota in patients with atopic dermatitis and normal subjects using T-RFLP method

I Dekio,[1,2,3] M Sakamoto,[2] H Hayashi,[2,5] T Ebihara,[1] E Morita,[3] M Suematsu,[4] M Amagai[1] and Y Benno[2] *1 Department of Dermatology, School of Medicine, Keio University, Shinjuku-ku, Japan, 2 Microbe Division / Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Japan, 3 Department of Dermatology, School of Medicine, Shimane University, Izumo, Japan, 4 Department of Biochemistry & Integrative Medical Biology, School of Medicine, Keio University, Shinjuku-ku, Japan and 5 Faculty of Engineering, Maebashi Institute of Technology, Maebashi, Japan*

Our previous study using bacterial 16S rRNA gene-based clone libraries revealed that the microbiota in healthy human skin include uncultured microorganisms, although the microorganisms in the skin exposed to the disease conditions remain to be examined. To compare the profiles of skin microbiota in 13 patients with atopic dermatitis (AD) and 10 healthy controls, terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA gene was applied to 23 swab-scrubbed samples from facial skin. T-RFLP method successfully revealed the complex bacterial members of the skin microbiota as peak patterns of capillary electrophoresis of terminal restriction fragments (T-RFs). Each T-RF peak reflected a microorganism, and the microorganism to which each peak was assigned could be predicted by computer simulation of T-RF length using the nucleotide sequence data of bacterial species residing in the skin. Among 18 species detected in the present study, Stenotrophomonas maltophilia was detected significantly more common in AD patients (5/13 for AD patients vs 0/10 for controls), whilst Dietzia maris was detected significantly more common in normal controls (8/10 for controls vs 2/13 for AD patients). Moreover, Streptococcus species, which are considered uncommon in uninfected skin, were detected in 7 patients and 8 normal controls. Although further study should be undertaken to investigate the roles of these microorganisms in AD, the microbiota were presumed to include hitherto uninvestigated bacterial species in the major population of patients with AD and healthy controls.

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The lysate of allogenic apoptotic non-infected macrophages diminishes *Leishmania major* burden by involvement of Foxp3-positive regulatory T-cells

OE Akilov,[1,2] Y Jin,[1,2] Z Zhou,[1,2] MX Wu[1,2] and T Hasan[1,2] *1 Dermatology, Harvard Medical School, Boston, MA and 2 Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA*

Parasites of the genus *Leishmania* cause substantial mortality and morbidity in the developing world. While numerous treatment modalities exist, there is no ideal therapy for leishmaniasis. The purpose of or study was elucidate the mechanisms of regulation of the intracellular parasite clearance by macrophages (Mϕ) during cutaneous leishmaniasis (CL). BALB/c mice received 3 injections subcutaneously with 2-week intervals in between with cell lysate obtained from non-infected bone marrow-derived Mϕ after photodynamic therapy with EtNBSe (PDT). The PDT regimen was chosen in this way that death of Mϕ occurred through apoptotic pathway. The control group of mice received PBS injections. Two weeks after the last injection mice were infected intradermally in the ear with 10^6 metacyclic promastigotes of *L. major*. Mice of the Mϕ+PDT group showed a delayed onset of disease and contained ~ 3.1-logs less parasites than did the controls ($p < 0.05$). Lower levels of cytokines TNF-α and IL-6 (cytometric bead array), and lower levels of chemokines CCL2/MCP-1 and CCL22/MDC (ELISA) were observed in the ear tissues of the mice that received a lysate than did the controls. A percentage of IFN-γ-producing T cells in the draining lymph nodes of the Mϕ+PDT mice was higher than in the control group. We found that a Mϕ death in CL lesions was a result of the attack of the immune system (caspase-3 activation on IHC) than cytopathic effect of the intracellular parasitism. Flow cytometric analysis revealed higher number of F4/80+ (Mϕ) and CD4+CD25+Foxp3+ T regulatory (T reg) cells in the lesions of the Mϕ+PDT mice than did the controls. Correlation analysis showed that the percentage of Mϕ in the skin directly correlates (r = 0.93) with number of T reg. These results demonstrate that T reg may play a role in a reduction of the parasite burden through before unknown mechanisms of the regulation of the Mϕ survival in the inflammatory infiltrate.

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Assessing microbial burden in chronic wounds: comparison of routine cultures, quantitative microbiology, and molecular techniques

YM Frankel,[1] JH Melendez,[2] L Price,[2,3] N Wang,[1] J Zenilman[2] and GS Lazarus[1] *1 Dermatology, Johns Hopkins Medical Institutions, Baltimore, MD, 2 Infectious Diseases, Johns Hopkins Medical Institutions, Baltimore, MD and 3 TGEN, Flagstaff, AZ*

Wounds account for >50% of direct medical costs for skin diseases in the U.S. Expensive systemic antibiotics are commonly prescribed based on qualitative culture (few/moderate/heavy growth). We studied microbial burden in chronic wounds determined by qualitative, quantitative microbiology, real-time PCR (RT-PCR), and metagenomic analysis. We evaluated microbial populations across wounds. 41 chronic wounds in 28 out-patients were assessed. We curetted leading wound edge using a 3mm curette. 12 wounds were evaluated at multiple sites (rim/rim; rim/center). 12 samples were analyzed using 16S clone libraries. Quantitative culture (CFU/gm) was used as gold standard for analyses. Minimum tissue for reproducible measurements was 20mg. 11/14 (79%) of negative qualitative cultures were positive on quantitative microbiology. Qualitative results (Not present/Few/Moderate/Heavy) did not correlate with quantity of organisms on quantitative culture. Our RT-PCR analysis was 92% sensitive. In 8/11 (73%) specimens negative by quantitative culture, RT-PCR identified organisms which could not be grown on culture. 16S clone library analysis demonstrated substantially more complex bacterial communities than culture or PCR. Probability for rim-center pair bacterial counts within 1 log was 95.9% (95% CI: 78.6%-99.3%, p=.0008). MRSA (44.8%) was the most common pathogen, followed by Pseudomonas aeruginosa (27.6%) and Group B Streptococcus (27.6%). There was remarkable consistency of bacterial populations across a wound. Qualitative culture results did not correlate with quantitative results. RT-PCR was a sensitive tool for rapid identification and characterization of bacteria in chronic wounds. 16S clone library analyses make it clear that bacterial populations in wounds are much more complex than demonstrated by culture. The role of microbial flora in wound healing needs to be reassessed using these new analytic methods.

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Pathomorphological characteristics of Malassezia folliculitis

V Kazlouskaya, O Myadeletz and U Adaskevich *Vitebsk State Medical University, Vitebsk, Belarus*

Malassezia folliculitis is a common pathology seen in young patients. The condition is frequently misdiagnosed or mixed with acne. Administration of antibiotics in such cases does not help to improve the clinical status. Usually small multiple whitish pustules are seen, comedons are absent. Malassezia folliculitis is often associated with seborrhoeic dermatitis of the scalp and skin. Frequently the condition causes psychological problems and sometimes severe itch. Biopsy is performed in severe cases with resistance to therapy and unusual location of folliculitis. Although some investigators founded Malassezia cells in the hair follicules, the pathogenesis of folliculitis development is not fully known. We observed 157 patients (133 men and 24 women) with the clinical picture of folliculitis. Middle age was 21,2±8,5 years. Content of the follicles was extracted and studied by direct microscopic examination and cultured in the Saboraud agar with addition of sterile olive oil. Malassezia was found to be a reason of folliculitis in 47 patients (29,9%). Skin biopsy with STIEFEL 5 mm diameter punch was performed in the skin of the back. Specimen where stained with Gimza staining method. In the surface of the stratum corneum basophilic cells of fungi of different shape are founded (round, oval, S-ahaped). Several cells are founded in stratum spinosum but their size is usually smaller than of those in the upper layers. There is no cell reaction around Malassezia in the epidermis. In derma hyphae of Malassezia are founded, surrounded by infiltrates composed of lymphocytes, macrophags, neutrophyls, and plasmocytes. Malassezia cells are founded also in the ducts of seborrhoeic and sweet glands, and around fat cells situated under the arteriols. This pathomorphological picture suggests that Malassezia yeasts affect not only superficial skin layers but also dermal structures. In Malassezia folliculitis not only yeasts but also hyphae that is a pathogenic form are seen in the specimens. Inflammation infiltrate is seen in deeper layers of the skin that explains the effect of systemic antifugal drugs.

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TLR2 and Nod2 activation increases HIV susceptibility and augments viral replication in Langerhans cells, but not in dermal type Dendritic cells: implications for sexual transmission of HIV

Y Ogawa,[1] T Kawamura,[1] A Blauvelt[2] and S Shimada[1] *1 Dermatology, University of Yamanashi, Yamanashi, Japan and 2 Dermatology, Oregon Health & Science University, Portland, OR*

Numerous studies have shown a higher risk of acquiring HIV infection in the presence of other sexually transmitted diseases (STD), however, biologic mechanisms responsible for enhanced HIV acquisition are unclear. Recent studies have documented Langerhans cells (LC) as initial cellular targets in sexual transmission of HIV. Here, we studied whether microbial components augment HIV infection in LC by activating TLR and Nod pattern recognition receptors. Monocyte-derived LC (mLC) expressed TLR1, 2, 3, 6, and Nod1 and 2, and induced maturation and cytokine production in response to ligand activation through these receptors. Next, mLC were incubated with TLR1-9 and Nod1 and 2 ligands for 24 h before or after HIV infection to assess effects on HIV susceptibility and replication in these cells. Surprisingly, TLR2 (heat-killed *Listeria monocytogenes*) and Nod2 (MDP) ligands dramatically enhanced both HIV susceptibility and replication in mLC, whereas other TLR and Nod ligands did not affect HIV infection. The same infection-enhancing effects were observed when mLC were incubated with other related bacterial components (lipoprotein, LTA, PGN, and whole Gram+ bacteria). Importantly, TLR2 and Nod2 ligands also significantly increased HIV susceptibility in freshly isolated human epidermal LC. In contrast, these ligands decreased both HIV susceptibility and replication in monocyte-derived dendritic cells (mDC). Regarding mechanisms underlying increased HIV infection in LC, we found that TLR2 and Nod2 activation resulted in significant up-regulation of cell surface CD4 on mLC, but not on mDC. Furthermore, in mLC, the activation of these ligands significantly decreased mRNA expression of APOBEC3G/3F, an HIV suppressor factor. Given these data, we hypothesize that ligation of TLR2 and Nod2 by Gram+ bacterial products may underlie enhanced sexual transmission of HIV in the setting of concomitant bacterial STD infection.

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Detection and quantification of gene expression in archival formalin-fixed, paraffin-embedded skin tissue

CB Bunker,[1] S Singh,[1,2] S Kaye,[2] ND Francis[3] and MO McClure[2] *1 Dermatology, Chelsea and Westminster Hospital, ICSM, London, United Kingdom, 2 Infectious Diseases, ICSM, London, United Kingdom and 3 Histopathology, Charing Cross Hospital, ICSM, London, United Kingdom*

Archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens are an excellent resource for retrospective studies. However, the reliable quantification of gene expression in FFPE tissue has previously been subject to limitation due to the quality of RNA that can be extracted from such samples. We aimed to develop a sensitive and reproducible method to analyse the expression of Human Endogenous Retrovirus K (HERV-K) mRNA in archival tissue. This method consisted of real time quantitative TaqMan PCR using primers spanning an amplicon range of 94 to 117 base pairs. Because of the small target size, this approach was optimal for quantitative determination of gene transcript levels in FFPE tissue in which RNA is fragmented. RNA was extracted from FFPE samples using the RNeasy FFPE kit, including on-column DNase digestion (Qiagen). RNA quality and normalisation of gene expression were controlled for by quantification of the spliced mRNA of the housekeeping gene *Hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) that has been shown previously to be ubiquitously and consistently expressed in all human cells. Primers and probes for *HPRT* were designed to span an intron to prevent annealing to genomic DNA. Using this method, expression of *HPRT* was detected in 100% of archival tissues analysed comprising 8 benign naevi, 8 primary melanoma and 8 normal skin FFPE samples (n=24). Relative target gene expression was calculated using the formula $2^{-\Delta Ct}$ where ΔCt (Cycle threshold) = Ct *target gene* -Ct *HPRT*. HERV-K full length mRNA was found to be expressed at significantly higher levels than *HPRT* in all samples. Good intra- and inter-assay reproducibility was observed. These results suggest that quantitative real time PCR utilising short amplicons is an ideal tool for retrospective analysis of gene expression in archival skin tissue.

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Expression of human endogenous retrovirus K in melanoma

S Singh,[1,2] S Kaye,[2] ND Francis,[3] MO McClure[2] and CB Bunker[1] *1 Dermatology, Chelsea and Westminster Hospital, ICSM, London, United Kingdom, 2 Infectious Diseases, ICSM, London, United Kingdom and 3 Histopathology, Charing Cross Hospital, ICSM, London, United Kingdom*

Human Endogenous Retroviruses (HERVs) have been implicated in the pathogenesis of cutaneous malignant melanoma (MM). HERV sequences comprise about 8% of the human genome and represent a reservoir of potentially pathogenic genes. The aim of this study was to detect and quantify the expression of HERV-K *pol*, *env* and *rec* genes in human MM cell lines and archival primary MM. A new real-time quantitative PCR method to analyse the expression of HERV-K mRNA in archival, formalin-fixed paraffin-embedded (FFPE) tissue was developed, utilising short amplicons to avoid problems of RNA fragmentation. Specificity of the PCR-amplified products was confirmed by sequencing. Studies of HERV-K gene expression in two MM cell lines (WM2664 and A375) showed expression of full-length but not spliced *env* or *rec* mRNA in WM2664, whereas expression of full-length and spliced *rec* mRNA, but not spliced *env* mRNA, was observed in A375. Full-length mRNA and unspliced *env* mRNA was found to be expressed in all archival tissue samples studied, comprising benign naevi (n = 8) and primary MM (n = 8). No correlation was observed between the levels of expression and the clinico-pathological subtype or Breslow thickness of MM. There were no significant differences in the levels of expression of *pol* and unspliced *env* between naevi and MM. None of the naevi but 2/8 MM expressed *rec*. The expression of spliced *rec* mRNA in primary MM and benign naevi has not previously been reported. Rec, a viral accessory protein, physically and functionally interacts with the promyelocytic leukemia zinc finger (PLZF) tumour suppressor and it has been suggested that Rec may be oncogenic by derepressing c-myc through the inhibition of PLZF. This work has successfully explored a new real-time PCR method that can quantify HERV-K gene expression in FFPE samples. It has been used to observe full length HERV-K mRNA expression in MM cell lines and all primary MM and benign naevi studied. The expression of *rec* by some MM requires further investigation.

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CD147/basigin plays an important role in tumor glycolysis in association with monocarboxylate transporter

J Su,[1,2] X Chen[2] and T Kanekura[1] *1 Department of Dermatology, Field of Sensory Organology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan and 2 Department of Dermatology, XiangYa Hospital, Central South University, Changsha, China*

Cancer cells require glycolysis for cellular energy resulting in excessive levels of production and the secretion of lactate. Lactate reduces extracelluar pH and then contributes to the invasion, angiogenesis, metastasis, and multi-drug resistance of tumor cells. The human cell-surface molecule CD147/basigin is over-expressed in many tumor cells and has been implicated in a specific- and strong interaction with the monocarboxylate transporter-1 (MCT1) that mediates the transport of lactate through the plasma membrane. Here, we examined whether CD147/basigin, highly expressed in melanoma cells, is involved, via the association with MCT1, in the transport of lactate, the end product of glycolysis. We used RNAi technology to knock down the expression of CD147 in A375 melanoma cells, and RT-PCR and Western blots to study its expression. The co-localization of CD147 and MCT1 on the cell membrane was determined by confocal microscopy analysis. The rate of glycolysis was assessed by measuring the extracelluar lactate level spectrophotometrically. We found that A375 cells manifested remarkably higher CD147 and MCT1 expression than normal human melanocytes (NHMC), and CD147/basigin and MCT1 were co-localized on the A375 cell membrane. The silencing of CD147/basigin in A375 cells clearly down-regulated the expression of MCT1 and abrogated the co-localization of CD147/basigin and MCT1. The glycolysis rate was remarkably higher in A375 cells than NHMC ($p<0.001$); it was significantly decreased ($p<0.001$) by inhibiting the expression of CD147. Our findings strongly suggest that highly-expressed CD147 interacts with MCT1 on the plasma membrane and plays a key role in tumor-cell glycolysis. The novel function of CD147/basigin in tumor-cell glycolysis may be fundamental in tumorigenesis and may provide new insights into the prevention of tumorigenesis and progression of malignant melanoma in association with CD147/basigin.

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The involvement of p53 in senile lentigo

D Murase, Y Amano, A Ohuchi and T Kitahara *Kao Biological Science Laboratories, Tochigi, Japan*

Benign pigmentations were once a focus in the cosmetic field as aesthetic defects, but now, this phenomenon draws more attention from outside the cosmetics world. Recent studies revealed the mechanism that p53, a tumor-suppressor protein, promotes pigmentation following UV irradiation by direct transcriptional activation of pro-opiomelanocortin (POMC) in the skin. Given that p53 can be activated by various kinds of cellular stress including aging, this finding may explain the occurrence of age spots (senile lentigo). The aim of this study was to confirm the involvement of p53 in the hyperpigmentation in senile lentigo (SL). First, we investigated the presence of p53 by immunohistochemistry in lesional and perilesional skins. As a result, we found stronger expression of p53 in the lesional epidermis than in the perilesional epidermis. To examine the impact of the difference in p53 expression levels, we analyzed gene expressions of p53 transcriptional targets (CDKN1A, GADD45A, MDM2) in both epidermis and determined that expression levels of these genes were significantly higher in the lesional epidermis than in the perilesional epidermis. We then speculated that p53 contributes to melanogenesis via the paracrine pathway involved in the epidermal hyperpigmentation. Therefore, we assessed the expression of stem cell factor (SCF) and endothelin-1 (ET-1) in cultured keratinocytes treated with and without 5-fluorouracil (5-FU), a known inducer of p53. As a result, 5-FU activated p53, which in turn resulted in increased gene expressions of these cytokines. Our findings suggest that accumulation of p53 caused by long-term sun exposure and aging regulates chronic hyperpigmentation via the paracrine melanogenic cytokines.

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Persistent molecular changes after repetitive *in situ* UV exposure of human skin

M Brenner,[1] SG Coelho,[2] JZ Beer,[3] SA Miller[3] and VJ Hearing[2] *1 Department of Dermatology, Ludwig-Maximilian University of Munich, Munich, Germany, 2 Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD and 3 Center for Devices and Radiological Health, Food and Drug Administration, Silver Spring, MD*

The incidence of melanoma has tripled in the past 4 decades, and epidemiological and laboratory data provide strong evidence that UV radiation is a major causative factor for this malignancy. We investigated long-term consequences of repetitive UV exposures of human skin in situ. We examined two sets of biopsies taken 1- 4 yrs after repetitive UV exposures to 95% UVA/5% UVB (Source 1, 6 subjects) or with Solar Simulated Radiation (Source 2, 4 subjects). Changes in protein expression of melanogenic factors (tyrosinase, MART-1, MITF), growth factors and their receptors (SCF, c-kit, bFGF, FGFR-1, ET-1, ETBR, HGF, GM-CSF), adhesion molecules (β-catenin, E-, and N-cadherins), cell cycle proteins (PCNA, cyclins D1 and E2) as well as Bcl-2, DKK-1 and DKK-3 were analyzed by fluorescence immunohistochemistry. Changes in RNA levels (for ETBR) were detected by tissue in situ hybridization. More than 1 yr after UV exposure 1/6 of Source 1 and 3/4 of Source 2-irradiated samples still showed a significantly increased expression of tyrosinase. Most of the molecular markers examined showed no detectable changes in their expression at >1 yr after UV irradiation. While an increase in ETBR protein expression was detected in 3/10 UV-treated (2/6 Source 1 and 1/4 Source 2) subjects, ET-1 protein expression and ETBR mRNA expression showed no change. In summary, among 20 factors examined, only tyrosinase and ETBR protein expression, and only in some subjects, were elevated at >1 yr post UV exposure. This suggests that persistent post-UV exposure changes are very minor and show a considerable subject-to-subject variation. A possibility that changes in the expression of the ETBR protein trigger downstream activation of abnormal melanocyte proliferation (and, hence, might potentially lead to melanoma) deserves further investigation.

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Tris (dibenzylideneacetone) dipalladium (Tris DBA), an N-myristoyltransferase 1 inhibitor, is effective against melanoma growth *in vitro* and *in vivo*

JL Arbiser,[1] S Bhandarkar,[1] J Bromberg,[2] P Selvakumar,[3] RK Sharma,[3] L Fried[1] and B Govindarajan[1] *1 Emory University, Atlanta, GA, 2 Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York, NY and 3 Pathology, Saskatoon Cancer Center, Saskatoon, SK, Canada*

Melanoma is a solid tumor that is notoriously resistant to chemotherapy, and its incidence is rapidly increasing. Recently, several signaling pathways have been demonstrated to contribute to melanoma tumorigenesis, including constitutive activation of MAP kinase, Akt and Stat-3. The activation of multiple pathways may account in part for the difficulty in treatment of melanoma. In a recent screen of compounds, we found that an organopalladium complex showed significant antiproliferative activity against melanoma cells. This complex, tris (dibenzylideneacetone) dipalladium (Tris DBA), has activity against B16 murine and A375 human melanoma *in vivo*. Tris DBA inhibits several signaling pathways including activation of MAP kinase, Akt, Stat-3 and S6 kinase activation, suggesting an upstream target. Tris DBA was found to be a potent inhibitor of N-myristoyltransferase 1 (NMT-1), which is required for optimal activity of membrane based signaling molecules. Perhaps the most well studied membrane signaling molecule is c-src, which may be upstream of MAP kinase, Akt, Stat-3 and S6 kinase. Tris DBA treatment decreased expression of c-src. Tris DBA is thus a novel compound that is a member of a class of noble metal complexes with potential antitumor activity. Further preclinical evaluation of Tris DBA and related complexes is warranted.

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Effective immunotherapy with α-GalCer-loaded embryonic stem cell-derived dendritic cells expressing multiple antigens against B16 melanoma

S Fukushima,[1,2] H Ihn,[1] Y Nishimura[2] and S Senju[2] *1 Dermatology & Plastic and Reconstructive Surgery, Kumamoto University, Kumamoto, Japan and 2 Immunogenetics, Kumamoto University, Kumamoto, Japan*

Genetically manipulated dendritic cells (DCs) expressing cancer antigens are considered to be a promising means for cancer immunotherapy. However, many cancer vaccine trials using DCs expressing cancer antigens have been reported without a satisfactory result. It is expected that in vivo transfer of DCs simultaneously presenting cancer antigens and α-galactosylseramide (α-GalCer) stimulate both tumor reactive T cells and NKT cells, thus resulting in a potent anti-cancer immunity. We have established a method to generate dendritic cells from murine embryonic stem cells (ES-DCs). Genetic modification of murine ES-DCs can be accomplished without the use of viral vectors, by the introduction of gene-expression vector plasmids into undifferentiated ES cells by electroporation and subsequent induction of differentiation of the transfectant ES cell clones to ES-DCs. ES-DC technology therefore provides us a novel means for cancer immunotherapy. We examined the effects of multiple antigen-targeted immunotherapies by ES-DCs expressing melanoma associated antigens (GPC3, SPARC, TRP2 and hgp100) expressed in B16 melanoma. CTLs specific to each antigen were sensitized by the *in vivo* transfer of ES-DCs transfected with either murine GPC3, SPARC, TRP2 or hgp100 gene. The simultaneous *in vivo* transfer of these ES-DCs protected the recipient mice from a high dose subcutaneous challenge with B16-F10 melanoma. Then, the effect of α-GalCer-loaded ES-DCs on peritoneal dissemination and spontaneous lung metastasis of B16-BL6/Luc, a melanoma stably expressing luciferase, was evaluated. α-GalCer-loaded ES-DCs expressing multiple antigens showed a significantly greater effect on inhibiting the growth of tumor cells than vehicle loaded ES-DCs or α-GalCer loaded ES-DCs without antigens. In conclusion, α-GalCer-loaded ES-DCs expressing multiple antigens induce a potent therapeutic effect against B16 melanoma.

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Effect of nrf2 on the pigmentation

T Yoon,[1] S Jung,[2] T Choi,[2] J Kim,[2] M Kim,[2] S Kim,[1] J Lee[2] and C Kim[2] *1 Department of Dermatology, Gyeongsang National University Hospital, Jinju, Korea, South and 2 Department of Dermatology, Chungnam National University, Daejeon, Korea, South*

Nrf2 is a redox-sensitive basic leucine zipper transcription factor, which is essential for the ARE (antioxidant responsive element)-mediated induction of phase II detoxifying genes. In normal state, Nrf2 is negatively regulated by the Keap1, which targets the Nrf2 to proteasomal degradation. However, in response to oxidative stress, Nrf2 dissociates from Keap1 and then binds to the AREs in the promoters of various phase II detoxifying genes. Since it has been well established that UV-induced oxidative stress is the trigger for the pigmentation process in melanocytes, we investigate the effect of Nrf2 on the melanogenesis using adenoviral gene delivery system. When melanoma cell line MNT-1 was transduced with adenovirus expressing GFP-tagged Nrf2, unexpectedly the pigmentation was inhibited in terms of tyrosinase activity and total melanin content. Consistents with, the protein level for tyrosinase was markedly decreased by overexpression of Nrf2. However, protein levels for MITF, Trp1 and Trp2 were not affected significantly by Nrf2. Interestingly, the mRNA levels for tyrosinase was not decreased significantly, suggesting that Nrf2-mediated downregualtion of tyrosinase was at the translational and/or posttranslational levels. To delineate the mechanism underlying this phenomenon, we next examined the activation status of several intracellular signaling cascades. Interestingly, overexpression of Nrf2 led to the phosphorylation of Akt, which has been known to reduce tyrosinase level. Together, these results suggest that Nrf2 play a negative role in melanogenesis through the modulation of intracellular signaling pathway.

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Knockdown of flotillin-2 by siRNA inhibits melanoma cell growth *in vitro* and *in vivo*

P Hazarika,[1] P Richards,[1] PE Vivas-Mejia,[2] X Zhang[1] and M Duvic[1] *1 Dermatology, UT MD Anderson Cancer Center, Houston, TX and 2 Experimental Therapeutics, UT MD Anderson Cancer Center, Houston, TX*

Flotillin-2 (flot-2) is a 41.7 kDa, highly conserved, lipid raft associated protein that induces filopodia formation [J Cell Biochem 75:147-59, 1999]. We have previously demonstrated that Flot-2 interacts with thrombin receptor, PAR-1, a G protein coupled receptor and over-expression of flot-2 enhanced sensitivity to thrombin activation including cell proliferation, actin cytoskeleton reorganization and Matrigel invasion. Flot-2 over-expression transformed SB2 melanoma cells from non-tumorigenic, non-metastatic to a highly tumorigenic and metastatic phenotype in a nude mouse xenograft model [Cancer Res 64: 7361-9, 2004]. High flotillin-2 expression is associated with lymph node metastasis and Breslow depth in melanoma (Melanoma Research 16:461-463, 2006). The aim of this study was to investigate the effects of suppressing flot-2 in human melanoma cell lines *in-vitro* and *in vivo*. WM2664 melanoma cells were transfected with flot-2 siRNA or control siRNA. After 48 hours of transfection, cell proliferation was measured by MTT assay and *in vitro* invasion was assessed by Matrigel invasion assay. Flot-2 siRNA treatment resulted in decreased cell proliferation (40%) and Matrigel invasion (80%). Athymic nude mice were injected subcutaneously with 1x106 WM2664 melanoma cells and after one week, Flot-2 siRNA or control siRNA incorporated into neutral liposome [1,2-dioleoyl-sn-glycero-3-phosphatidyl choline(DOPC)] were injected into tail vein of mice (N=10), twice weekly for 4 weeks. Tumor volumes were measured twice weekly. Tumor growth was decreased in flot-2 siRNA treated group compared to control siRNA treated group (mean tumor volume 255 mm3 vs 552 mm3, p=< .04). After 4 weeks of therapy, the mice were sacrificed to harvest the tumors and individual tumor weights were recorded. Tumor weights were decreased in flot-2 siRNA treated group (mean tumor weight 0.32g vs 0.63g, p= < .05). Our data suggest that flot-2 may serve as a target for therapeutic intervention in melanoma patients.

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Bayesian recognition of dermoscopic structures in skin lesions

P Wighton,[1,2,3] TK Lee,[1,2,3] H Lui,[2,3] DI McLean[2] and MS Atkins[1] *1 School of Computing Science, Simon Fraser University, Burnaby, BC, Canada, 2 Photomedicine Institute, Department of Dermatology and Skin Science, University of British Columbia and Vancouver Coastal Health Research Institute, Vancouver, BC, Canada and 3 Cancer Control Research Program and Cancer Imaging Department, BC Cancer Research Centre, Vancouver, BC, Canada*

The purpose of this research is to develop a generic framework for the automatic classification and visualization of dermoscopic structures in skin lesions. We formulate the framework and test is efficacy at classifying and visualizing pigmentary networks. Our dataset is a selection of images from Argenziano et. al.'s *Interactive Atlas of Dermoscopy*. The dataset was created by considering a subset of images for which the illumination and magnification was constant. From this set, images were removed if the entire lesion was not visible, or was heavily occluded by either hair or oil. The final dataset consisted of 94 images. Each image is labeled as having an absent, regular or irregular pigment network. We begin by computing several pixel-wise measurements of each image such as L*a*b*, filter bank convolutions, wavelet decompositions, etc. A total of 52 measurements are made at each pixel. Linear discriminant analysis is then used to reduce the dimensionality while maintaining saliency. Bayesian methods are used to estimate the probability that a given pixel is indicative of an absent, regular and irregular pigment network respectively. Maximum likelihood estimation is then used to assign a label to each image. Visualizations of where regular and irregular pigment networks are found within the lesion are also constructed. Leave-one-out cross validation was used to evaluate the method. The framework can correctly identify the presence of a pigment network with 87% accuracy. It can also identify the type of pigment network with a 72% accuracy. Convincing visualizations of the location and type of pigment network are also generated. In conclusion, our Bayesian based framework seems to be a promising technique for the automatic identification and visualization of dermoscopic structures in skin lesions.

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High throughput integrated analyses for the tyrosinase-induced melanogenesis: Microarray, proteomics and interactomics studies

Y Park,[1] D Park,[2] J Bhak,[2] D Lee[1] and J Yang[1] *1 Dermatology, Sungkyunkwan University Samsung Medical Center, Seoul, Korea, South and 2 Korean Bioinformation Center, Daejeon, Korea, South*

Tyrosinase is a key enzyme in producing melanin pigment. We aimed to detect the genes that have shown altered expression levels in the tyrosinase-mediated melanogenesis by using high-throughput screening methods. The tyrosinase gene was overexpressed in HEK293 cells, and then a DNA microarray coupled with proteomic tools was applied to detect the dysregulated genes and proteins in highly pigmented cells. The candidate genes were obtained from the significance analysis of microarray (SAM), and these data were compared to the yeast two-hybridization results. Computational prediction via protein-protein interaction (PPI) mapping suggested the existence of hub genes in melanogenesis. The results of SAM showed that more than 700 genes were up- and down-regulated compared to the control samples. From a proteomic study, we detected 33 dysregulated proteins. PPI mapping using bioinformatic tools revealed 66 genes that formed an interaction hub. The approach of combining the expression data analysis and predicted protein interaction partners performed in large scales can bring valuable and more reliable gene targets for understanding pigmentation. Most importantly, RNA binding motif protein 9 is newly detected as a putative critical melanogenesis-associated gene in this study.

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The effect of trifluoroethanol on tyrosinase activity and conformation: Inhibition kinetics and computational simulations

Y Park,[1] D Park,[2] J Bhak,[2] D Lee[1] and J Yang[1] *1 Dermatology, Samsung Medical Center, Seoul, Korea, South and 2 Korean Bioinformatics Center, Daejeon, Korea, South*

We studied the inhibitory effects of trifluoroethanol (TFE) on the activity and conformation of tyrosinase. TFE increased the degree of secondary structure of tyrosinase, which directly resulted in enzyme inactivation. A reciprocal study showed that TFE inhibited tyrosinase in a slope-parabolic mixed-type inhibition manner (KI = 0.5 ± 0.096 M). Time-interval kinetic studies showed that the inhibition was best described as first-order with biphasic processes. Intrinsic and ANS-binding fluorescences were also measured to gain more insight into the supposed structural changes; these showed that TFE induced a conspicuous tertiary structural change in tyrosinase by exposing hydrophobic surfaces. We also predicted the tertiary structure of tyrosinase and simulated its docking with TFE. The docking simulation was successful with significant scores (binding energy for Autodock4: -4.75 kcal/mol; for Dock6: -23.07 kcal/mol) and suggested that the TRP173 residue was mainly responsible for the interaction with TFE. Our results provide insight into the structure of tyrosinase and allow us to describe a new inhibition strategy that works by inducing conformational changes rather than targeting the active site of the protein.

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Expression of human endogenous retrovirus k (HERV-K) mRNA and production of putative viral particles by melanoma cell lines

S Singh,[1,2] CB Bunker,[1] S Kaye,[2] M Tristem,[3] ME Gore[4] and MO McClure[1] *1 Dermatology, Chelsea and Westminster Hospital, Imperial College (IC), London, United Kingdom, 2 Infectious Diseases, IC, London, United Kingdom, 3 Biological Sciences, IC, London, United Kingdom and 4 Cancer Medicine, Royal Marsden Hospital, London, United Kingdom*

The human endogenous retrovirus K (HERV-K) provirus family is fixed in the human genome and has been implicated in the pathogenesis of melanoma (MM). The aims of this study were to quantify the expression of full-length and spliced HERV-K mRNA in human MM cell lines, to characterise expressed sequences and to determine whether MM cells release virus particles. A real-time quantitative PCR methodology was developed to quantify the expression of full-length (*pol* and unspliced *env*) and spliced (*env*, *rec* and *np9*) HERV-K mRNA in the human MM cell lines A375 and WM2664, and the human embryonic kidney cell line 293T. Expressed HERV-K *env* sequences from A375 cells were cloned for sequence and phylogenetic analysis. Viral pellets were obtained from MM cell line supernatants by ultracentrifugation and analysed by PCR for HERV-K viral RNA. Full-length and *np9* mRNA were expressed in all three cell lines investigated, but at higher levels in the MM cell lines. In all cell lines, the relative expression of *pol* was lower than that of unspliced *env*. Spliced *env* and *rec* mRNA were expressed in A375 cells only. In all cases the relative expression was less than 1, thus HERV-K genes were expressed at lower levels than the housekeeping gene *hprt*. Sequence analysis of expressed *env* cDNA clones derived from A375 cells revealed that independent isolates had nucleotide sequence differences. Putative viral particles were detected in A375 cell supernatants and sequence analysis revealed that these particles contain sequences with homology to the *env* region of HERV-K 101 and 109. Expression of HERV-K mRNA in MM cell lines has previously been demonstrated by our group and other researchers, but this is the first study to quantify and characterise expressed sequences.

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Quantification of human endogenous retrovirus k (HERV-K) expression in primary melanoma, normal skin and benign naevi

S Singh,[1,2] S Kaye,[2] ND Francis,[3] D Peston,[3] ME Gore,[4] MO McClure[2] and CB Bunker[1] *1 Dermatology, Chelsea and Westminster Hospital, Imperial College (IC), London, United Kingdom, 2 Infectious Diseases, IC, London, United Kingdom, 3 Histopathology, IC, London, United Kingdom and 4 Cancer Medicine, Royal Marsden Hospital, London, United Kingdom*

Human endogenous retroviruses (HERVs), remnants of ancestral exogenous retroviral infections fixed in the germ-line DNA, have been implicated in melanomagenesis. The HERV-K provirus family is the most likely to be pathogenic because some members have open reading frames for all viral genes. Genes for two potentially oncogenic proteins, Rec and Np9, have been described in the HERV-K genome. In this study the expression of HERV-K mRNA in archival primary melanoma (MM), normal skin (NS) and benign naevi (BN) has been investigated by real-time quantitative PCR. The MM group comprised *in situ* (n=6), superficial spreading (n=12), nodular (n=7), acral (n=9) and lentigo maligna (n=5) sub-types. Full-length HERV-K mRNA expression was observed at high levels in all tissues (*pol*: n=47 and unspliced *env*: n=40). There were no differences in relative expression between MM, BN and NS (*pol*: p=0.267 and unspliced *env*: p=0.823). No differences were observed between lesion site (*pol*: p=0.970 and unspliced *env*: p=0.796) and males and females (*pol*: p=0.418 and unspliced *env*: p=0.766). However, the relative expression of *pol* increased with the Breslow thickness of MM (n=19; p=0.458). *np9* was expressed in 14/39 MM, 4/16 BN and 9/31 NS and there was no difference in relative expression between the groups (p=0.964). Spliced *env* was expressed in 8/39 MM, but only 1/16 BN and 0/31 NS (p=0.003). *rec* was expressed in 9/39 MM but not in BN, nor in NS (p=0.009) nor in surrounding extra-lesional skin in 5 *rec*-positive cases. *rec*-positive MM were all histologically in vertical growth phase and thicker than *rec*-negative MM (median Breslow 2.1mm v 1.05mm). This is the first detailed study of HERV-K mRNA expression in primary MM, BN and NS. Expression of the potentially oncogenic *rec* mRNA in some primary MM, but not in BN or NS merits further evaluation.

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Improvement of keratinocyte-melanocyte interactions by PAR2 receptor activation

F Labarrade,[1] M Bonnans,[1] C Serre,[1] C Plaza,[1] J Botto,[1] C Dal Farra[2] and N Domloge[1] *1 Vincience, ISP Global Skin Research Center, ISP, Sophia-Antipolis, France and 2 ISP Corporate Research Center, ISP, Wayne, NJ*

Protease-Activated Receptors (PARs) are G-protein-coupled receptors, activated by proteolysis of the extra cellular N-terminus, thus exposing the specific agonist ligand. The purpose of this study was to investigate the activation mechanism of PAR2 and its possible functions in the skin pigmentation process. First, we investigated the expression of PAR1 to PAR4 mRNAs in different skin cell types, by RT-PCR studies. PAR2 mRNA was highly expressed in primary human keratinocytes but no transcript was observed in human fibroblasts or melanocytes. Next, we analyzed the activity of the MAP kinase signaling pathway in relation to PAR2 stimulation. Normal human keratinocytes in culture were treated with IV08.006, a molecule designed to act selectively on the PAR2 receptor. In this study, MAP kinase ERK1/2 phosphorylation was observed ten minutes after PAR2 activation. During the pigmentation process, melanosomes are transferred to keratinocytes via melanocyte dendrites. Hence, we investigated whether PAR2 activation with IV08.006 could enhance the interactions between melanocytes and keratinocytes, and therefore melanosome uptake. For this purpose, normal human keratinocytes and melanocytes were cultured in two separate compartments of a silicon culture insert. Cell migration and contact formation between the two cell types were visualized 24 hours after insert removal. This experiment was performed in the presence of IV08.006. Our data suggested that PAR2 activation led to an increase in keratinocyte-melanocyte contacts and improved melanocyte dendrite elongation. Finally, DNA microarray analysis of transcript levels revealed gene expression patterns 6h and 48h after PAR2 stimulation. In conclusion, these studies show that activation of PAR2 improves interactions between keratinocytes and melanocytes, favoring enhancement of pigmentation.

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Modulation of skin pigmentation by modulation of the PAR2 receptor

M Bonnans,[1] F Labarrade,[1] C Plaza,[1] J Botto,[1] C Gondran,[1] F Portolan,[1] C Dal Farra[2] and N Domloge[1] *1 Vincience, ISP Global Skin Research Center, ISP, Sophia-Antipolis, France and 2 ISP Corporate Research Center, ISP, Wayne, NJ*

Protease-activated receptor-2 (PAR2) belongs to the large superfamily of G-protein-coupled receptors, is implicated in melanosome transfer, and can thus modulate cutaneous pigmentation. In this domain, we used IV08.006, a designed molecule to act selectively on PAR2 receptor, and tested its ability to modulate skin pigmentation. Through immunofluorescence (IF) staining of the PAR2 receptor, we observed an increase in PAR2 expression in NHK treated with 1% of IV08.006 for 48 hours. This result was confirmed by Western blot. The effect of PAR2 modulation on melanosome uptake was evaluated by looking at fluorescent microsphere ingestion by NHK. For this purpose, NHK were pretreated, or not, with the PAR2-inducing agent for 48 h. Fluorescent microspheres were added to the culture medium for the last 16 hours. Fluorescence observation and quantification were then performed, and showed that microsphere ingestion was significantly increased in NHK where PAR2 was up-regulated. As this activity has been previously linked with actin cytoskeleton reorganization, we performed actin staining on NHK. This study showed cytoskeletal modifications in PAR2-induced NHK, in association with extended membrane protrusions. This observation corroborated the phagocytosis mechanism implicated in the ingestion of melanosomes by keratinocytes. Furthermore, we investigated the effect of the association of IV08.006 with a previously described tanning compound on skin pigmentation. IF studies on normal human *ex vivo* skin showed that PAR2 is similarly up-regulated in skin samples treated with the PAR2 enhancer. Melanin intensity was revealed by Fontana-Masson staining of human skin sections, after 48 hours of treatment. The association of the two compounds enhanced skin pigmentation to a greater extent, compared to the effect observed with the tanning compound alone. In conclusion, these studies show that enhancing melanin transfer by PAR2 activation can be a useful approach to boosting skin tanning.

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Narrow-band UVB stimulates the migration and functional development in Vitiligo IgG Antibody treated melanoblast cells

H Yu[1] and C Wu[2] *1 Dermatology, Kaohsiung Medical University, Kaohsiung, Taiwan and 2 Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan*

Vitiligo is characterized by the destruction of melanocytes. Currently, the autoimmune component is believed to play an important role in the pathogenesis of the disease due to strong associations of vitiligo with multiple autoimmune diseases and the presence of autoantibodies in vitiligo. Our previous studies revealed that in addition to anti-melanocyte IgG antibodies, non-cytotoxic anti-keratinocyte IgG antibodies exist in vitiligo. Furthermore, we demonstrated that the anti-keratinocyte IgG antibodies displayed a UVB protective effect in vitiligo skin. The inactivated melanocytes (melanoblasts, MBs) residing in the outer root sheath of hair follicle serve as the pigment cell reservoir for repigmentation. Narrow-band UVB (NBUVB) has been considered to be an effective and well-tolerated treatment for vitiligo. Accumulating results from clinical observations demonstrated that narrow-band UVB mostly resulted in follicular pattern of repigmentation, suggesting that MBs were essential in NBUVB irradiation-induced repigmentation. We investigated the effects of NBUVB on vitiligo IgG antibodies treated NCCmelan5 cells, a neural crest derived mouse melanoblast cell, in terms of proliferation, migration, and melanin formation in this study. Our results showed that NBUVB irradiation increased the proliferation of vitiligo IgG antibodies treated NCCmelan5. In addition, NBUVB enhanced the mobility of vitiligo IgG antibodies treated NCCmelan5 via enhanced phosphorylated focal adhesion kinase (phosphorylated p125FAK) expression. The melanogenesis and tyrosinase expression in vitiligo IgG antibodies treated NCCmelan5 were promoted by NBUVB. In conclusion, our findings provided in vitro evidence demonstrating the interaction between NBUVB irradiation and vitiligo IgG antibodies on proliferation, migration and functional development in NCCmelan5 cells. Furthermore, our study provides a theoretical basis explaining the effectiveness of NBUVB in treating vitiligo.

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Photoprotective roles of endothelin-1/stem cell factor induced heme oxygenase-1 expression in cultured human melanocytes

E Akasaka,[1] H Nakano,[1] K Sato-Jin,[1] K Kimura,[1] D Rokunohe,[1] D Sawamura[1] and G Imokawa[2,1] *1 Department of Dermatology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan and 2 School of Bioscience and Biotechnology, Tokyo University of Technology, Tokyo, Japan*

Melanocytes protect the skin from the solar damage. Endothelin-1 (ET-1), stem cell factor (SCF), and alpha-melanocyte stimulating hormone (α-MSH) are cytokines secreted by UVB-exposed kerationcytes, playing pivotal roles in regulating the melanogenesis in normal human melanocytes (NHM). It has recently been reported that ET-1 or α-MSH promotes survival of NHM against the sun damage and inhibits the UVB-induced apoptosis. However, the signaling mechanisms involved in the photoprotective effects are poorly understood. In this study, we demonstrated that expression of heme oxygenase-1 (HO-1), an anti-oxidative protein, is up-regulated by ET-1 or SCF, but not by α-MSH in NHM. Furthermore, ET-1 and SCF synergistically increased HO-1 expression. In addition, the ET-1/SCF-induced HO-1 expression was abolished by MEK and PI3K inhibitors. We then determined whether ET-1 and/or SCF elicit photoprotective effects against UVB irradiation. Treatment with ET-1, but not with SCF, significantly suppressed the UVB-induced cell death, and the photoprotective effect was significantly up-regulated by concomitant addition of ET-1 and SCF compared with ET-1 alone. Moreover, overexpression of HO-1 was able to promote cell survival of UVB-exposed pigment cells and a pharmacological HO-1 inhibitor diminished the photoprotective effect. Taken together, these results indicate that ET-1/SCF augments cellular antioxidant potential through HO-1 induction via the Raf/MEK/ERK and PI3K/Akt signaling pathways, thereby protecting melanocytes from UVB induced oxidative stress.

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MKK6 increases the melanocyte dendricity through the regulation of Rho family GPTases

J Lee,[1] M Kim,[1] J Kim,[1] T Choi,[1] S Jung,[1] Y Kim,[1] Y Lee,[1] Y Lee,[1] C Kim[1] and T Yoon[2] *1 Chungnam National University, Daejeon, Korea, South and 2 Gyeongsang National University, Jinju, Korea, South*

Dendrite formation requires actin polymerization, and Rho family GPTases including RhoA, Rac1 and Cdc42 are well implicated in this process. It has been documented that RhoA regulates the formation of the actin stress fibers though bundling of pre-existing filaments, whereas Rac1 and Cdc42 are required for the formation of lamellipodia and filopodia, respectively, via de novo actin polymerization. In other systems, Rac1 and Cdc42 are shown to be related with the p38 MAPK pathway. To investigate the involvement of p38 MAPK in melanocyte dendrite formation, we made the recombinant adenovirus expressing MKK6, an upstream MAPKK for p38 MAPK. Interestingly, the activation of p38 MAPK by overexpression of MKK6 resulted in increase of dendrite number in normal human melanocytes cultured *in vitro*. When MKK6 was overexpressed in melanoma cell line SK-mel-24, dendrite length was dramatically increased. To examine whether the activation of p38 MAPK by MKK6 expression affects the Rho family GTPases, we perfomed Western blot analysis against RhoA, Cdc42 and Rac1. As expected, overexpression of MKK6 led to the upregulation of Cdc42 and Rac1, while RhoA was slightly decreased by MKK6 overexpression. These results suggest that activation of p38 MAPK pathway by MKK6 is an important step to regulate Rho family GPTases, thereby influencing the dendricity of melanocytes.