OESOPHAGUS

Impaired transforming growth factor β signalling in Barrett's carcinogenesis due to frequent SMAD4 inactivation

B A Onwuegbusi, A Aitchison, S-F Chin, T Kranjac, I Mills, Y Huang, P Lao-Sirieix, C Caldas, R C Fitzgerald

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See end of article for authors' affiliations

Correspondence to: Dr R Fitzgerald, MRC Cancer Cell Unit, Hutchison/MRC Research Centre, Hills Rd, Cambridge, UK; rcf@hutchison-mrc.cam.ac.uk

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Background and aims: Transforming growth factor β (TGF-β) is frequently involved in gastrointestinal carcinogenesis although its contribution to oesophageal adenocarcinoma (AC) and its precursor Barrett's oesophageal epithelium (BE) metaplasia are unclear.
Methods: Expression of TGF-β signalling components was assessed by reverse transcription-polymerase chain reaction (PCR), western blot, and immunohistochemistry in oesophageal endoscopic biopsies and cell lines. Genomic alterations in *SMAD4* were characterised by fluorescence in situ hybridisation, methylation specific PCR, and sequencing. Functional integrity of TGF-β signalling was assessed by characterisation of p21 and proliferation status. Smad4 negative BIC-1 cells were transiently transfected with *smad4* and TGF-β responsiveness evaluated.
Results: *smad4* mRNA expression was progressively reduced in the metaplasia-dysplasia-adenocarcinoma sequence ($p<0.01$). A quarter of AC samples displayed an abnormal Smad4 protein isoform, with no corresponding changes in gene sequence or organisation. Methylation of *smad4* has not been described previously but we found promoter methylation in 70% of primary AC samples. In 6/8 oesophageal cell lines, chromosomal rearrangements affected the *smad4* locus. Lack of *smad4* expression in BIC-1 cells occurred secondary to loss of one copy and extensive deletion of the second allele's promoter region. TGF-β dependent induction of p21 and downregulation of minichromosome maintenance protein 2 was lost in >80% of BE and AC. TGF-β failed to inhibit proliferation in 5/8 oesophageal cell lines. In BIC-1, the antiproliferative response was restored following transient transfection of *smad4* cDNA.
Conclusions: In BE carcinogenesis, downregulation of Smad4 occurs due to several different mechanisms, including methylation, deletion, and protein modification. Frequent alterations in TGF-β signalling lead to a functionally significant impairment of TGF-β mediated growth suppression.

The incidence of oesophageal adenocarcinoma (AC) has risen sharply since the 1970s,[1] and the five year survival rate is less than 20% unless diagnosed at an early stage.[2] Early intervention is in theory possible because AC usually arises via the formation of columnar metaplasia, termed Barrett's oesophageal epithelium (BE). BE patients have a 30-fold increased risk of progressing to cancer through a multistep metaplasia-dysplasia-adenocarcinoma sequence.[3] During the metaplasia-dysplasia sequence there is accumulation of genetic abnormalities which result in loss of proliferative control.[4] The pleiotropic cytokine, transforming growth factor β (TGF-β), exerts profound regulatory effects on the differentiation and proliferation of epithelial cells. An alteration in TGF-β signalling may thus contribute to the development and progression of BE, in keeping with other cancers.[5]

TGF-β signalling is initiated by activation of type I and II transmembrane serine/threonine kinase receptors (TβRI and TβRII)[6] which leads to phosphorylation of the intracellular signalling molecules, Smad2 and Smad3. This complex, in association with Smad4, is then translocated into the nucleus resulting in transcriptional regulation of target genes such as downregulation of c-Myc and upregulation of the cyclin dependent kinase inhibitors $p15^{INK4B}$ and $p21^{CIP/WAF1}$.[6] Hence, in combination with induction of apoptosis in certain cell types, TGF-β can function as a negative growth factor.[5]

Gastrointestinal malignancies frequently display inactivating mutations of the TGF-β signalling cascade. In microsatellite unstable tumours, 81% of colon cancers and 69% of gastric cancers undergo frameshift mutations of TβRII.[7] In addition, functional inactivation of *smad4* (DPC4, deleted in pancreatic carcinoma, locus 4) has been described in 50% of pancreatic adenocarcinomas,[8] 30% of metastatic colorectal cancers,[9] and 25% of small intestinal carcinomas.[10]

The contribution of perturbed TGF-β signalling in AC is poorly understood. It has been suggested that mutations in TβRII are an infrequent event,[11 12] presumably as a result of microsatellite instability being uncommon.[13] In one study, 3/11 ACs demonstrated loss of TβRII expression.[14] BE associated ACs have been shown to undergo loss of heterozygosity at chromosome 18q, the location of *smad2* and *smad4*, in up to 69% of patients, and in as many as 46% of patients with non-dysplastic BE.[15 16] However, further analysis did not reveal any inactivating mutations of *smad4*.[15]

This study was designed to test the hypothesis that there are abnormalities in TGF-β signalling in Barrett's carcinogenesis.

Abbreviations: AC, adenocarcinoma; BE, Barrett's oesophageal epithelium; FISH, fluorescence in situ hybridisation; HGD, high grade dysplasia; LGD, low grade dysplasia; MSP, methylation specific polymerase chain reaction; Mcm2, minichromosome maintenance protein 2; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NE, normal squamous epithelium; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TGF-β, transforming growth factor β; TβRI and TβRII, type I and II transmembrane serine/threonine kinase receptors

Table 1 Alteration of genes associated with cell cycle control in oesophageal cell lines

	p16 expression	p53 expression	p53 base change	p53 amino acid change	p53 exon	p53 codon	Reference
C hTRT	−	M	CGC-CAC	Arg-His	5	175	Palanca-Wessels,[20] own observations
Gi hTRT	−	M	CGG-TGG	Arg-Trp	7	248	Palanca-Wessels,[20] own observations
Go hTRT	+	M	Coding shift	Gly-coding shift	8	302	Palanca-Wessels,[20] own observations
OE33	+	M	TGC-TAC	Cys-Tyr	5	135	Own observations
TE7	−	−	−	−	−	−	Own observations
SEG	−	W	−	−	−	−	Own observations
BIC	−	M	GAG-TAG	Glu-Ter	6	204	Own observations
FLO	−	M	TGT-TTT	Cys-Phe	8	277	Own observations

−, no mRNA expression; +, expression of mRNA; M, expression of mutant p53; W, expression of wild-type p53.

The specific aims of this research were firstly to examine expression of the TGF-β signalling components in clinical specimens and cell lines from the metaplasia-dysplasia-carcinoma sequence; and secondly, to determine the genetic basis and functional significance of the observed reduction in Smad4.

METHODS

Patient samples

Paired endoscopic oesophageal biopsy samples for research and routine histopathology were obtained prospectively following local research ethics committee approval by Addenbrooke's Hospital NHS Trust. Samples were classified by an expert upper gastrointestinal histopathologist, as part of the routine diagnostic service, using internationally agreed criteria.[17 18] Specimens were classified into the following groups: normal squamous epithelium (NE, n = 30); BE (endoscopically visible segment with columnar lined epithelium and specialised intestinal metaplasia on biopsy) without dysplasia (n = 30); low grade dysplasia (LGD, n = 15); high grade dysplasia (HGD, n = 15); and adenocarcinoma arising from BE (AC, n = 35). All cases of HGD were confirmed by a second independent consultant pathologist within the clinical department. Different patient samples were used for different analyses. All data points within an experiment were derived from separate patients rather than multiple biopsies taken from within the same patient. For immunohistochemistry, paraffin sections were selected from the tissue archive at Addenbrooke's Hospital from BE and AC patients. All tissue sections fulfilled the diagnostic criteria specified above.

Cell lines

BE HGD cell lines transduced with the human catalytic subunit of telomerase reverse transcriptase (hTERT), C hTRT, Gi hTRT, G0 hTRT (gift from P Rabinovitch, University of Washington, Washington, USA) were maintained in MCDB 153 medium.[19] These cell lines were all derived from patients with histopathologically confirmed HGD.[19] Barrett's associated adenocarcinoma cell lines OE33 (European Collection of Cell Cultures, Wiltshire, UK), TE7 (gift from T Nishihira, Kurokawa County Hospital, Japan), SEG-1, BIC-1, and FLO-1 (gift from D Beer, University of Michigan, Michigan, USA) were used. OE33 and TE7 cells were maintained in RPMI-1640 medium, and SEG-1, BIC-1, and FLO-1 in Dulbecco's modified Eagle's medium, all supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. A transitional cell bladder carcinoma cell line RT4 was used as a control for genomic analysis of *smad4*. Details of all of these cell lines with regard to their p53 and p16 status, which affect cell cycle control, are given in table 1.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Paisley, UK). RNA (2 μg) was reverse transcribed, and 2 μl of cDNA amplified as follows: 30 cycles of 94℃ for 30 seconds, 55℃ for 30 seconds, and 72℃ for 45 seconds for TβRI, TβRII, *smad2*, *smad3*, *smad4*, and β actin, as previously described,[21] 30 cycles of 94℃ for 45 seconds, 60℃ for 45 seconds, and 72℃ for one minute for GAPDH, using the primers 5′-GCA GGG GGG AGC CAA AAG GG-3′ (forward) and 5′-TGC CAG CCC CAG CGT CAA AG-3′ (reverse). PCR products were analysed on 1.5% agarose gels, stained with ethidium bromide, and quantified by densitometry using Kodak Electrophoresis Documentation and Analysis System 120 (EDAS) software (Eastman Kodak Company, Rochester, New York, USA).

Western blotting

Protein lysates were prepared with ice cold lysis buffer (20 mM Tris pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% NP40) containing protease inhibitors (Complete Tablet; Roche, Germany). Following quantification (BCA protein assay; Sigma-Aldrich Company Ltd, Dorset, UK), 50 μg of protein were separated by gel electrophoresis on 8% polyacrylamide gels and transferred to PVDF membranes (Hybond-P; Amersham Biosciences, Amersham, UK). Membranes were incubated overnight at 4℃ with the following antibodies: TβRII (1:500; clone C-4), Smad4 (1:500; clone B-8), p-Smad2/3 (1:500), Smad2/3 (1:500, clone E-20), actin (1.500, clone I-19) (all Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA), and SUMO-1 (1:500; Alexis Corporation, Nottingham, UK).

Tissue immunohistochemistry

Archival paraffin sections from BE and AC patients were deparaffinised, rehydrated, and then exposed to sodium citrate buffer for three minutes in a pressure cooker. Slides were blocked with 10% goat serum for one hour at room temperature and incubated with either TβRII (Santa Cruz Biotechnology; 1:100), Smad4 (Santa Cruz Biotechnology; 1:100), or Smad2 (clone S-20; Santa Cruz Biotechnology;

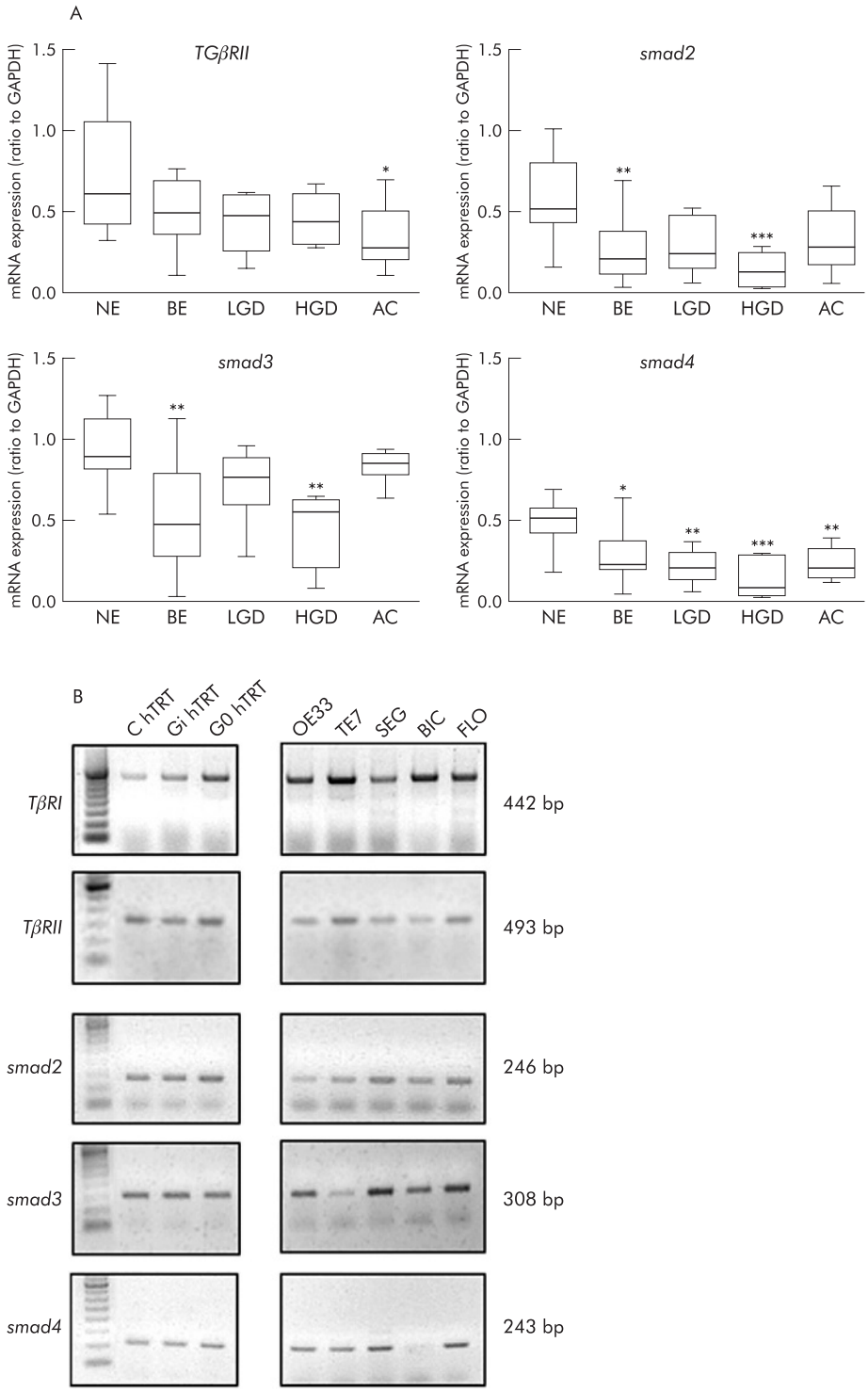

Figure 1 mRNA expression of transforming growth factor β (TGF-β) signalling pathway components in the metaplasia-dysplasia-carcinoma sequence. (A) Reverse transcription-polymerase chain reaction (RT-PCR) was performed for primary samples of normal squamous epithelium (NE), Barrett's oesophageal epithelium (BE), low grade dysplasia (LGD), high grade dysplasia (HGD), and adenocarcinoma (AC), and compared with GAPDH expression for *TGFβRII* and *smad* molecules 2, 3 and 4. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with NE. (B) RT-PCR was also performed for a panel of cell lines. Representative gels are shown for cell lines derived from HGD in BE (hTRT immortalised) in the left hand panel and from BE adenocarcinoma cell lines in the right hand panel.

1:100) antibodies overnight at 4℃. Antibody detection was achieved with antimouse or antigoat horseradish peroxidase (Dako Ltd, Ely, UK) followed by 3,3′ diaminobenzidine and a haematoxylin counterstain. Qualitative analysis was then performed.

Methylation analysis

Genomic DNA (1 μg) was denatured with sodium hydroxide and incubated with sodium bisulphite.[22] DNA was then purified using Wizard DNA Clean Up System (Promega, Madison, Wisconsin, USA), desulphonated with sodium

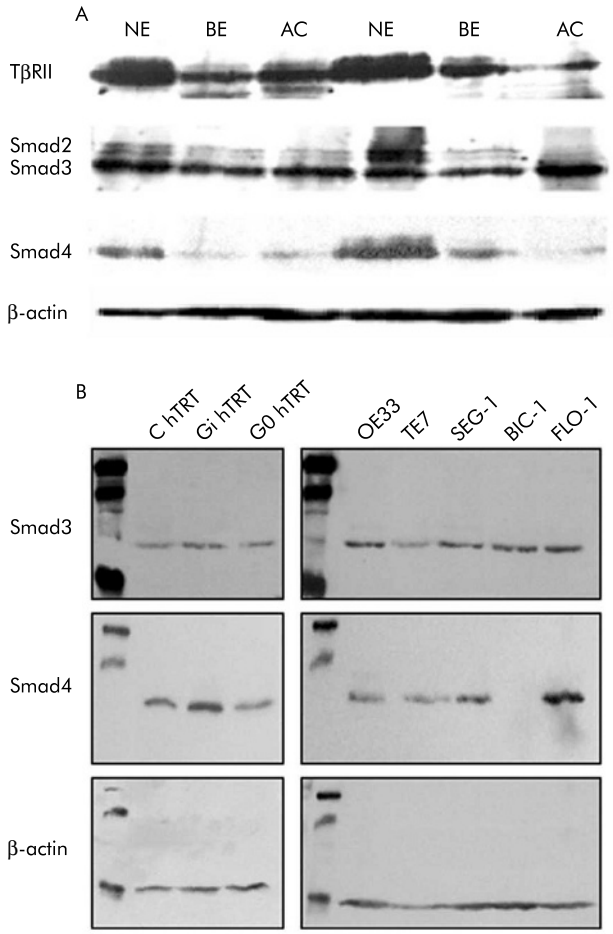

Figure 2 Protein expression of transforming growth factor β (TGF-β) signalling pathway components in the dysplasia-carcinoma sequence in vitro. (A). Western blot on 25 μg of cell lysate using primary samples of normal squamous epithelium (NE), Barrett's oesophageal epithelium (BE), and adenocarcinoma (AC) for TGFβRII, Smad2/3, and Smad4 compared with β-actin (upper panel). (B). Western blot for Smad3 and Smad4 in BE oesophageal cell lines. Left hand panel shows cell lines derived from high grade dysplasia in BE (hTRT immortalised) and the right hand panel from BE adenocarcinoma cell lines.

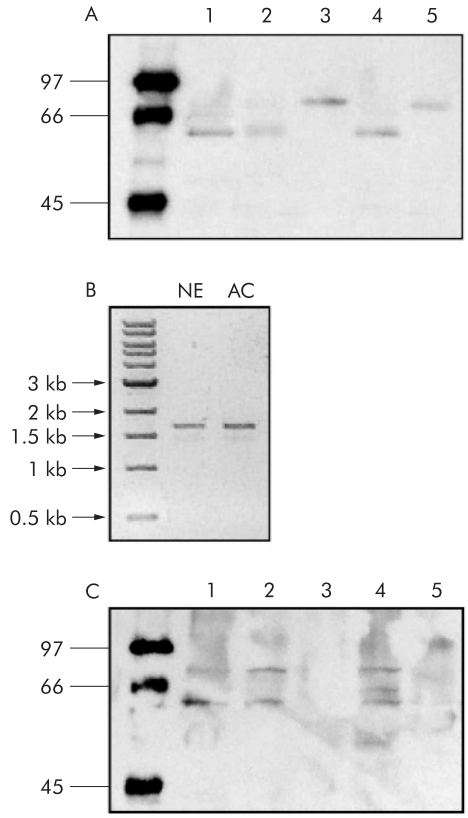

Figure 3 Smad4 alterations in adenocarcinoma (AC). (A) Representative western blot from a denaturing gel of Smad4 expression in Barrett's oesophageal epithelium (BE) adenocarcinoma. Sample 3 and 5 display a 10–15 kDa difference in normal Smad4 mobility. This abnormality was seen in 25% of biopsies examined. (B) Expression of Smad4 mRNA in control normal squamous epithelium (NE) and in a patient sample (AC) which had exhibited an alteration in Smad4 mobility when examined by western blot. The AC sample expressed full length Smad4 mRNA. (C) Smad4 western blot in (A) stripped and reprobed for evidence of sumoylation using an antibody SUMO1. Samples 1, 2, and 4 show the presence of SUMO1 at the molecular weight of Smad4. Samples 3 and 5 do not show the presence of SUMO1 at the level Smad4 of altered mobility.

hydroxide, ethanol precipitated, and resuspended in Tris/EDTA. Treated DNA was analysed by methylation specific PCR (MSP) for the *Smad4* promoter using the following primers: for the methylated reaction, 5′-GTA ATA ATA CGG TTT TGG TCG TC-3′ (forward) and 5′-CTC CCA CCC CCT AAA CGA CCG CG-3′ (reverse); for the unmethylated reaction, 5′-GTA ATA ATA TGG TTT TGG TTG TT-3′ (forward) and 5′-CTC CCA CCC CCT AAA CAA CCA CA-3′ (reverse). PCR was carried out for 40 cycles with annealing temperatures of 65℃ for the methylated reaction and 59℃ for the unmethylated reaction. MSP products were further analysed by cloning into pCR2.1 vector (Invitrogen, Paisley, UK) and sequencing of individual clones using a BigDye Terminator protocol and the ABI 3100 Genetic Analyser (Applied Biosystems, Warrington, UK).

Fluorescence in situ hybridisation (FISH)

The *smad4* gene was identified from Ensembl to be contained within the RP11-729G3 BAC clone. BAC DNA were isolated by the alkaline-lysis method and labelled with biotin-16-dUTP (Roche Biochemicals, UK) using nick translation (Vysis, UK). Labelled BAC was precipitated together with a probe for centromere 18 (gift from M Rocchi, Molecular Cytogenetics Resources, Bari, Italy) or a whole chromosome 18 paint labelled with FITC (Roche Biochemicals, UK) in the presence of unlabelled human COT-1 DNA. Metaphase preparations were obtained using standard potassium chloride/Carnoy's fixative protocols and stored at −20℃. Metaphases were made from peripheral blood lymphocytes from normal individuals for reference. Probes were applied to slides for a 72 hour hybridisation, detected with strepavidin-Cy3 (Amersham, UK) and counterstained with DAPI in mounting medium (Vectashield, Vector Labs, UK). Slides were examined under a Zeiss Axioplan 2 epifluorescence microscope and digital images taken using a Hamamatsu ORCA II camera (Hamamatsu, Japan).

smad4 promoter analysis

For BIC-1 cells, the 300 kb region of chromosome 18 around the *smad4* promoter was studied using a series of markers. A bladder cell line RT4 known to contain the region was used as a control. Markers shown in fig 7C are taken from Yanaihara and colleagues,[23] except *smad4*+3 kb: 5′-CAT CCG GGA ACA TTT GTT TT-3′ (forward) and 5′-TTG GGC TGA ACG GTC TTT AC-3′ (reverse).

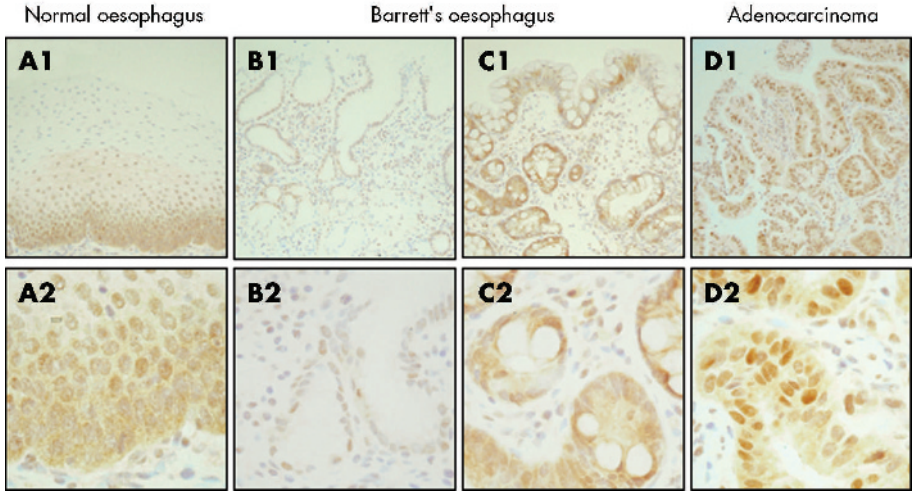

Figure 4 Immunohistochemical staining for Smad4 in sections of normal squamous epithelium, non-dysplastic Barrett's oesophageal epithelium, and adenocarcinoma. Positive immunostaining is denoted by brown colouration in contrast with the blue haematoxylin counterstain. The top panel (A1–D1) is at 100× magnification and the bottom panel shows the same fields at 400× magnification.

PCR and sequence analysis

Genomic DNA was isolated using Trizol reagent (Invitrogen). To analyse exons 1–11 of the *smad4* gene, 250 ng of DNA were amplified in a 100 µl volume using primers, as previously described,[24] 2.5 U of *Pfu* DNA polymerase (Stratagene), and conditions of hot start followed by 30 cycles of 45 seconds at 94℃, 45 seconds at 58℃, and 45 seconds at72℃. PCR product (20 µl) was analysed on a 1.5% agarose gel and the remaining PCR products were then purified and directly sequenced using a BigDye Terminator protocol and the ABI 3100 Genetic Analyser (Applied Biosystems, Warrington, UK).

Organ culture

Biopsy samples from NE, BE without dysplasia, and AC (n = 9) were cultured in serum free organ culture media (medium 199) in a 95% O_2/5% CO_2 atmosphere, as described previously.[25] Following a three hour equilibration period, samples were cultured with or without 20 ng/ml of recombinant TGF-β1 protein (Tebu-bio, Cambridgeshire, UK) for a further 24 hours. Samples were then snap frozen and stored at −80℃ until required

Quantitative real time PCR

cDNA (1 µl) was amplified in a 50 µl volume containing 25 µl of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, West Sussex, UK) and 0.2 µM of each primer. Primers used were 5′-GGA AGA CCA TGT GGA CCT GT-3′ (forward) and 5′-GGC GTT TGG AGT GGT AGA AA-3′ (reverse) for p21, 5′-TTC CCT GGG ATT CTG GTT-3′ and 5′-AAG CAG GCT TGG AGA AAC AA-3′ (reverse) for minichromosome maintenance protein 2 (MCM2), and 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′ (forward) and 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′ (reverse) for β-actin. Triplicate reactions were performed in a DNA Engine Opticon thermal cycler with initial enzyme activation of 15 minutes at 95℃, followed by 30–35 cycles of 10 seconds at 95℃, 20 seconds at 60℃, and 20 seconds at 72℃. Following PCR the threshold cycle was obtained and relative quantities determined for each sample normalised to β-actin.

Cell proliferation assay

Cells seeded at a density of 5000 cells/well were cultured with 0.05–10 ng/ml recombinant TGF-β1 protein (Tebu-bio, Cambridgeshire, UK). After 72 hours of incubation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich) (5 mg/ml) was added and the plate incubated for a further four hours. Culture media was then removed and MTT crystals dissolved using MTT solvent (0.1 N HCl in anhydrous isopropanol).[26] The optical density of each well was read at 570 nm.

smad4 transfection, immunoprecipitation, and cell proliferation

BIC-1 cells were seeded at 2×10^5 cells/well for protein expression or at 2×10^4 cells/well for the MTT assay. Cells were transfected with the pRK5-*smad4* FLAG expression vector (gift of A Chantry, School of Biological Sciences, University of East Anglia, UK) using transfection reagent (Genejuice; Novagen, Merck Biosciences, Nottingham, UK). Twenty four hours after transfection, cells were cultured in TGF-β (0.05–10 ng/ml) for 72 hours. For protein experiments, 50 µg of protein from lysed cells were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and membranes probed with Smad4 antibody, then stripped, and reprobed with actin antibody. In separate experiments, 24 hours after transfection, cells were incubated with 10 ng/ml TGF-β1 for 24 hours. Cells were lysed and Smad4 was immunoprecipitated from 200 µg of cell lysate using 2 µg of Smad4 antibody (Santa Cruz Biotechnology) overnight at 4℃. Sample (15 µl) was separated by SDS-PAGE and probed with anti-FLAG antibody (1:5000; Abcam Ltd, Cambridgeshire, UK). Membranes were stripped and reprobed with p-Smad2/3 antibody (Santa Cruz Biotechnology) for one hour at room temperature.

Immunofluorescence staining

Following transfection, cells were treated with 10 ng/ml TGF-β1 for two hours, fixed with methanol/acetone for five minutes at −20℃, washed with phosphate buffered saline, and blocked with 10% horse serum in phosphate buffered saline for 30 minutes. Slides were incubated with a 1:50 dilution of anti-Smad4 monoclonal antibody (Santa Cruz Biotechnology) for one hour at room temperature, washed, and incubated with a 1:100 dilution of FITC conjugated antimouse IgG (Vector Laboratories, Cambridgeshire, UK) and examined by confocal microscopy.

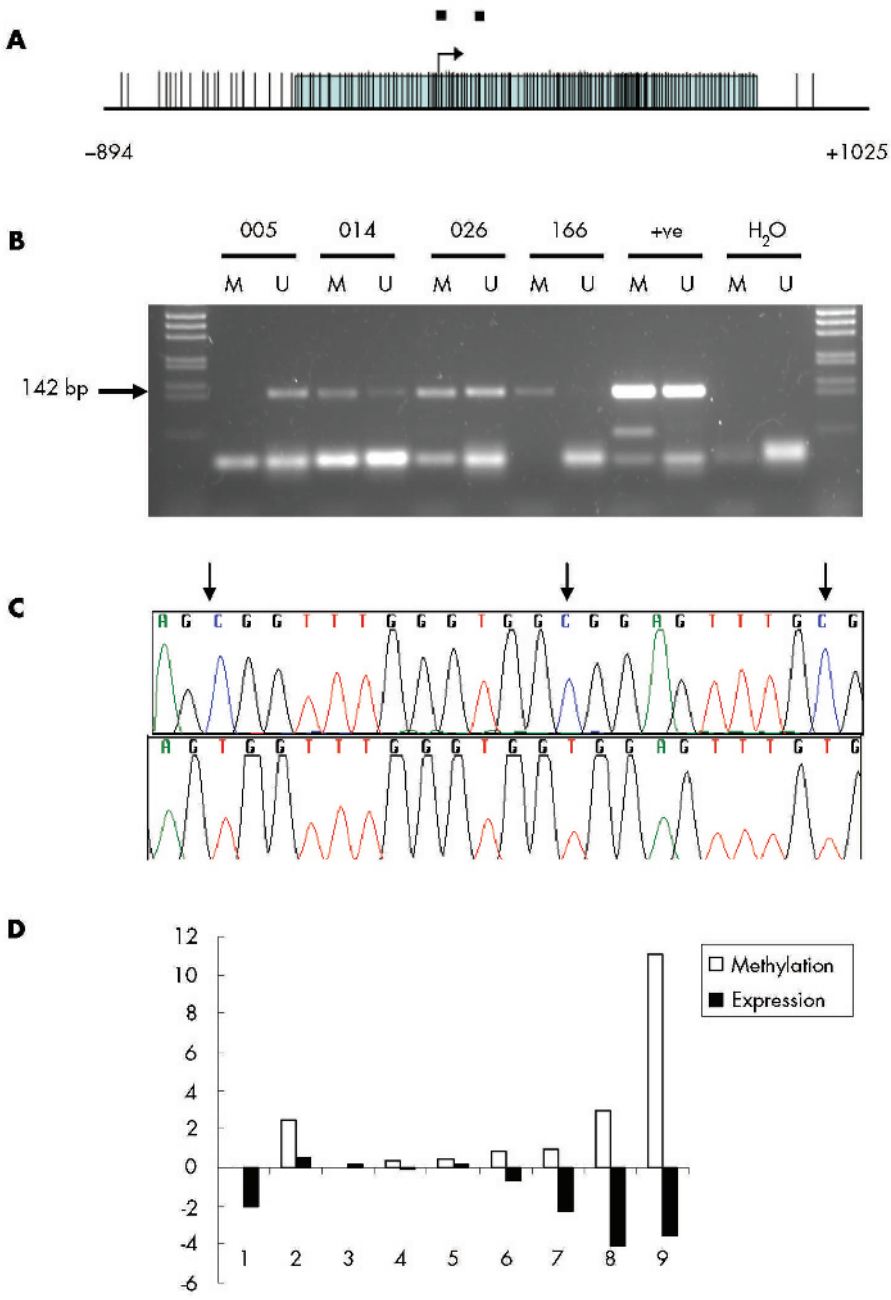

Figure 5 Methylation of the *smad4* promoter in adenocarcinoma. (A) The Smad4 promoter region is shown with the shaded area representing the extent of the CpG island. Vertical lines represent CpG sites; two black boxes represent methylation specific polymerase chain reaction (MSP) primer positions. The transcription start site corresponds to GenBank Accession No AB043547, base 119575. (B) MSP analysis of adenocarcinoma DNA showing the Smad4 MSP band at 142 bp. M, methylated specific reaction; U, unmethylated specific reaction. (C) Sequence analysis of clones of methylated (top) and unmethylated (bottom) MSP products. Black arrows indicate the location of CpG sites. (D) Methylation of the *smad4* promoter in adenocarcinoma samples expressed as a ratio of the intensities of the methylated MSP product against the unmethylated product. Matched expression data are shown as log10 of the quantitative polymerase chain reaction values.

Statistics

Data were analysed using Graphpad Prism software. The Kruskal-Wallis test was used to compare values between different groups, and Dunn's multiple comparison test was used to identify specific differences. Regression analysis was used to examine the correlation between *Smad4* methylation status and mRNA expression.

RESULTS

Expression changes in TGF-β signalling components in the metaplasia-dysplasia-carcinoma sequence

Expression of TGF-β signalling components was analysed in primary endoscopic tissues representative of the BE-AC sequence from a prospective cohort of patients. A panel of cell lines were also characterised to enable downstream functional studies to be performed. A significant reduction in *smad2* ($p<0.01$), *smad3* ($p<0.001$), and *smad4* ($p<0.05$) mRNA expression was seen in BE patient samples compared with primary NE (fig 1A). Although dysplastic samples displayed a reduction in *smad2* and *smad3* expression, this only reached significance in HGD ($p<0.001$ *smad2*; $p<0.01$ *smad3*). Expression of *smad4* mRNA was significantly decreased in BE, dysplastic BE, and AC ($p<0.01$, LGD; $p<0.001$, HGD; $p<0.01$, AC). Reduced expression of *TβRII* was only observed in AC ($p<0.05$). Expression of signalling components was more variable in cancer derived cell lines compared with levels seen in the dysplastic hTRT cell lines. The most noticeable abnormality was that *smad4* mRNA expression was completely absent in BIC-1 adenocarcinoma cells (fig 1B).

Changes in protein levels for TGF-β pathway components correlated with changes observed at the mRNA level for *smad2* and *smad4* (fig 2A). Furthermore, no Smad4 protein

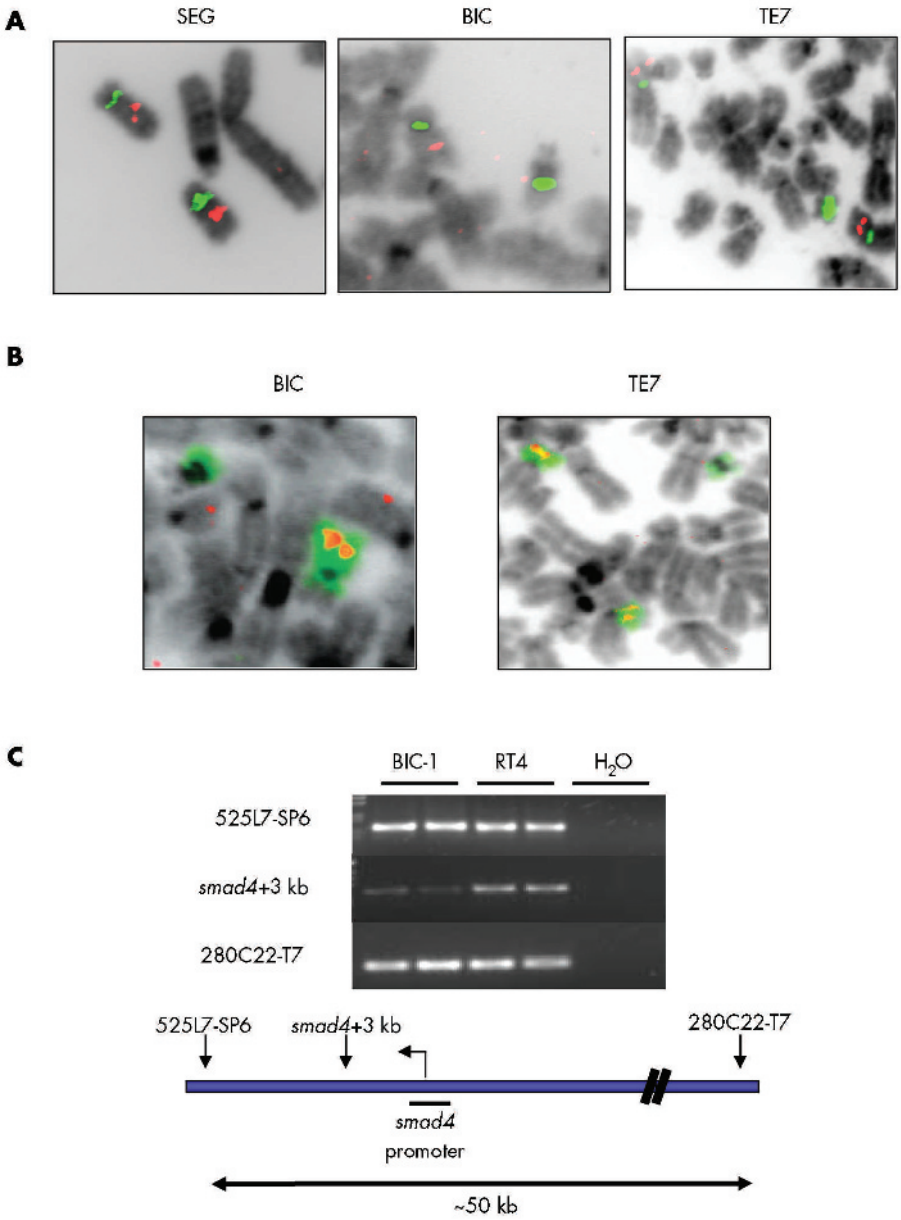

Figure 6 Chromosome 18 and *smad4* analysis in cell lines. (A) Fluorescence in situ hybridisation images using probes for the centromere (green) and *smad4* (red) in SEG-1, BIC-1, and TE7. (B) Structural rearrangements involving chromosome 18 identified using whole chromosome 18 paint (green) and SMAD4 (red) in BIC-1 and TE7. SEG-1 cells have two copies of chromosome 18 and two copies of Smad4. Chromosome 18 rearrangements in TE7 are balanced and no loss of *smad4* has occurred. BIC-1 cells contain only one copy of chromosome 18 and subsequently only one copy of *smad4*. (C) Polymerase chain reaction analysis of BIC-1 and control RT4 DNA showing that the remaining allele of *smad4* in BIC-1 is affected by a deletion spanning a 30–50 kb region of chromosome 18, including the *smad4* promoter.

Table 2 Chromosome 18 and Smad4 analysis by fluorescence in situ hybridisation in oesophageal cell lines

	CEP 18	*smad4* copy No	Translocation	Description of chromosome 18 translocations
C hTRT	2	2	None	
Gi hTRT	3	3	None	
Go hTRT	4	4	None	
OE33	4	2	Yes	2×18, 2× t(18;?)(p10;?)
TE7	3	2	Yes	i(18)(p10), 2×t(18;?)(q10;?)
SEG	2	2	None	
BIC	2	1	Yes	del 18, t(18;?)(q10;?)
FLO	4	4	None	

CEP 18, centromere 18 probe; t, translocation; I, iso-chromosome; p, short arm of chromosome; q, long arm of chromosome.

was expressed in BIC-1 cells, in keeping with the lack of *smad4* RNA expression (fig 2B). TβRII levels were reduced in BE and AC compared with NE (fig 2A) although no alteration was demonstrated in the cell lines, in keeping with the mRNA expression data (data not shown). Analysis of an additional series of AC samples by western blot revealed that seven of 28 (25%) samples displayed a band for Smad4 at 75–80 kDa, which is 10–15 kDa heavier than expected (fig 3A). Exons 1–11 of the *smad4* gene for these samples did not reveal any sequence alteration (data not shown), and there was a normal 1.6 kB product corresponding to the full length coding sequence of *smad4* when analysed by RT-PCR (fig 3B). Hence the abnormality in Smad4 protein mobility is likely to be a post-translational protein modification. The change in mobility is too great to be accounted for by phosphorylation or ubiquitination. Sumoylation was considered but no evidence for this was demonstrated (fig 3C).

Immunohistochemistry, from separate archival specimens, demonstrated that the observed decrease in expression of the signalling components reflects alterations within the

Table 3 Response of biopsy samples to transforming growth factor β in organ culture

	Normal oesophagus	Barrett's oesophagus	Adenocarcinoma	Duodenum
p21 increase	6/9 (67%)	2/9 (22%)	3/9 (33%)	7/9 (78%)
Mcm2 decrease	7/9 (78%)	2/9 (22%)	1/9 (11%)	8/9 (88%)

epithelium, rather than the lamina propria. Representative images are shown for Smad4 (fig 4). In all sections of NE examined, there was significant Smad4 expression within the basal layers of the epithelium (fig 4A) whereas staining in Barrett's oesophageal sections was highly variable and some patient sections had little discernable Smad4 staining (fig 4B compared with 4C). In AC, expression was heterogeneous but overall there was decreased expression compared with the homogeneously high levels of Smad4 expression within the normal squamous epithelium. This is in keeping with the RNA expression data. As expected, anti-Smad4 staining was variably localised to the cytosol or the nucleus, consistent with its translocation depending on its activation status.

Hence in both primary tissue and cell lines from the metaplasia-dysplasia-adenocarcinoma sequence there is a reduction in mRNA and protein expression of TGF-β signalling components, which is particularly marked for Smad4. In addition, there is aberrant Smad4 protein detected in 25% of AC samples.

Genomic alterations of SMAD4 in the metaplasia-dysplasia-carcinoma sequence

The *smad4* promoter region contains multiple CpG sites (fig 5A) and analysis of this region by MSP demonstrated methylation in 3/10 BE samples and in 7/10 AC samples (fig 5B). MSP products were sequenced to investigate the 17 CpG sites within the products. All CpG sites within all methylated MSP products sequenced were methylated and all sites within unmethylated products were unmethylated (fig 5C). Quantitative real time PCR and quantitation of MSP demonstrated some correlation between the degree of methylation and mRNA expression ($p = 0.086$) (fig 5D). The majority of cell lines did not show a significant degree of Smad4 methylation (data not shown).

FISH studies were then undertaken to determine whether copy number changes accounted for the variability in *smad4* expression in oesophageal cell lines (fig 6A,B; table 2). The most interesting cell line was BIC-1 where loss of one copy of chromosome 18 had occurred and the remaining copy was involved in a translocation where the breakpoint had occurred within the centromere. Only one copy of *smad4* was detected in BIC-1 (fig 6A,B for TE7, SEG, and BIC data).

Mutational analysis of *smad4* in the cell lines demonstrated a deletion of between 30 and 50 kb in BIC cells which

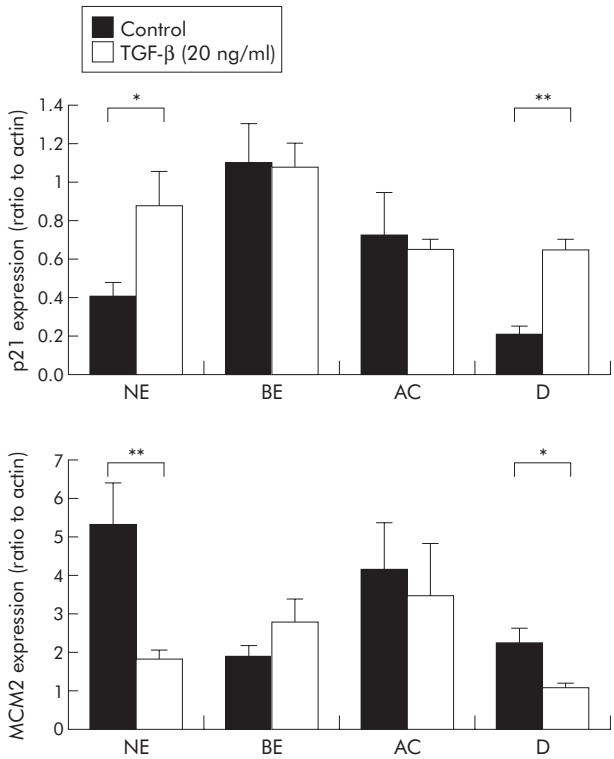

Figure 7 Transforming growth factor β (TGF-β) signalling in the metaplasia-dysplasia-carcinoma sequence. Quantitative real time reverse transcription-polymerase chain reaction for *p21* (A) and minichromosome maintenance protein 2 (*Mcm2*) (B) from normal squamous epithelium (NE), Barrett's oesophageal epithelium (BE), BE associated adenocarcinoma (AC), and duodenal (D) samples cultured for 24 hours with or without 20 ng/ml TGF-β1. For *p21*, *p<0.03, **p<0.002; for *Mcm2*, *p<0.03 , **p<0.009.

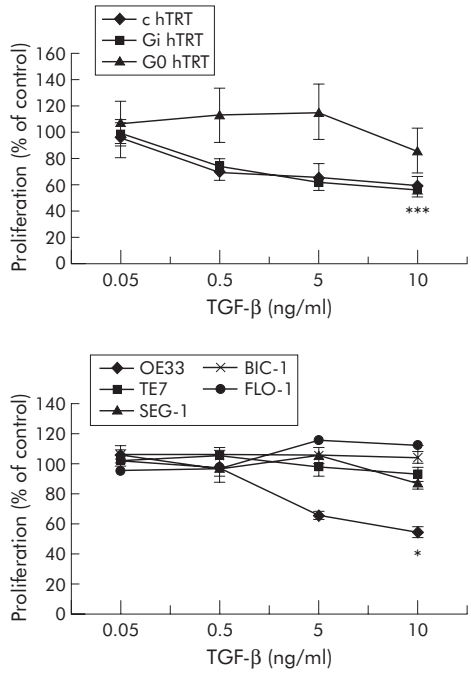

Figure 8 Transforming growth factor β (TGF-β) signalling in Barrett's oesophageal epithelial (BE) dysplasia and adenocarcinoma cell lines. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) proliferation assay on oesophageal cell lines cultured in serial dilutions of TGF-β (0.05–10 ng/ml) for 72 hours. Results are expressed as mean (SEM) of three separate experiments. The responsive cell lines are c hTRT and Gi hTRT BE high grade dysplasia cell lines in the upper graph and OE33, a BE adenocarcinoma cell line in the lower graph. *p<0.05, ***p<0.001 compared with TGF-β 0.05 ng/ml.

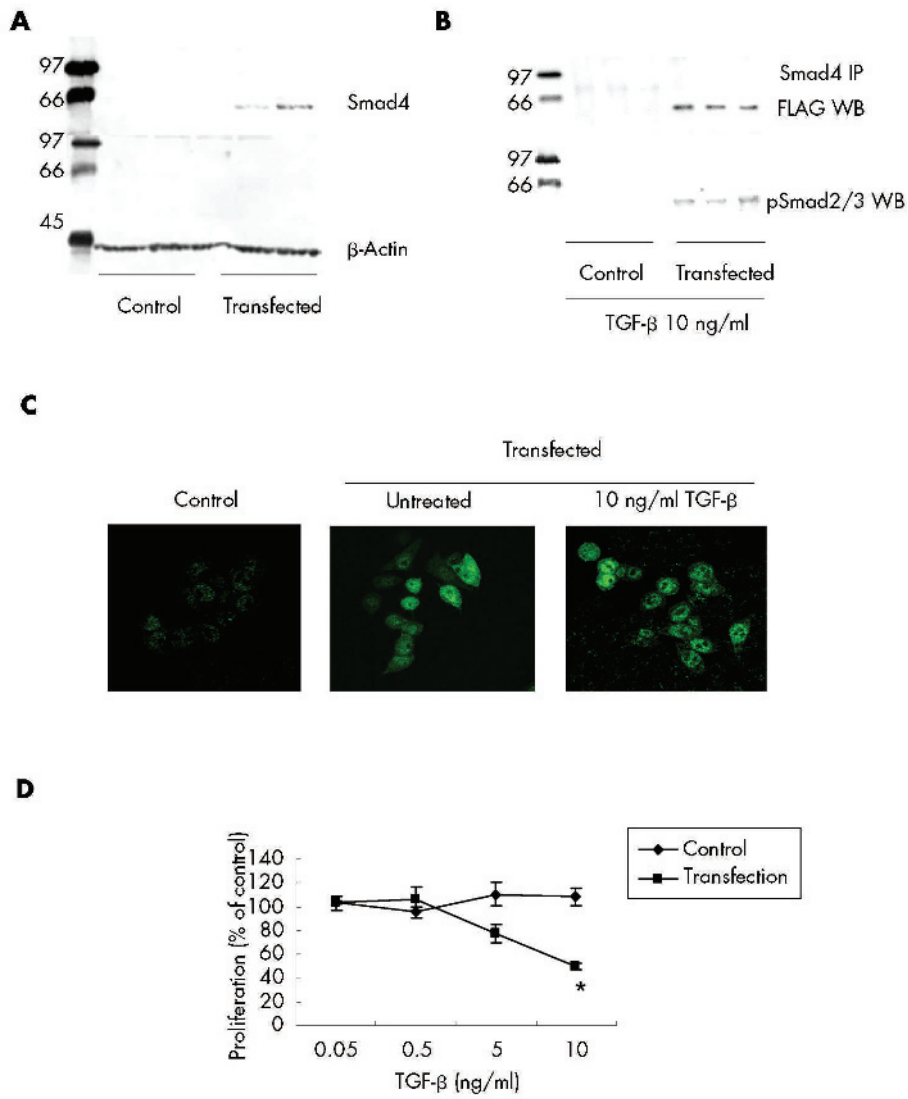

Figure 9 Restoration of Smad4 expression and transforming growth factor β (TGF-β) signalling in BIC-1 cells following transient transfection with pRK5-Smad4 FLAG. (A) Western blot for Smad4 in control cells and transfected cells (upper band). Blots were stripped and reprobed with β-actin as a loading control (lower band). (B) Immunoprecipitation for Smad4 (upper band) followed by western blot for FLAG in control and transfected cells treated with 10 ng/ml TGF-β for 24 hours. Blots were stripped and reprobed with phosphorylated Smad2/3 (lower band). There is the expected association between Smad 4 and Smad2/3 in the transfected cells. (C) Far left panel demonstrates that Smad 4 expression is absent by immunofluoresence in untransfected BIC-1 cells. Following transient transfection, Smad4 is present in the cytoplasm (middle panel). Following treatment with 10 ng/ml TGF-β for two hours there is relocalisation of Smad 4 from a mainly cytoplasmic location (middle panel) to the nucleus (right panel). (D) Proliferation in control and transfected BIC-1 cells treated with TGF-β for 72 hours and assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. *$p<0.05$ compared with 0.05 ng/ml TGF-β.

included the promoter region for *smad4* but not the downstream exons (fig 6B). The ratio of the band for this region in BIC compared with a control cell line RT4 was 1:544, reflecting a small degree of clonal heterogeneity. Hence BIC-1 cells contain only one copy of *smad4*, which in >90% cells contains a deletion at the promoter that would result in loss of mRNA and subsequently protein expression.

Functional consequences of alterations in expression of TGF-β signalling components

TGF-β signalling was measured in endoscopic biopsies maintained as organ cultures.[25] In the control samples, 67% of NE and 78% of duodenum exhibited upregulation of the cell cycle kinase inhibitor *p21* in response to TGF-β compared with 22% of BE and 33% of AC samples. Similar responses were seen with respect to downregulation of the proliferative marker *Mcm2*, following TGF-β stimulation (table 3). These results were confirmed by quantitative analysis for *p21* (NE, $p<0.03$; duodenum, $p<0.002$), and *Mcm2* (NE, $p<0.009$; duodenum, $p<0.03$) (fig 7).

In the cell lines the effect of TGF-β on proliferation was assessed by the MTT assay. This assay is quantitative but not feasible for primary tissues. TGF-β failed to inhibit the growth of most cells, including a BE dysplasia cell line (G0 hTRT) and 4/5 adenocarcinoma lines (TE7, SEG-1, BIC-1 and FLO-1) (fig 8). BE HGD lines C hTRT and Gi hTRT, and the BE adenocarcinoma cell line OE33 (10 ng/ml TGF-β: $p<0.001$, C hTRT; $p<0.001$, Gi hTRT; $p<0.05$, OE33) were TGF-β responsive.

Following transfection with the expression vector pRK5-*smad4* FLAG in BIC-1 cells (fig 9A), Smad4 protein was able to complex with, and coimmunoprecipitate with, activated Smad2/3 (fig 9B), as well as relocate from the cytoplasm to the nucleus (fig 9C). In the absence of TGF-β, transfected cells were unable to bind p-smad2/3 with ectopic Smad4 (data not shown). Furthermore, in comparison with untransfected cells, BIC-1 cells expressing Smad4 exhibited a significant decrease in the rate of proliferation when treated with 10 ng/ml TGF-β for 72 hours ($p<0.05$) (fig 9D). Overexpression of Smad2, Smad3, or Smad4 in other unresponsive cell lines did not restore TGF-β unresponsiveness (data not shown). Hence the lack of Smad4 was functionally significant in BIC-1 cells.

DISCUSSION

TGF-β responsiveness is reduced during all stages of the BE metaplasia-dysplasia-carcinoma sequence secondary to abnormalities at several points in the TGF-β pathway; in particular, Smad4. In the clinical samples, Smad4 expression

levels were profoundly reduced at all stages of the Barrett's-dysplasia-carcinoma sequence (figs 1, 2); 70% of AC samples had hypermethylation at the promoter region and 25% had an abnormal protein band (figs 3A, 5). In BIC-1 cells, there was complete lack of Smad4 expression due to loss of one allele and extensive deletion of the promoter region in the remaining allele (fig 6). Functional complementation of impaired TGF-β signalling occurred in BIC-1 cells when Smad4 expression was re-established (fig 9).

smad4 was first identified as a candidate tumour suppressor in pancreatic cancer,[8] and since then mutations have been detected in one third of metastatic colorectal cancers,[9 27] two thirds of gastric carcinomas,[28] and recently a complex pattern of mutations and abnormal splicing of Smad4 has been demonstrated in thyroid tumours.[29] Furthermore, mice heterozygous for *smad4* develop gastric polyps months after birth that progress to invasive carcinoma.[30] With regard to understanding the biological effects of *smad4* silencing on carcinogenesis, the genes that are involved in cell proliferation, adhesion, and motility have been shown to be differentially regulated with respect to Smad4 status.[31]

Although mutations in *smad4* are an infrequent event in AC,[15] our findings suggest that promoter methylation may be a significant cause of reduced *smad4* mRNA expression in BE carcinogenesis. To date, this represents the first example of methylation of the *smad4* promoter in any malignancy. Other mechanisms that have been proposed for suppression of *smad4* transcription include base pair substitutions in the promoter region in endometrial carcinoma.[32] Protein alterations may also contribute to the reduction in Smad4 via the Ras signalling pathway,[33] which is amplified in 40% of AC.[34] The 10–15 kDa heavier Smad4 protein observed in a subset of AC indicates another possible modulation of Smad4 activity, although the limited amount of endoscopic material available precluded full characterisation. One possibility is protein interaction with proteins such as SMIF and Jab1,[35 36] or post-translational modification, especially as the altered mobility band was seen on a denaturing gel. Another possibility is sumoylation[37–39]; however, there was no evidence of sumoylation of Smad4 in these samples (fig 3C).

Overall, the proportion of samples in this series having some abnormality in Smad4 expression at the gene or protein level suggests that this may be important, albeit via a variety of different mechanisms compared with colon and pancreatic cancer.[40 41] However, the limitations of this study should be taken into account. For example, due to the small size of the endoscopic biopsy specimens it was not possible to perform all of the experiments on the same sample. Therefore, each experiment was performed on separate patients selected from the prospective cohort, apart from the methylation study in which mRNA expression and methylation status were directly compared. Ideally, it would have been helpful to have long term outcome data for the Barrett's patients. In addition, the impact of abnormalities in other genes, such as p16 and p53, which control the G1/S checkpoint and that could affect our results should also be considered. However, C hTRT, Gi hTRT, and OE33 exhibit alterations in the expression of p16 and p53 (table 1) yet are still growth inhibited by TGF-β. This implies that in AC, TGF-β can still control proliferation despite the presence of alterations in p16 and p53.

In summary, reduction of Smad4 expression frequently occurs in BE carcinogenesis via a variety of mechanisms. The resulting functional effects of impaired TGF-β signalling are profound and occur throughout the Barrett's metaplasia-dysplasia-adenocarcinoma sequence.

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Authors' affiliations

B A Onwuegbusi, Y Huang, P Lao-Sirieix, R C Fitzgerald, MRC Cancer Cell Unit, Hutchison/MRC Research Centre, Cambridge, UK
A Aitchison, I Mills, CR-UK Uro-oncology Group, Department of Oncology, University of Cambridge, Hutchison/MRC Research Centre, Cambridge, UK
S-F Chin, T Kranjac, C Caldas, Cancer Genomics Program, Department of Oncology, University of Cambridge, Hutchison/MRC Research Centre, Cambridge, UK

Conflict of interest: None declared.

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EDITOR'S QUIZ: GI SNAPSHOT

Robin Spiller, *Editor*

Intermittent fever in a patient with apparent fatty liver

Clinical presentation

A 41 year old female presented with non-specific abdominal pain and intermittent hyperpyrexia (38°C); she had been recently treated for apical granulomas with an otherwise negative medical history and clinical examination, including a normal body mass index. At presentation the patient had not received any medication for the past two weeks.

Laboratory investigations were within normal limits apart from: erythrocyte sedimentation rate 30 mm (normal range 5–20), aspartate aminotransferase/alanine aminotransferase 49/62 U/l (normal range 13–37/7–43), and serum alkaline phosphatase 168 U/l (normal range 44–132). Blood cultures were negative.

A chest *x* ray revealed no lesion and liver sonography demonstrated a patchy hypo-hyper-echoic polycyclic area in the anterior-superior parenchyma compatible with segmental fatty liver. Thoracic abdominal contrastographic computed tomography confirmed a large regular polycyclic borders area, hypodense compared with the remaining parenchyma, with no mass effect and/or any displacement of the vessels, resembling focal fatty areas (fig 1A, B); no other pathological signs were found except for small lymphoadenopathies adjacent to frenic pillars.

Question

Is it really fatty liver? What is your diagnosis?
See page 823 for answer
This case is submitted by:

S Colagrande
Sezione di Radiodiagnostica, Dipartimento di Fisiopatologia Clinica, Italy
S Santini
Azienda Ospedaliera USL 11-Ospedale San Giuseppe, Firenze, Italy
M Pinzani
Dipartimento di Medicina Interna, Università degli Studi di Firenze, Azienda Ospedaliero-Universitaria di Careggi, Firenze, Italy

Correspondence to: Professor S Colagrande, Sezione di Radiodiagnostica, Dipartimento di Fisiopatologia Clinica, Università degli Studi di Firenze, Azienda Ospedaliero-Universitaria di Careggi, Viale Morgagni 85, 50134 Firenze (FI), Italy; stefano.colagrande@unifi.it

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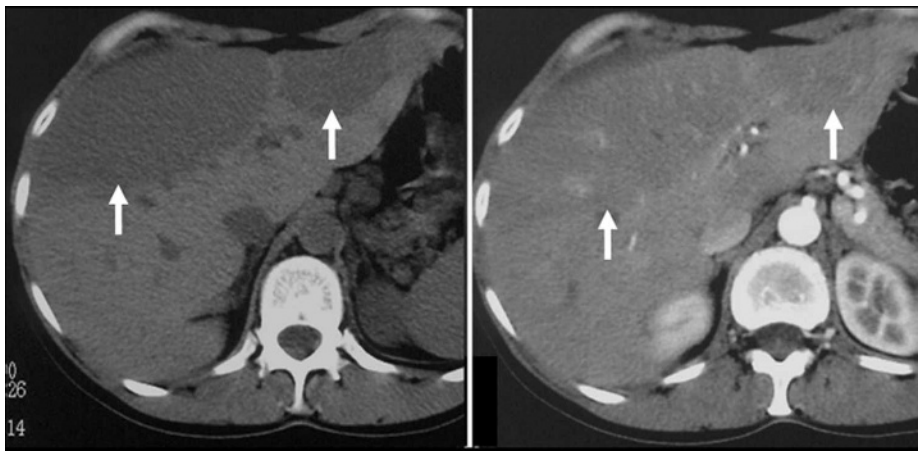

Figure 1 Direct (A) and iodinated (B) spiral computed tomography scans revealed regular polycyclic borders area (white arrows), hypodense compared with remaining parenchyma, with no mass effect and/or any displacement of the parenchymal vessels.