Review

Title:

Role of SMAD proteins in colitis associated cancer: from known to the unknown

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Abstract

Small mothers against decapentaplegic (SMAD) proteins are a family of signal transduction molecules in transforming growth factor β (TGF β) ligand pathways that have been found to play a key role in the pathogenesis of inflammatory bowel disease (IBD). Longstanding IBD predisposes individuals to colitis-associated colorectal cancer (CAC), an entity that possess unique characteristics compared to hereditary and sporadic cancer. The ligands of the TGF β super family along with SMADs have also been implicated in several aspects of colorectal cancer (CRC) formation. SMAD proteins are shown to be involved in a number of potentially carcinogenic mechanisms such as altering gene transcription, controlling stem cell differentiation to causing epigenetic changes. Modulation of these proteins has emerged as a novel therapeutic intervention for IBD although its effect on carcinogenesis remains elusive. This account reviews available evidence linking SMAD proteins to CAC and explores the potential areas for future research in this area.

Colitis associated cancer

Association between chronic inflammation and cancer has long been recognized, and a causal relationship has been shown in relation to multiple organ systems (1), (2). Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), have a well-established predisposition to develop colitis associated cancer (CAC) (3), (4). In UC, the risk of cancer exponentially increases with the duration of disease, with a cumulative probability of 2% and 8.3% respectively at 10 and 30 years (4). Although the available data in Crohn's disease is less and conflicting, the accumulated risk at 10 years is reported to be 2.9% and increasing to 8.3% at 30 years with a combined relative risk 4.5 times that of a normal population (5). However the risk of CAC in ulcerative colitis has been reported to be declining in the recent past due to the advancement in surveillance techniques, maintenance therapy and surgery (6), (7), (8). The key

risk factors for CAC are the severity, extent and duration of inflammation of the intestine (9). This could be explained by the action of pro inflammatory cytokines which not only promote angiogenesis and cell proliferation but, by creating oxygen free radicals and nitrogen species, hastens the tumorigenic process through mutations in key growth regulatory genes(10). Silencing of tumour suppressor genes, such as INK4, APC, MLH, MINT1 and RUNX3 through promoter methylation, histone modification and miRNAs (miR-122, miR-181a, miR-146b-5p, let-7e, miR-17 and miR-143) have also been identified during CAC tumorigenesis (11)· (12) . However recent findings by Yeager et al suggests a mutational signature more compatible with rapid cell turnover rather than direct DNA damage (13). They found TP53, MYC and IDH 1 to be mutated at a significantly higher frequency in CAC along with *Wnt*, MYC and TGF β pathways being altered in approximately half of those tumours.

The sequence in the occurrence of key gene mutations in CAC also differ from that of sporadic CRC (14), (15) (Figure 1). The common mutation in *APC* (Adenomatous Polyposis Coli) is an early occurrence in sporadic CRC although is characteristically a late phenomenon in CAC (16), while *P53* mutations appear to be a more prominent early event in CAC (17). On the other hand, *DCC* (Deleted in Colon Cancer) and *k-RAS* (Kirsten- Rat Sarcoma gene) mutations occur in a similar sequence (18). More recent evidence using next generation sequencing suggest further dissimilarities in the mutational signatures of these two groups (13). These phenomena lead to the hypothesis that inflammation may drive tumorigenic pathway through a different mechanism to sporadic CRC and require deeper understanding.

SMAD proteins

Products of the gene, *mothers against decapentaplegic* (*Mad*) were first identified during a mutational screen for the decapentaplegic (*dpp*) gene responsible for the formation of *Drosophila* wings (19). Identification of three homologues of *mad* in *C. elegans* causing small body size earned it the name *Sma*; the subsequently discovered vertebrate counterpart was therefore called SMAD (SMA/ *MAD*) (20). Structurally SMAD consists of two globular proteins (MH1 and MH2) linked by a non-structural linker. The MH1 domain contains a hairpin

structure with DNA binding capability (21); preference for specific base sequences differs between different SMADs. The MH2 domain contains hydrophobic elements that bind to trans-membrane receptors TGF β R and BMPR. Members of the TGF β superfamily, which includes TGF β 1, bone morphogenic protein (BMP), Nodal and activin, exert their canonical effect through phosphorylation of SMADs triggered by the threonine kinase activity of the trans-membrane heteromeric ligand receptors TR β I and TR β II. SMAD2 and SMAD3 act as signal transduction molecules in the TGF β 1 pathway while SMAD1, SMAD5 and SMAD8 has a similar function in the BMP signalling pathway (21). The cofactor SMAD4 combines with the activated complexes in both pathways and migrates to the nucleus to bind directly to chromatin and regulate transcription (Figure 2). Inhibitory SMAD7, and SMAD6 to a lesser degree, antagonise both signalling pathways by inhibiting phosphorylation of the substrate SMADs (21). In addition to the canonical pathway, TGF β also acts through a myriad of pathways that are beyond the scope of this review.(22) However, notable molecular pathways such as mitogen activated protein kinases (PAPK) p38 and c-jun N-terminal kinase (JNK) are now also believed to be regulated by SMADs (23).

SMAD proteins in inflammatory bowel disease

The role of TGF β 1 in IBD has been clearly described in multiple studies, whereby the absence of TGF β 1 causes inflammation of the colonic mucosa, while the induction of its activity results in the inhibition of pro-inflammatory cytokines produced by the T cells (24), (25), (26) . In particular, Monteleone and colleagues have shown deficiency of TGF β 1 activity in immune and non-immune cells of IBD-affected colon by observing reduced concentrations of phosphorylated SMAD3 (24). Significant elevation of SMAD7 levels in IBD tissue further indicates a connection of TGF β 1 and SMAD7 to its pathogenesis (24), also validated by resistance of SMAD7-expressing CD4 T cells to regulatory T (Treg) cell mediated proliferation in colitis (27). Even though this latter study mainly focused on lamina propria mononuclear cells (LPMC), the epithelium of the large bowel mucosa is believed to show similar changes (28). Interestingly, *SMAD7* mRNA expression levels within LPMC and whole mucosal biopsies was found to be

similar to control samples, leading to investigate the competition between acetylation and ubiquitination of SMAD7 in the cytoplasm (29). As already widely studied, ubiquitination allows the molecule to be degraded in a proteasome mediated mechanism, while acetylation resists this process. Increased acetylation of SMAD7 in IBD tissue therefore allows the protein to evade proteasome-mediated degradation(29). Models with SJL, BALB/c and SCID mice, show that inhibition of SMAD7 synthesis using an antisense oligonucleotide could restore TGF β 1 activity and result in colonic mucosal healing in both UC and CD(30). This observation has been exploited with the introduction of an orally administered anti sense oligonucleotide (21 bases) complementary to *SMAD7* mRNA and prevent its transcription in patients with CD. The new medication, mongersen (GED-0301, Celgene), is designed to achieve only adequate luminal concentrations in the intestine with no systemic effect (31)· (32). The drug is listed to be tested at clinical trial stage III, after showing promise in phase I and II trials.

SMAD proteins and carcinogenesis

Both TGF β 1 and BMP molecules have shown biphasic behaviour during carcinogenesis, with anti-carcinogenic activity during the early phase and pro-carcinogenic activity during the late phase of the process (33). The explanation of this bi-phasic action remains elusive.

P53 and SMAD proteins

Evidence is accumulating to show that the function of this pivotal tumour suppressor gene is closely related to SMADs. *P53* is mutated early not only in CAC, but also in non-cancerous chronically inflamed colonic mucosa (34). The convergence between TGF signalling pathway and p53 has been suggested by some authors as the underlying mechanism behind the biphasic role of TGF β ligands in carcinogenesis (35). This interaction may be able to link the effects of TGF β on inflammation-associated neoplasia and needs to be further explored. Early studies on *Xenopus* developmental models proposed p53 to be an essential element for activated SMAD complexes to be recruited by a target promoter (36). Gain-of-function mutant p53 (p53H175) inhibits

phosphorylation of SMAD2/3, inhibits translocation of SMAD4 in to the nucleus and induces attenuation of expressing TGF β RII along with several other inhibitory effects (37), highlighting the interaction between SMADs and p53 (Figure 3). Phosphorylation of p53 by interaction with MAPK p38, a component of TGF β non-canonical pathway, was shown to be directly mediated by SMAD7 (23). The latter acts as a scaffold facilitating the interaction between p38, Ataxia-telangiectasia mutated (ATM) and p53 promoting apoptosis and cell death in HEK 293 and HTC 116 cell lines (23). Multiple interactions between p53 and SMAD pathways, including those via microRNA and Drosha complex, that influence gene transcription have been unearthed indicating their complementary action on carcinogenesis (38).

Effects of SMADs on stem cell proliferation and epigenetic changes

BMP arm of the TGF β pathway is a key regulator involved in intestinal stem cell replication and differentiation. Evidence in human intestinal stem cells in this area is not abundant. Recently Qi et al has shown that BMP inhibits the proliferation of Lgr5+ stem cells in colonic crypts (39). Vil-CreER;Lgr5-EGFP;Bmpr1afl/fl mice were used to demonstrate the hyper proliferation of Lgr5+ stem cells following complete ablation of BMPRI receptors. They propose that this function prevents premalignant hyper proliferation of the stem cells following injury. This study also contradicts previously proposed mechanism of BMP controlling stem cell proliferation via an inhibition of the *Wnt* pathway (40). In the current study the researches failed to observe a rise in nuclear β -catenin levels in the stem cells indicating an intact *Wnt* pathway (39). BMP and Nodal, part of TGF β superfamily, have shown to modulate embryonic stem cell differentiation in a complex multifaceted mechanism; specifically, BMP pathway in embryonic stem cells to be regulated by SMAD7 under the influence of Nodal (41). Elevated SMAD7 levels divert these cells to differentiate into mesoderm by blocking the action of BMP, which usually influence them to maintain the pluripotent state. This observation has also been validated in murine embryonic stem cells, where a more efficient differentiation into mesoderm or neuroectoderm is shown in the absence of BMP-SMAD signalling (42). A link between SMADs and epigenetic changes has been reported by Papageorgis, et al

in human breast cancer cells (43). The hypermethylation of CDH1 was observed to be dependent on an intact TGF β 1 signalling resulting in epithelial mesenchymal transition (EMT). Enhanced expression of SMAD7 was observed to reverse this action and the cells to regain epithelial characteristics in a MCF10 breast cancer line, including enhanced production of E-cadherin for increase cell adhesion. Similarly higher methylation levels of CDH1 has been observed in colonic epithelium of patients with UC (44). BMP arm of the TGF β pathway has also been linked to epigenetic changes in key growth regulating genes. Reduction of BMP signalling in mouse embryonic stem cells after SMAD1/SMAD5 double KO was linked to higher methylation level in the SMAD1/SMAD5 deficient cell lines, which ultimately affected the enrichment of gene ontology categories involved in cell development and differentiation (42). This finding may open a whole new area of future research looking in to effect of BMP-SMAD signalling on epigenetic changes in stem cells in the colonic crypts.

Role of BMP in carcinogenesis

BMP's inhibitory role in colorectal carcinogenesis is well described in relation to *juvenile polyposis syndrome* where the epithelial mesenchyme transformation appears to be influenced by the absence of BMP (45). In particular, hamatomatous polyps, which cause the shift to malignancy, are characterised by *BMPRIA* and *SMAD4* gene mutations which cause loss of BMP function (46). BMP signalling also reduces during the transformation of sporadic colorectal adenomas to carcinoma (47). However, recent evidence indicates that BMP induces *Wnt* pathway in the presence of aberrant *SMAD4* and/or mutated *p53*, which is paradoxical to its native action (48). Furthermore, mutated *SMAD4* converts BMP action from tumour suppressor to promoter, and patients harbouring this mutation in CRC have shown to have poor overall survival (49). As the activation of the *Wnt* pathway and *p53* mutation are early events (50), (34) in the neoplastic process associated with IBD, this could suggest that BMP may be an early driver of this phenomenon. The detailed functioning of the BMP pathway in IBD or the effect of manipulating *SMAD7* on the BMP pathway and its correlation to neoplasia has therefore not been explored adequately.

Observations in sporadic CRC

Although the pathological process leading CAC differs from that of sporadic CRC, understanding the influence of SMAD proteins on specific aspects of CRC pathogenesis helps to place the available data in context. Moreover although the processes differ in the sequence they occur there are events in the carcinogenic process that are common to both (17). Riggins et al in an early attempt to describe this biphasic behaviour evaluated the SMAD 2,4,5 and 6 genes in a number of human cancers including CRC (51). They did not find significant mutations in these genes that could explain the resistance of these tumours to TGF β although SMAD 2 and 4 were mutated in a minority of CRC. Relationship between the risk of CRC with single nucleotide polymorphisms (SNP) in SMAD7 gene is observed in several studies although the findings are inconsistent (52), (53), (54). Both higher and lower risks have been attributed to these genomic variations, although whether the SNPs significantly affect gene transcription of SMAD7 with an effect on TGF β signalling is not clear. A recent meta-analysis identified several SNPs (rs4464148, rs4939827 and rs12953717) consistent throughout most studies, with a higher risk of CRC in specific ethnic groups (55). Deletion of the SMAD7 gene has been associated with better overall and disease free survival outcome, while copy number gains showed the converse (56). SMAD7 expression was shown to be up regulated in tissue from 14 sporadic CRC compared to adjacent tissue as matched controls (57). Furthermore, two CRC cell lines (HCT-116 and DLD-1) showed marked reduction in DNA replication after inhibition of SMAD7 synthesis with an antisense oligonucleotide (57). Both cell lines demonstrated a reduction in the proliferating population on cytometry. Same cell lines have been used to demonstrate the cancer cell death associated with knockdown of SMAD7 via activation of eukaryotic translation initiation factor 2A (eIF2 α) (58). Development of colorectal tumours in *APC*^{-/+} mice could also be down regulated by the same SMAD7 antisense, highlighting the tumorigenic effect of SMAD7 protein on CRC. Analysis of 600 colorectal cancer specimens in microarrays underlined the correlation between lack of SMAD2, phosphorylated-SMAD2 and SMAD4 with advanced tumour stage and poor overall outcome (59). From a structural point of view, the effector function of

SMAD2 and SMAD4 resides in their C-terminal, while the inhibitory function is associated with the N-terminal of the proteins. The N-terminal was shown to acquire an auto-inhibitory activity due to mutations resulting in the loss of tumour suppressor activity (60). Recent evidence have emerged linking the SMAD activity to micro RNA and specifically of SMAD7. Wang et al has demonstrated Nuclear reporter subfamily 2 (NR2F2) induced miR 21 mediated inhibition of SMAD7 promoting TGF β dependant epithelial mesenchymal transition (EMT) in CRC (61). This indicates the lack or inhibition of that of SMAD7 could promote cancer metastasis. These available evidence from sporadic CRC in this regard is non convergent, in that some SMADs appear to have tumour suppressor activity while others promote tumour growth. These findings may provide direction into future CAC research.

Role of SMADs in CAC

The close relationship of TGF-SMAD pathways with the pathogenesis of IBD and CRC carcinogenesis may suggest that SMAD could be playing a central role in CAC as well. Although evidence from human clinical research is scarce relating SMAD proteins to CAC, studies done on animal models provide some insight. From available evidence one can argued that changes in SMADs would alter intracellular signalling of the TGF β family ligands, which affect transcription of apoptosis and cell cycle genes, ultimately having a direct effect on carcinogenesis. Gorelik and colleagues have observed that blocking TGF β in T cells could exert an anti-tumour immune response towards thymoma and melanoma in mice (62). Similarly a high level of SMAD7 expression (blocking TGF β) in LPMCs is protective against CAC in murine models; SMAD7 Transgenic mice showed significantly less tumour scores (both size and number) compared to wild type mice with dextran sodium sulphate (DSS) and azoxymethane (AOM) induced colitis, despite having higher inflammatory scores (63). Moreover, protection from developing CAC is dependent on interferon γ (IFN γ), indicating that this protection could be due to SMAD7 expressing T cells. Furthermore, dysplastic epithelial cells from WT mice show higher SMAD7 expression, although whether the accumulation of SMAD7 in epithelium is driving the dysplasia or it is a protective response to inflammation related neoplasia

remains unanswered (63). Interestingly, SMAD7-expressing T cells are down regulated in a small cohort of patients with CAC compared to those with inflammation alone (63). Therefore, it would be interesting to evaluate *p53* mutations in the same samples and correlate them with SMAD7 levels, as mutant *p53* has been shown to attenuate the ability to produce SMAD7 (negative feedback loop) in response to TGF β (37). In a novel attempt to analyse correlation between tumorigenesis and mediators in IBD using a network model, Lu and colleagues identified SMAD activation (along with five other molecules) as an inhibitor of cell proliferation and apoptosis (64). The group developed a Boolean network model to elucidate the development of neoplasia amid chronic inflammation. This interesting *in silico* model is however still to be validated in *in vitro* or *in vivo* settings. Furthermore, the absence of SMAD3 expression in combination with the presence of *H. pylori* has also been observed to cause colon cancer preceded by an inflammatory phase in a mouse model (65). Although a possible involvement of *H. pylori* in a background of absent TGF signalling could lead to the development of CAC, these results are yet to be reproduced.

Effects of manipulating SMAD proteins in IBD

The interest in SMAD pathways in IBD has been renewed by the introduction of a new drug targeting SMAD7. Theoretically, based on the available knowledge, an increased activity of both TGF β 1 and BMP pathways is expected when SMAD7 is blocked in colonic epithelial cells (24). The alteration in the concentration gradient of BMP in the colonic crypts due to the inhibition of SMAD7, could affect the differentiation of stem cells, as previously suggested (42), with resulting implications in the neoplastic process. Although reducing SMAD7 in LPMC in order to reduce inflammation is expected to reduce the risk of neoplasia, the fact that SMAD7-expressing LPMCs are protective against CAC (63) could result in a paradoxical effect with regard to an existing neoplastic lesion. Furthermore, if SMAD7 is a facilitator of *p53*(23), inhibiting its action could promote cell perpetuation due to the loss of tumour suppressor activity. However, it should be noted that evidence is also available to the contrary, indicating SMAD7 sustains colorectal cancer cells lines and enhance their invasiveness(57), which eventually confirm this area needs further evaluation before a targeted therapy

could be implemented. An interesting review by Massagué et al on the signalling context of TGF β describes epigenetics as one of the three determinants in differential function of the pathway (22). Variations in signal transduction molecules and partner transcription factors such as p53 are the two other determinants by which the author attempts to untangle the mystery behind paradoxical effects of TGF β at different occasions. With evidence emerging to indicate the close relationship of SMAD pathways to epigenetic changes in embryonic cells (43) and p53 function, it could be postulated that inhibiting SMAD7 can have direct or indirect implication on colorectal tumorigenesis. While non-converging evidence exists in colorectal cancer, inhibition of SMAD7 has been shown to favour carcinogenesis in the liver, prostate, pancreas and breast (66).

Direction for future research

Decades after the TGF-SMAD pathway was first described, it still remains an 'unsolved puzzle' at the centre of many biological processes including inflammation, fibrosis, wound healing and cancer. Several well-identified stages of dysplasia precede CAC and the effects of TGF-SMAD pathways may be paradoxical at different stages. It is difficult to predict the end effect of blocking SMAD7 with an antisense and increasing TGF β activity when patients have these preceding changes alongside inflammation. The changes that take place in the epithelial cells of the colon during the neoplastic process have not yet being well studied and the developers of mongersen (GED-0301, Celgene) have also indicated the need for this (28). Authors' unpublished observations show high expression of SMAD7 on immune histochemical staining (R&D system; monoclonal Mouse IgG_{2B}; MAB2029) in the epithelial layer of ulcerative colitis associated cancers (Figure 4). An in-depth analysis of SMAD7 pathways would be therefore valuable in providing answers to many queries regarding the role of SMAD proteins in CAC. Development of human colonic organoids have confirmed the need of low BMP for colonic stem cells to replicate(67). Recent findings by Qi et al further substantiate the role of BMP in restricting the proliferation of Lgr5⁺ stem cells in colonic crypts. Effects of a high BMP concentration, in the back drop of inhibiting

SMAD 7, on the reparative process of the intestinal epithelium require further evaluation. Epigenetic changes that could occur in IBD mucosa due to chronic inflammation may also affect the SMAD binding elements in DNA altering transcription of apoptosis and cell cycle regulatory genes. Therefore, a deeper understanding of the epigenetic changes that may be affected by the TGF β ligand pathways in the colon is required. If the high SMAD7 expression is sustaining inflammation in colonic epithelium, the same expression might also inhibit the BMP pathway within the cells, affecting promoter methylation as described by Gomes et al (42). In conclusion, the role of SMADs in CAC has been largely unexplored as yet. Further studies are required to envisage the probable effects of altering the multifaceted SMAD related pathways on CAC.

Conflict of interest

Authors; Pramodh Chandrasinghe, Biancastella Cereser, Ibrahim Al Bakir, Neha Tabassum, Morgan Moorghen, Ailsa Hart, Justin Stebbing and Janindra Warusavitarne have no conflict of interest to be declared with regard to the work in this publication.

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Figure legend

Figure 1

Colorectal cancer tumorigenic process: adenoma carcinoma sequence in sporadic CRC versus colitis associated cancer based on available evidence (14–17)

MSI – microsatellite instability

Figure 2

SMAD dependent canonical TGF β ligand pathways

Figure 3

Interaction between SMAD proteins and components of colorectal carcinogenic process

Figure 4

Colitis associated colon cancer with high expression of SMAD7 in epithelial cells compared to non-neoplastic epithelium.

a1- H&E staining , **b1** – IHC staining for SMAD7 in non-neoplastic colonic tissue of a 52 year old patient with longstanding UC (scale bar – 1mm). **a2** and **b2** – magnified view of the region in-set (scale bar: 100 μ m). **a3**- H&E staining and **b3**- IHC staining for SMAD7 of a sigmoid colon cancer of the same patient (pT2, moderately differentiated adenocarcinoma); **a4** and **b4** – magnified view of the region in-set (scale bar: 100 μ m).

c1 – H&E staining and **d1**- IHC staining for SMAD7 in non-neoplastic colon tissue of a 49 year old male with long standing UC (scale bar – 1mm). **c2** and **d2** – magnified view of the region in-set (scale bar – 100 μ m). **c3** – H&E staining and **d3**- IHC staining for SMAD7 of a transverse colon cancer of the same patient (pT3, moderately differentiated adenocarcinoma); **c4** and **d4** - magnified view of the region in-set (scale bar: 100 μ m).

H&E- haematoxylin and eosin, IHC - immune histochemistry, UC – Ulcerative colitis