Epigenetic regulation of the homeobox gene *MSX1* associates with platinum resistant disease in high grade serous epithelial ovarian cancer

N.A. Bonito¹*, J. Borley¹*, C.S. Wilhelm-Benartzi¹, S. Ghaem-Maghami¹, R. Brown¹,²

¹ Department of Surgery & Cancer, Imperial College London, London W12 0NN, UK
² Institute of Cancer Research, Sutton, London SM2 5NG, UK

* Contributed equally to the work

Translational Relevance

Although considered a chemo-responsive disease, a proportion of high grade serous ovarian cancer (HGSOC) patients do not respond to platinum-based chemotherapy at initial presentation. Clinically validated biomarkers of lack of response would enable alternative treatment stratification for these patients and identify novel mechanisms of resistance. We have identified CpG sites at contiguous genomic locations within the homeobox *MSX1* gene as significantly associated with lack of response of HGSOC patients in discovery, test and validation patient cohorts. A decrease in methylation was significantly correlated with decreased *MSX1* gene expression. Increased expression of *MSX1* in three *TP53* mutant or wild-type expressing, platinum-resistant ovarian cancer lines led to cisplatin sensitisation and increased p21 expression. However, in two *TP53*-null cell lines, *MSX1* failed to change cisplatin sensitivity. In conclusion, hypomethylation and expression of *MSX1* is a biomarker of resistant HGSOC disease at presentation and identifies a novel mechanism of platinum drug resistance.
Abstract

Purpose: Although High Grade Serous Ovarian Cancer (HGSOC) is frequently chemoresponsive, a proportion of patients do not respond to platinum-based chemotherapy at presentation or have progression-free survival of less than 6 months. Validated predictive biomarkers of lack of response would enable alternative treatment stratification for these patients and identify novel mechanisms of intrinsic resistance.

Experimental Design: DNA methylation was investigated in independent tumour cohorts using Illumina HumanMethylation arrays and validated by bisulphite pyrosequencing. Gene expression was examined by Affymetrix arrays and qRT-PCR. The role of Msh homeobox 1 (MSX1) in drug sensitivity was investigated by gene reintroduction into ovarian cancer cell lines.

Results: CpG sites at contiguous genomic locations within the MSX1 gene have significantly lower levels of methylation in independent cohorts of HGSOC patients which recur by 6 months compared to after 12 months and/or with poor RECIST response (p<0.05, q<0.05). A decrease in methylation at these CpG sites correlates with decreased MSX1 gene expression. Low expression of MSX1 is associated with poor progression-free survival independent of known clinical prognostic features (p=0.014). Three mutant or wild-type TP53 expressing ovarian cancer cell lines, resistant to cisplatin, have reduced MSX1 expression compared to matched parental, platinum sensitive, lines. Re-expression of MSX1 in resistant lines led to cisplatin sensitisation and increased cisplatin induced p21 expression. However, in two TP53-null cell lines, MSX1 failed to change cisplatin sensitivity or p21 expression.

Conclusions: Hypomethylation of MSX1 is associated with resistant HGSOC disease at presentation and identifies a novel mechanism of platinum drug resistance.
Introduction

Epithelial ovarian cancer (EOC) is the second commonest, but most lethal, of the gynaecological malignancies, with overall 5 year survival rates around 45% [Reviewed in (1, 2)]. Adjuvant chemotherapy is recommended for those with stage 1C disease or higher and normally comprises of platinum based agent with or without taxol (3). Despite this, approximately 20% of patients will be resistant to first line platinum agents and prognosis in these platinum resistant patients is particularly poor (2). Currently there are no reliable methods to determine or predict platinum resistance and thus patients who will gain little clinical benefit undergo treatment regimes associated with high morbidity and side effects. Defective Homologous Recombination shows promise as a marker of platinum response, although has limited sensitivity and specificity (4) and ultimately combinations of independent predictive biomarkers of response are likely to be required. Biomarkers which predict treatment response may identify novel targets for therapies aimed at reversal of resistance and will facilitate stratification of patients who should be considered for alternative treatment with non-cytotoxic, molecularly targeted agents.

The use of DNA methylation as a biomarker has several advantages over other molecular endpoints, including sensitivity and stability of DNA methylation, as well as less confounding effects of contaminating normal tissue in patient-derived biopsies. Furthermore, DNA methylation measurements can be compared to absolute reference points (for instance, completely methylated or completely unmethylated DNA), which provides a quantitative assays (5). Additionally, tumour DNA methylation can be detected in body fluids of patients, potentially leading to measurement of tumour-derived biomarkers non-invasively (5). There are now numerous examples of the potential of DNA methylation as a therapy stratification biomarker or as marker of acquired resistance, although few methylation biomarkers have been clinically validated or used as stratification biomarkers in clinical studies (6).

We have examined DNA methylation in high grade serous ovarian cancer using Illumina 27K HumanMethylation array data in independent tumour cohorts for cross-validation. This analysis identified methylation at the Homeobox gene *MSX1* as associated with platinum resistant disease. Dysregulation of HOX genes have been associate with a variety of human cancers [summarised in (7)]. Msh homeobox 1 (*MSX1*), also known as *HOX7*, is a homeobox gene with critical roles in the control of cellular differentiation in development (8) (9). MSX1 has been shown to cause a change in cell morphology and a reduction in cell growth by induction of apoptosis in HeLa cancer cells (10). We have examined the role of MSX1 in chemosensitivity by gene reintroduction into platinum resistant ovarian tumour cell
lines that have reduced MSX1 expression compared to matched parental, non-platinum exposed, lines.
Methods

Patient samples

Fresh-frozen EOC tissue was collected and stored at -80 °C at Imperial College NHS Trust Tissue Bank, Hammersmith Hospital (“Hammersmith Cohort”). Tumours selected were from primary debulking surgery (no previous chemotherapy), advanced stage disease (FIGO stage 3 and 4) with high grade (G2-3) serous histology. Patients were excluded with mixed cell or borderline pathology. In order to assess the quality of the individual fresh frozen tissue samples used for DNA extraction, adjacent tissue was histopathologically examined and samples excluded with low tumour cell content.

Overall survival (OS) and progression free survival (PFS) were defined as the interval from the date of initial surgical resection to the date of last known contact, or death for overall survival and the interval from the date of initial surgical resection to the date of progression or recurrence for progression free survival. Stage was defined using FIGO criteria.

Response to chemotherapy was defined by RECIST 1.1 (11) criteria using scans done at two distinct time points, CT chest, abdomen and pelvis scan post-surgery but prior to adjuvant chemotherapy, and after 6 cycles of chemotherapy. Patients were categorised into two groups, those who responded to chemotherapy (complete or partial response) or with stable disease were compared to those with progressive disease only.

The clinical characteristics of the Hammersmith Cohort and the The Cancer Genome Atlas samples used for validation (“TCGA Cohort”) are shown in Supplementary Table S1.

Bisulphite conversion

Up to 500mg of tumour tissue was used per sample and DNA extracted by the chlorinated Nucleon extraction method (Gen-Probe Life Sciences Ltd.) and resuspended in TE buffer (10mM Tris, 1mM EDTA, pH8). Tumour DNA was bisulphite converted using the EZ-96 DNA Methylation™ Kit (Zymo) as per manufacturers protocol. 1μg of genomic DNA was used for each sample. Successful bisulphite conversion was confirmed by successful conversion of cytosines at calponin locus (12).

Infinium HumanMethylation27 beadchip

Samples were processed as per the Infinium Assay Methylation Protocol Guide (Illumina) (http://www illumina com/products/infinium humanmethylation27 beadchip kits ilmn#documentation). Following bisulphite conversion, 200 ng per samples were resuspended at 50ng/μl. Methylation data was summarized as β values, calculated as M/(M+U) where M is
signal from methylated beads and U is signal from unmethylated beads at the targeted CpG site. θ values were adjusted for background and data was log transformed to achieve a normal distribution for both TCGA and the beadchip. Probes with a detection p value of >0.05 were removed. Quality control checks were performed through Genome Studio.

TCGA dataset analysis

Illumina Human Methylation27 Beadchip data on HGSOC from The Cancer Genome Atlas data portal (http://cancergenome.nih.gov/dataportal) (“TCGA Cohort”) was used for independent validation of correlations observed in the Hammersmith cohort. Level 2 expression data on Affymetrix HGU133A microarrays, level 3 methylation data and annotated clinical data were obtained. The expression microarray data was pre-processed and normalised across samples.

Plasmid transfection

Cell lines used are described in Supplementary Table 2. Cells were stable transfected either with MSX1 expression plasmid obtain from Origene (RC205682) or the corresponding empty vector (PS100001) using FUGENE HD transfection reagent (Promega) as per respective protocols. Cells were grown and maintained under 500µg/ml of Geneticin® (Life technologies).

Real Time-PCR and gene expression profiling

To analyse gene expression, two-step reverse transcription PCR was performed. Total RNA was extracted using the QIAshredder and RNAeasy Mini Kits (Qiagen) according to manufacturers protocol. Total RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using Fast SYBR® Green Master Mix (Applied Biosystems) using specific primers. Each reaction was performed following the manufactory recommendations using 10 ng of converted RNA. The data was analysed using the \( \Delta\Delta\text{Ct} \) method. For MSX1 gene expression, qRT-PCR was performed using Taqman primers (MSX1 Hs 00427183_m1 (Applied Biosystems)) compared to endogenous control (beta-actin) and StepOnePlus Real-Time PCR Systems (Applied Biosystems).

Cell proliferation assays

Cells were seeded at 10,000 cells per well in triplicate, after 24 hours cells were treated with Cisplatin at concentrations of 0 µM to 50 µM for 24 hours. Cell proliferation was quantified
with MTT assay after a further 48 hours of culture after 24 hours of cisplatin treatment using CellTitre 96 Aqueous One Solution Proliferation Assay (Promega).

Statistics

All statistical analyses were performed using the R statistical package (version 2.10 at http://www.r-project.org). Individual generalized linear regression models were performed adjusting for array batch, grade and residual disease in order to determine significant differentially methylated loci and associated differential gene expression of Affymetrix gene expression data with RECIST chemotherapy response, and PFS <6months versus >12months in both datasets. A significant relationship between TCGA DNA methylation and gene expression in the tumour tissue was determined through Spearman correlation.

For gene expression using FastSYBR Green, the standard error of mean (SEM) was calculated for three independent experiments. To determine the statistical significance of the differences observed, an unpaired student t-test was used. The differences were considered significant when a two-sided P-value was below 0.05. (* - P<0.05, ** - P<0.01, *** - P<0.005)
Results

Hypomethylation of MSX1 in HGSOC is associated with progressive disease and short progression free survival

Discovery analysis across all loci on the 27K Illumina bead array, comparing HGSOC patients from a Hammersmith Cohort (n=78) with progressive disease (PD) versus those who responded to chemotherapy or had stable disease, identified multiple CpG sites at the MSX1 gene which demonstrated significant differential methylation, even after taking multiple comparisons into account (p<0.05, FDR <5%). We further evaluated this relationship in a cohort (n=61) of Hammersmith patients with response data defined by RECIST 1.1 criteria (Table 1). We observe 8 contiguous CpG sites spanning the first intron and 2nd exon of MSX1 to be significantly (p<0.05) associated with RECIST response (Figure 1). For 7 out of 8 significant probes methylation was significantly lower in the group with progressive disease versus those with response to chemotherapy or stable disease, with a methylation difference of >15% (Supplementary figure S1). Progression-free survival (PFS) of less than 6 months is widely used to clinically define platinum resistant disease, while PFS greater than 12 months is often used to define platinum sensitive disease (13). Six of the 8 CpG sites within the MSX1 gene identified as associated with response have significantly lower levels of methylation in HGSOC from the Hammersmith cohort which recur by 6 months compared to after 12 months (p<0.05, q<0.05), with the remaining two showing a similar trend (Table 1).

Primary chemotherapy response was not defined by RECIST criteria in the TCGA dataset, and therefore could not be used to validate the association with response observed in the Hammersmith cohort. However, PFS data was available in the TCGA dataset for 146 patients and methylation data available for 7 of the 8 MSX1 probes identified from the Hammersmith Cohort (Table 1 and Figure 1). Linear regression models adjusting for array batch, grade and residual disease found methylation levels at 4 probes to be significantly associated with PFS and a further 2 showing strong trend. Consistent with the Hammersmith dataset, methylation at these CpGs demonstrated a corresponding lower level of methylation in patients with PFS <6 months.

MSX1 gene expression is associated with DNA methylation and progression-free survival

DNA methylation and gene expression data was available for 252 samples in the TCGA data. This data demonstrated that a decrease in methylation at 6 of the identified CpG sites
were significantly correlated (p<0.05) to a decrease in gene expression (Table 1 and Figure 1). MSX1 expression and progression free survival (PFS) were significantly associated in the TCGA cohort dataset using univariate Cox proportional hazard regression analysis (Hazard Ratio, 0.92, 95% CI 0.85-0.99, p=0.029, n=309). Patients with low expression of MSX1 had poorer PFS than those with higher MSX1 expression. A significant association was retained in multivariable Cox analysis when age, grade, stage and residual disease were included in the model (p=0.014), demonstrating that MSX1 expression is an independent biomarker from clinical factors known to be associated with PFS.

**MSX1 sensitises cells expressing TP53, but not null-TP53 cells**

Relative MSX1 gene expression was examined by qRT-PCR on three matched pairs of cisplatin sensitive and resistant ovarian cancer cell lines: PEA1/PEA2, PEO1/PEO4 and PEO14/PEO23 pairs derived from HGSOC patients before treatment and at relapse after platinum-based chemotherapy (14) and A2780/A2780cp70 sensitive and in vitro derived resistant line (15). Gene expression was markedly lower in the platinum resistant cell lines derived from patients following chemotherapy or in vitro derived compared to their platinum sensitive pair (Figure 2). However, no significant difference in DNA methylation could be detected between the pairs of cell lines (data not shown). In order to examine whether increased MSX1 expression would increase the cells sensitivity to cisplatin, the ovarian cell lines A2780cp70, PEA2, and PEO4 were stably transfected either with an MSX1 containing plasmid (MSX1) or with the corresponding empty vector (EV). As shown in Figure 3, all three ovarian cancer cell lines transfected with MSX1 showed an increase in cisplatin sensitivity compared to the empty vector control. The HGSOC cell lines PEA2 and PEO4 stable overexpressing either MSX1 or the corresponding EV were treated with cisplatin and the expression of p21 and MSX1 was analysed by qRT-PCR. Overexpression MSX1 increases the expression of p21 after cisplatin treatment, when compared to the EV transfectants (Figure 3).

MSX1 has previously been shown to interact with p53 and increased p53-dependent apoptosis (10). In HGSOC, p53 is mutated in about 97% of cases, although often will still express mutant forms of p53 (16). The TP53 mutations described in the literature for the cell lines used were confirmed and the ovarian cell lines examined all express either wildtype or mutant alleles of TP53 (Supplementary Table S2). MSX1 was also transfected into the ovarian adenocarcinoma SKOV3 (Supplementary Table S2). In SKOV3, no TP53 mRNA is detected and, consequently, the protein is absent (17). In SKOV3, MSX1 overexpression did not change the cisplatin sensitivity nor the expression of p21 after 24 hours of cisplatin
treatment (Figure 4). To further examine effects of MSX1 in a null p53 cell line, the p53-null lung cancer cell line H1299 was transfected with MSX1. Again in H1299, MSX1 overexpression did not change the cisplatin sensitivity nor the expression of p21 after 24 hours of cisplatin treatment (Figure 4).
Discussion

In independent tumour cohorts, differential methylation of multiple CpG sites at an intragenic region of the homeobox gene MSX1 spanning the intron and second exon is significantly associated with primary chemotherapy resistance and poor progression free survival in HGSOC. Increased methylation significantly correlates with increased gene expression. MSX1 encodes a member of the muscle segment homeobox gene family and its expression is associated with epithelia-mesenchymal interactions during embryogenesis (18). Intriguingly, the same 8 CpG sites we observe associated with response to chemotherapy in MSX1 have been associated with fetal heart malformation and again increased methylation is associated with an increase in MSX1 expression (19). Given the suggestion by the authors that DNA methylation at these CpG sites impairs MSX1-dependent mesenchymal embryogenesis it is tempting to speculate that MSX1 in ovarian cancer may influence epithelial-mesenchymal transition (EMT) which has been implicated in resistance of ovarian tumours to chemotherapy (20).

Binding of MSX1 in the genome of myoblasts promotes enrichment of the repressive H3K9me2 mark via recruitment of EHMT2 (G9a) histone methyltransferase (21), the enzyme responsible for maintaining this histone mark. Given the involvement of epigenetic regulation in drug resistance (6), it is therefore possible that MSX1 is mediating its effects on chemosensitivity through epigenetic regulation. An alternative explanation of MSX1 effects on chemosensitivity could be through p53 stabilisation. Previous studies have suggested that MSX1 decreased cell growth and induced apoptosis in HeLa cell lines through stabilisation of p53, decreased degradation and increasing nuclear localisation (10). We observe chemosensitisation of MSX1 in cell lines, which still express mutant or wild-type TP53, but not in TP53 null cell lines. Interestingly, EHMT2 methylates lysine 373 in p53 (22). Potential hypotheses to be examined in future studies would be whether MSX1 cooperates with G9a/EHMT2 in methylating p53 (and related p63 and p73 proteins) leading to changes in cisplatin sensitivity or whether MSX1/EHMT2 mediated changes in histone H3K9 methylation can directly influence expression of genes associated with drug resistance.

Both tumour cohorts use fresh frozen tumour that is not microdissected and heterogenous cell types within each sample may confound overall gene expression data, although will be expected to have less impact on DNA methylation data. There was no correlation between methylation data in the Hammersmith cohort and the percentage of tumour nuclei, therefore there is no bias in the proportions of tumour and normal cells in biopsies influencing the association observed (data not shown).
A limitation of the TCGA data is that the primary chemotherapy response was not defined by RECIST criteria and so could not be used to validate the association with response observed in the Hammersmith cohort. Instead we have used PFS to validate the associations observed. PFS of less than 6 months is widely used to clinically define platinum resistant disease, while PFS greater than 12 months is often used to define platinum sensitive disease (13, 23). Analysis of the TCGA data validated the association with PFS observed in the Hammersmith cohort at 4 CpG MSX1 sites, with others showing similar trend. Consistent with the Hammersmith cohort, CpG sites demonstrated a corresponding lower level of methylation in patients with PFS <6 months. It should be noted that the TCGA data represents analysis of tumours collected across multiple clinical sites and the robustness and reliability of clinical data from multiple centres may not be as well controlled as in the single site Hammersmith data. Indeed analysis of the TCGA data does not show some of the expected clinical correlations, such as association between survival and surgical debulking (data not shown).

We have identified methylation and expression of MSX1 as a biomarker of lack of response to primary chemotherapy in HGSOC. Clinical stratification based on such biomarkers could enable alternative treatment stratification for these patients at presentation or of maintenance therapies during remission. The functional role of MSX1 and its role potentially in epigenetic regulation or methylation of cellular proteins such as p53 needs further in-depth study.
References


Acknowledgements
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Figure Legends

Figure 1: Schematic of MSX1 locus showing CpG sites associated with clinical outcome.

Figure 2. MSX1 gene expression in cisplatin sensitive (Black bar) and resistant (white bar) cell line pairs. RNA levels of MSX1 gene were analysed by qRT-PCR and normalised against β-actin. Statistical analysis was performed using standard error of the mean (SEM) and Student t-test (* - P<0.05, ** - P<0.01 and *** - P<0.005).

Figure 3. MSX1 overexpression re-sensitises cells to cisplatin treatment. MSX1 expression vector and empty vector (EV) transfected cell lines PEA2 (A.), PEO4 (B.) CP70 (C.) were treated with cisplatin for 24h. MTT assay was performed 48h after treatment. Data is representative of at least three independent experiments. D. PEA2 and PEO4 cell were treated with the indicated cisplatin concentrations for 24 hours. p21 RNA levels were analysed relative to β-actin by qRT-PCR. Statistical analysis was performed using standard error of the mean (SEM) and Student t-test (* - P<0.05, ** - P<0.01 and *** - P<0.005).

Figure 4. MSX1 overexpression does not sensitise cells to cisplatin in p53-null cells. MSX1 expression vector and empty vector (EV) transfected cell lines SKOV3 (A.) and H1299 (B.) were incubated with the indicated cisplatin concentration for 24 after which either MTT assay was performed as described before. C. p21 expression was measured by qRT-PCR in SKOV3 and H1299 cells. Statistical analysis was performed using standard error of the mean (SEM) and Student t-test (* - P<0.05, ** - P<0.01 and *** - P<0.005).
Figure 1. Schematic of MSX1 locus showing CpG sites associated with clinical outcome.

- **MSX1**
- **CpG islands**

** Probe ID: cg22609784, cg35673795, cg27384339, cg0400986, cg09749775, cg03843978, cg01301548, cg019781588

- **Linear regression model associating differential DNA methylation with chemotherapy RECIST response or PFS, p<0.05**
- **Spearman correlation, p<0.05, decreased methylation associated with decreased gene expression**
- **Data not available**
- **Not significant**

**Data:**
- **RECIST response, HH n=61**
- **PFS < 6 month, HH n=39**
- **PFS < 6 month, TCGA n=146**
- **Correlation to gene expression, TCGA n=252**
Figure 2. **MSX1** gene expression in cisplatin sensitive (Black bar) and resistant (white bar) cell line pairs.
Figure 3. MSX1 overexpression re-sensitises cells to cisplatin treatment.

A. **PEA2**

![Graph showing survival fold change for PEA2 with and without MSX1 expression at varying concentrations of cisplatin.]

B. **PEO4**

![Graph showing survival fold change for PEO4 with and without MSX1 expression at varying concentrations of cisplatin.]

C. **A2780/cp70**

![Graph showing survival fold change for A2780/cp70 with and without MSX1 expression at varying concentrations of cisplatin.]

D. **p21 Expression**

![Bar graph showing p21 expression fold change relative to β-actin for PEA2 and PEO4 with and without MSX1 expression at varying concentrations of cisplatin.]
Figure 4. MSX1 overexpression does not sensitisce cells to cisplatin in p53-null cells.

A. SKOV3

B. H1299

C. Expression p21
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NA Data not available

HH: Hammersmith Cohort

TCGA: The Cancer Genome Atlas Cohort