Dysregulated TGF-ß1-induced Smad signalling occurs as a result of defects in multiple components of the TGF-ß signalling pathway in human head and neck carcinoma cell lines

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Abstract. This study examined Smad2- and Smad3-dependent transcription in 12 human head and neck squamous cell carcinoma (HNSCC) cell lines following treatment with transforming growth factor-ß1 (TGF-ß1). A markedly elevated level of TGF-ß1-induced Smad3 signalling was observed in one cell line (H357), whilst four cell lines (BICR31, H314, BICR56, BICR19) demonstrated absence of Smad3-dependent transcription that correlated with loss of TGF-ß1 growth inhibition; TGF-ß1-induced Smad2-dependent transcription was retained in two of these cell lines (H314, BICR31). Using transient expression of TGF-ß signalling components and a Smad3-dependent reporter assay, we show that BICR31 and H314 had defects of Smad4, BICR56 had abnormal TßR-II and BICR19 overexpressed Smad7. The results demonstrate that deregulated TGF-ß1-induced Smad signalling is common in HNSCC cell lines and can occur as a result of a variety of defects in the TGF-ß signal transduction pathway.

Introduction

Transforming growth factor-ß is the generic term for a family of highly conserved ubiquitous peptides (TGF-ß1, -ß2, -ß3 in humans) that show a remarkable diversity of biological action. TGF-ß signal transduction is initiated when active TGF-ß binds to type II TGF-ß receptors (TßR-II) and after heteromerisation, type I TGF-ß receptors (TßR-I) are transphosphorylated. Smad2 and Smad3 are then phosphorylated by TßR-I that enables them to form Smad2/4 and Smad3/4 complexes that are translocated to the nucleus to regulate the transcription of target genes in association with transcriptional co-activators/repressors (1). The inhibitory Smad, Smad7, resides within the nucleus in the basal state, is induced in response to TGF-ß, and functions via a negative feedback mechanism to control the intensity and duration of Smad signalling (1).

The role of TGF-ß in neoplasia is complex. TGF-ß functions to suppress epithelial tumor progression in the early stages via its ability to negatively regulate cell growth. Later, however, the ligand functions to promote invasion and metastases through paracrine mechanisms, such as the promotion of angiogenesis and the suppression of immune responses, and via autocrine signalling pathways (2). In a small number of cases, tumour cells escape the negative effects of TGF-ß through defects in the TGF-ß signalling pathway and to date, mutations of TßR-I (3), TßR-II (4,5) and Smad4 (6) have been reported in human head and neck squamous cell carcinoma (HNSCC). Decreased expression of TßR-II is thought to occur more commonly than gene mutation (7) and in oral carcinoma cells, leads to a less differentiated, more aggressive phenotype (8). The shift from Smad-dependent to more Smad-independent signalling pathways was thought to be pivotal in influencing the change from tumour suppressor to pro-metastatic tumour behaviour (2) but it is now recognised that Smad2/3 can mediate both of these functions (9).

In the present study, we have used Smad2- and Smad3-specific reporter plasmids to examine the integrity of the TGF-ß pathway in a series of 12 HNSCC-derived cell lines. Four cell lines were identified showing absence of TGF-ß1-induced Smad3/4-dependent transcription, which correlated with loss of TGF-ß1-induced growth inhibition. Then, we used a combination of reporter assays and vectors expressing key components of the TGF-ß signal transduction pathway to locate the specific abnormality within the pathway. The data demonstrate a broad spectrum of defects in the TGF-ß signal transduction pathway in HNSCC keratinocyte cell lines, including anomalies of TßR-II, loss of Smad4, and for the first time, overexpression of the inhibitory Smad7.

Materials and methods

Cell culture. The culture of the human HNSCC-derived keratinocyte cell lines and the spontaneously immortalised human skin keratinocyte cell line HaCaT has been described previously (10-12). All of the tumour-derived cell lines express normal TßR-I and TßR-II [BICR56 contains one normal and one mutant TßR-II allele (7)] and are either growth inhibited

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by exogenous TGF-ß (H103, H157, H357, H376, H400, H413, BICR3, BICR6) or are refractory to the growth inhibitory effects of the ligand [H314, BICR19, BICR31, BICR56 (13,14)]. HaCaT cells are growth inhibited by TGF-ß1 (15).

Smad-dependent reporter assays. The Distal Element (DE)-luc reporter was used to examine Smad2/4 activity (16) and the SBE-luc reporter was used to investigate Smad3/4 function (17). Briefly, cells were grown to ~50% confluence, the culture media replaced with media containing 1% FBS and then the cells were transfected with 0.45 μg DE-luc and 0.05 μg pEFflag/mixer or 0.5 μg SBE-luc, together with 0.5 μg of the Renilla luciferase control vector pRL-TK (Promega, UK) using Fugene6 transfection reagent (Roche Molecular Biochemicals, UK). Where appropriate, TGF-ß1 (1 ng/ml), or the vehicle control was added to the media after 6 h and the samples harvested 20 h later. Specific firefly and Renilla luminescence was measured using the Dual Luciferase assay reagents (Promega), according to the manufacturer's instructions.

Western blot analysis. To examine TGF-ß1-dependent phosphorylation of Smad2, cells were grown to 60-70% confluence and then treated for 30 min in media containing 1% FBS and either 2 ng/ml TGF-ß1 or vehicle control (4 mM HCl, 0.1% (w/v) BSA). Cells were lysed *in situ* in 1X sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue] and boiled for 5 min prior to protein separation in an 8% SDS-PAGE gel. Following transfer of proteins to Immobilon-P membrane (Millipore), the blots were placed in the appropriate blocking solution (1X TBS, 0.1% Tween-20, 5% fat-free milk) for 1 h and then incubated with the primary antibody (anti-phospho Smad2, 1:1000; cell signalling) overnight at 4˚C. The next day, membranes were washed in appropriate blocking solution (x3) for 10 min and exposed to secondary antibody (P-Smad2, anti-rabbit HRP, Sigma, 1:3000) for 1 h at room temperature. Further washes (x3) in blocking solution were carried out prior to ECL detection (Amersham, UK). Similar techniques were used for the examination of base-line levels of Smad7; although in these circumstances, the blocking solution was (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100, 150 mg NaCl, 3% BSA), the primary antibody was goat polyclonal anti-Smad7 (1:750; Santa Cruz) and the secondary antibody was donkey anti-goat IgG-HRP (1:3000; Santa Cruz).

To detect Smad4, cell pellets were washed in phosphate buffered saline (PBS), re-suspended in 50 μl of 1X sample buffer (2%[w/v] SDS, 10% [v/v] glycerol, 125 mM Tris HCl [pH 6.8], 5% [v/v] B-mercaptoethanol, 0.005% [w/v] bromophenol blue) and boiled for 5 min. Protein separation, transfer and detection were carried out as described above using the appropriate blocking solution (200 mM Tris, 137 mM NaCl, 3%[v/v] Tween-20, 5% [w/v] fat-free milk) and primary and secondary antibodies (mouse monoclonal anti-Smad4 B8, 1:750, Santa Cruz; goat anti-mouse 1gG-hRP, 1:1000, Sigma).

Expression plasmids and transient DNA transfection. The expression vectors used for the ectopic expression of TGF-ß signalling components were rat TßR-I cDNA in pcDNAI,
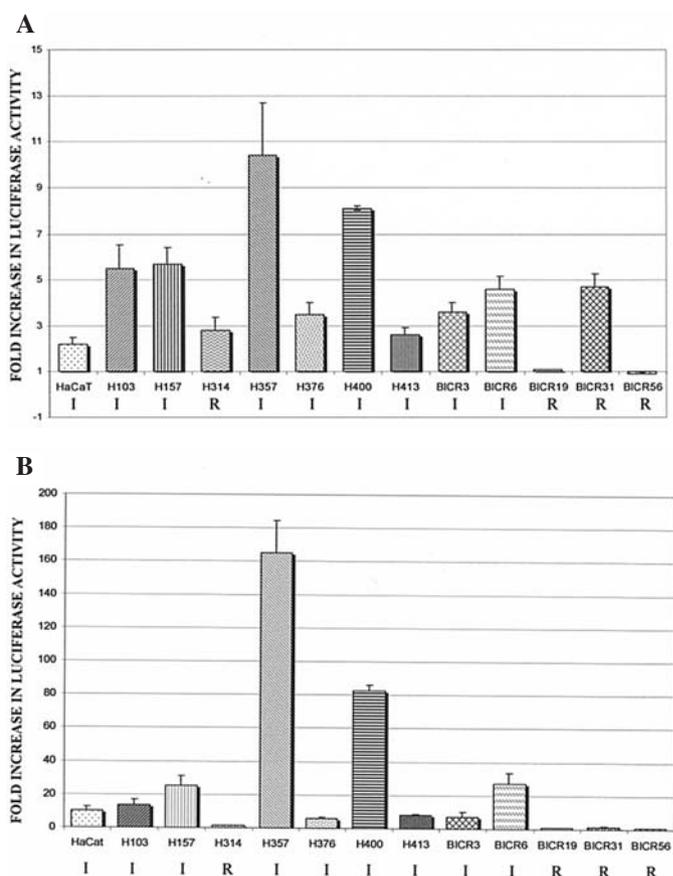

Figure 1. TGF-ß1-induced activation of the Smad-dependent reporters, DE-luc (A) and SBE-luc (B) in HNSCC cell lines. Cells were transfected with the plasmids and incubated for 6 h before the addition of TGF-ß1 (1 ng/ml) or diluent alone. Luciferase activity was measured after a further 20 h incubation. The data are representative of three independent experiments (± standard deviations). Growth inhibitory response to TGF-ß1: I, growth inhibited; R, refractory.

human TßR-II cDNA in pcDNAI, constitutively active rat TßR-I in pRK5F (pRK-R4[TD202]-FLAG) or a rat mutant TßR-I incapable of binding/activating Smads [TßR-ImL45TD] (18) and Myc-tagged Smad3 in pcDNA3.

For transient transfections, cells were grown to ~25% confluence in 12-well plates and transfected with a mixture of 2 μg plasmid DNA using Fugene6 transfection reagent (Roche Molecular Biochemicals). Cells were cultured for a further 16 h prior to the reporter assays.

Inhibition of Smad7 expression using antisense oligo-nucleotides. Phosphorothioate single-stranded oligonucleotides matching the region 107-128 of the human Smad7 cDNA (5'-GCTGCGGGGAGAAGGGGCGAC-3') were synthesised in the sense and antisense orientations and used as described previously (19). To examine the effect of the oligonucleotides on Smad7 protein levels, cells were grown to 40-50% confluence in 100 mm^2 dishes, transfected with 1 μg/ml oligo-nucleotides using Fugene6 and then cultured for a further 40 h before lysis. For the reporter assays, cells were transfected with 1 μg oligonucleotides, with or without 1 μg of expression plasmid, for ~20 h prior to the transfection of the reporter plasmids and a further 1 μg oligonucleotide.
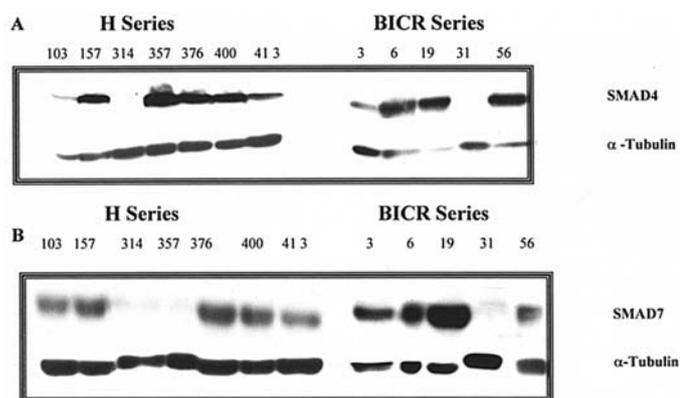
Figure 2. Expression of SMAD4 (A) and Smad7 (B) in HNSCC cell lines. Basal protein levels were examined by Western blot analysis using anti-Smad4 and anti-Smad7 antibodies. Membranes were re-probed with α-tubulin to control for protein loading and transfer.

Figure 4. TGF-ß1-induced transcriptional activation of SBE-luc in BICR31 and H314 following transient transfection with Smad4 or pcDNA3 vector control. Cells were treated with 1 ng/ml TGF-ß1 6 h after transfection of the luciferase reporter and harvested 20 h later. Data are presented as a fold increase in luciferase activity following application of TGF-ß1. Data are representative of three separate experiments (± standard deviations).
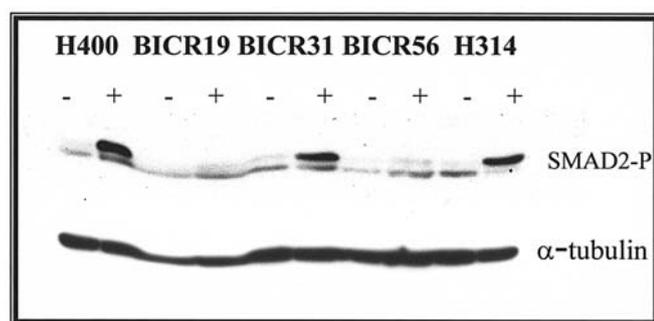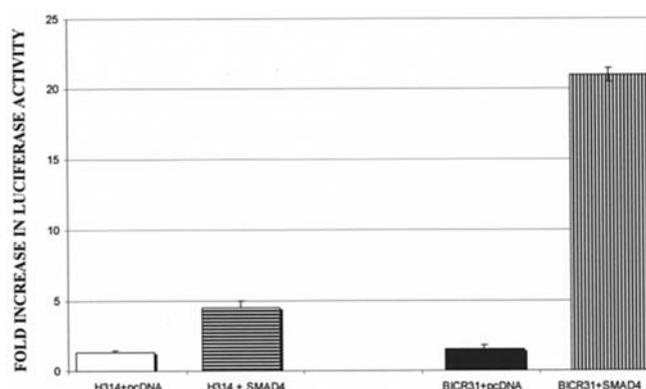

Figure 3. Western blot analysis of TGF-ß1-induced phosphorylation of Smad2 in cell lines that are refractory to the anti-proliferative effects of TGF-ß1. H400 (growth inhibited by TGF-ß1) was used as a positive control. Cells were treated for 30 min with either 2 ng/ml TGF-ß1 (+) or diluent control (-). Blots were probed with α-tubulin to control for protein loading and transfer.

Results

TGF-ß1-induced Smad signalling. TGF-ß1-induced Smad signalling was examined in luciferase reporter assays using the DE-luc and SBE-luc reporter constructs to investigate Smad2- and Smad3-dependent signalling, respectively. The activation of the reporters in the HNSCC cell lines, and the control HaCaT cells, following treatment with exogenous TGF-ß1 (1 ng/ml) is shown in Fig. 1A (DE-luc) and Fig. 1B (SBE-luc). TGF-ß1-induced activation of DE-luc was greater (H103, H157, H357, H400, BICR6, BICR31), similar (H314, H376, H413, BICR3) or absent (BICR19, BICR56) relative to HaCaT cells. TGF-ß-dependent activation of SBE-luc was again highly variable and, relative to the HaCaT cells, was markedly elevated (H357), increased (H157, H400, BICR6), similar (H103, H376, H413, BICR3) or absent/markedly reduced (H314, BICR19, BICR31, BICR56).

Expression of Smad4 and Smad7. With the exception of two cell lines (H314, BICR31), Smad4 was expressed in all cell lines, including two cell lines that were not growth inhibited by TGF-ß1 (BICR19, BICR56; Fig. 2A). Absence of Smad4 in H314 and BICR31 has been noted previously (20).

The expression of Smad7 protein was heterogeneous (Fig. 2B). Smad7 appeared to be overexpressed in one cell line that was refractory to the anti-proliferative effects of TGF-ß1 (BICR19) and was much reduced in H357, which showed elevated levels of Smad signalling, and H314 and BICR31, which failed to express Smad4.

TGF-ß1-dependent phosphorylation of Smad2 in cell lines refractory to ligand-induced growth arrest. To examine the ability of TGF-ß receptors to phosphorylate R-Smads, TGF-ß1-dependent phosphorylation of Smad2 was examined by Western blot analysis in the four cell lines that were refractory to the growth inhibitory effects of TGF-ß1 (BICR19, BICR31, BICR56, and H314); H400 was a positive control (Fig. 3). Treatment of cells with exogenous TGF-ß1 (2 ng/ml) for 30 min resulted in phosphorylation of Smad2 in the fully responsive control cell line H400 and also in Smad4-defective cell lines BICR31 and H314, but was absent in BICR19 or BICR56. The data demonstrate that BICR31 and H314 have functional TGF-ß receptors that are capable of phosphorylating R-Smads. By contrast, TGF-ß receptors were unable to phosphorylate Smad 2 in BICR19 and BICR56, indicating that defects of TGF-ß signal transduction were located above or at the level of receptor/R-Smad interaction in these cell lines.

Restoration of Smad signalling in cell lines refractory to ligand-induced growth arrest. To identify the exact nature of the signalling defect in H314, BICR31, BICR56 and BICR19, components of the TGF-ß signalling pathway were ectopically expressed in the above cell lines and TGF-ß1-induced activation of the SBE-luc reporter construct was measured.

Following the transient transfection of wild-type Smad4 and treatment with TGF-ß1 (1 ng/ml), a 21-fold and 4.5-fold increase in SBE-luc reporter activity was noted in BICR31 and H314, respectively; no significant increase was seen with the pcDNA vector control (Fig. 4). The results argue strongly for the absence of Smad4 protein in BICR31 and H314 being responsible for loss of Smad3/4-dependent signalling in these cell lines.
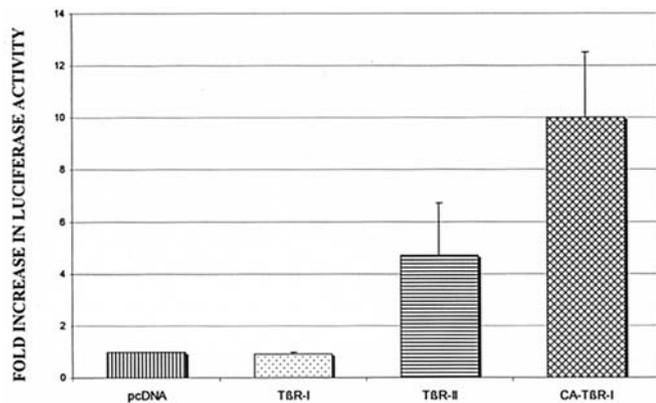
Figure 5. The effect of ectopically expressing TGF-ß signalling components in BICR56. Transcriptional activation of SBE-luc following transient transfection (2 μg/well) of TßR-I, TßR-II receptors, constitutively active TßR-I (CA-TßR-I) or pDNA3 vector 24 h prior to luciferase assays. Cells transfected with wild-type TßR-I, TßR-II or pcDNA3 were treated with 1 ng/ml TGF-ß1 6 h after transfection of the luciferase reporter and harvested 20 h later. Data are presented as a fold increase in luciferase activity following application of TGF-ß1. To examine CA-TßR-I, cells were transfected with either a mutant TßR-I incapable of binding/activating Smads (TßR-ImL45TD, not shown) or with the CA-TßR-I construct. Baseline transcriptional activity was compared between the two constructs and data is presented as a fold increase in luciferase activity. Data are representative of three separate experiments (± standard deviations).

To examine possible TGF-ß receptor defects in BICR56, cells were transiently transfected with wild-type TßR-I, TßR-II or the constitutively active TßR-I. Transcriptional activation of SBE-luc was seen following transfection of constitutively active TßR-I (10-fold) and TGF-ß1-induced activation of the reporter was observed following transfection of wild-type TßR-II (4-fold). Transfection of the empty pcDNA vector or wild-type TßR-I alone did result in any significant increase in transcriptional activity (Fig. 5).

To investigate the nature of the TGF-ß signal transduction defect in BICR19, cells were transiently transfected with wild-type TßR-I, TßR-II, constitutively active TßR-I (TD) and wild-type Smad3. Transfection of wild-type TßR-I, wild-type TßR-II or the empty pcDNA vector had no effect on TGF-ß-dependent activation of SBE-luc. Similarly, transfection of constitutively active TßR-I did not increase baseline levels of transcription when compared to controls. However, the ectopic expression of Smad3 resulted in an 8-fold increase in baseline transcriptional activity (Fig. 6A) suggesting that the signalling pathway distal to Smad3 activation in BICR19 was intact. To determine whether the elevated levels of Smad7 were responsible for the block in Smad3 signalling in BICR19, Smad7 protein levels were reduced through the use of anti-sense oligonucleotides (Fig. 6B). Transfection of BICR19 cells with constitutively active TßR-I, together with the antisense Smad7 oligonucleotides, but not the sense controls, resulted in an increase in luciferase activity (Fig. 6C).

Discussion

Smad2 and Smad3 are recognised as being the principle transducers of signals from TGF-ß receptors. Recent studies show that Smad2 and Smad3 contribute to unique patterns of gene activation (21,22) that modulate specific cell functions (23-27).

Currently, the role of Smad2 and Smad3 in tumour cell behaviour is under close scrutiny.

The present study examined Smad2/4-[pDE-luc (16)] and Smad3/4-[pSBE-luc (17)] dependent transcription in human HNSCC cell lines that exhibit a range of responses to the anti-proliferative effects of TGF-ß1. Whilst this methodology does not directly examine physiological endogenous gene activation, it provides an extremely sensitive assessment of functional Smad signalling capability. The results show that Smad3/4-dependent transcription is lost in cell lines that are refractory to the growth inhibitory effects of TGF-ß1 suggesting that TGF-ß1-mediated growth arrest is dependent on functional Smad3/4 complexes. The data are consistent with previous observations demonstrating an absence of Smad3/4-dependent transcription in HNSCC lines that are refractory to TGF-ß1 (28), interference with TGF-ß1-induced cell cycle arrest by inhibition of Smad3 and Smad3-dependent regulation of the cell cycle control molecule c-*myc* (29).

Activation of the Smad2-dependent reporter DE-luc was retained in two cell lines where Smad3/4-dependent transcription and Smad4 protein expression was absent (H314, BICR31). The results suggest that Smad2 is functional in these cell lines and capable of driving TGF-ß1-induced transcription independently of Smad4. AP-1 associated genes (20), fibronectin (30) and PAI-1 expression (31) are known to be regulated by Smad4-independent pathways, but whether this reflects Smad2 activity exclusively remains to be determined. It has been suggested that the retention of certain TGF-ß signalling pathways, but loss of TGF-ß-induced growth inhibition, is important in late stage metastatic disease (2). Whilst it is not certain whether Smad2 can regulate the expression of endogenous genes in the absence of Smad4, it is possible that Smad2 signalling may be critical for the metastatic dissemination of tumour cells; a thesis with some experimental support (32). We have shown recently that both H314 and BICR31 readily form metastases to loco-regional lymph nodes when transplanted to the floor of the mouth in athymic mice (33).

The integrity of the TGF-ß signal transduction pathway was investigated in cell lines with defective Smad3/4-dependent signalling. In H314 and BICR31, the functional activity of TßR-I/TßR-II was demonstrated by ligand-induced phosphorylation of Smad2 and the importance of the Smad4 defect in these lines was shown by the restoration of TGF-ß1-induced Smad3/4-dependent transcription following transient transfection of wild-type Smad4. The data extend previous observations documenting absence of Smad4 protein in H314 and BICR31 (20). In the present study, TGF-ß1-dependent phosphorylation of Smad2 was lost in BICR56 and BICR19, thereby demonstrating defects above, or at the level of receptor/R-Smad interaction. In BICR56, transient transfection of TßR-II, or constitutively active TßR-I, but not wild-type TßR-I, restored TGF-ß1-induced Smad3/4-dependent signalling indicating a TßR-II defect in this cell line. We have shown previously that BICR56 contains one mutated TßR-II allele (7) and, therefore, the results of the present study indicate that the level of expression of the wild-type allele is not sufficient to propagate the TGF-ß signal. In BICR19, Smad3/4-dependent signalling could not be restored by the ectopic expression of TßR-I, TßR-II or by mimicking the process of receptor
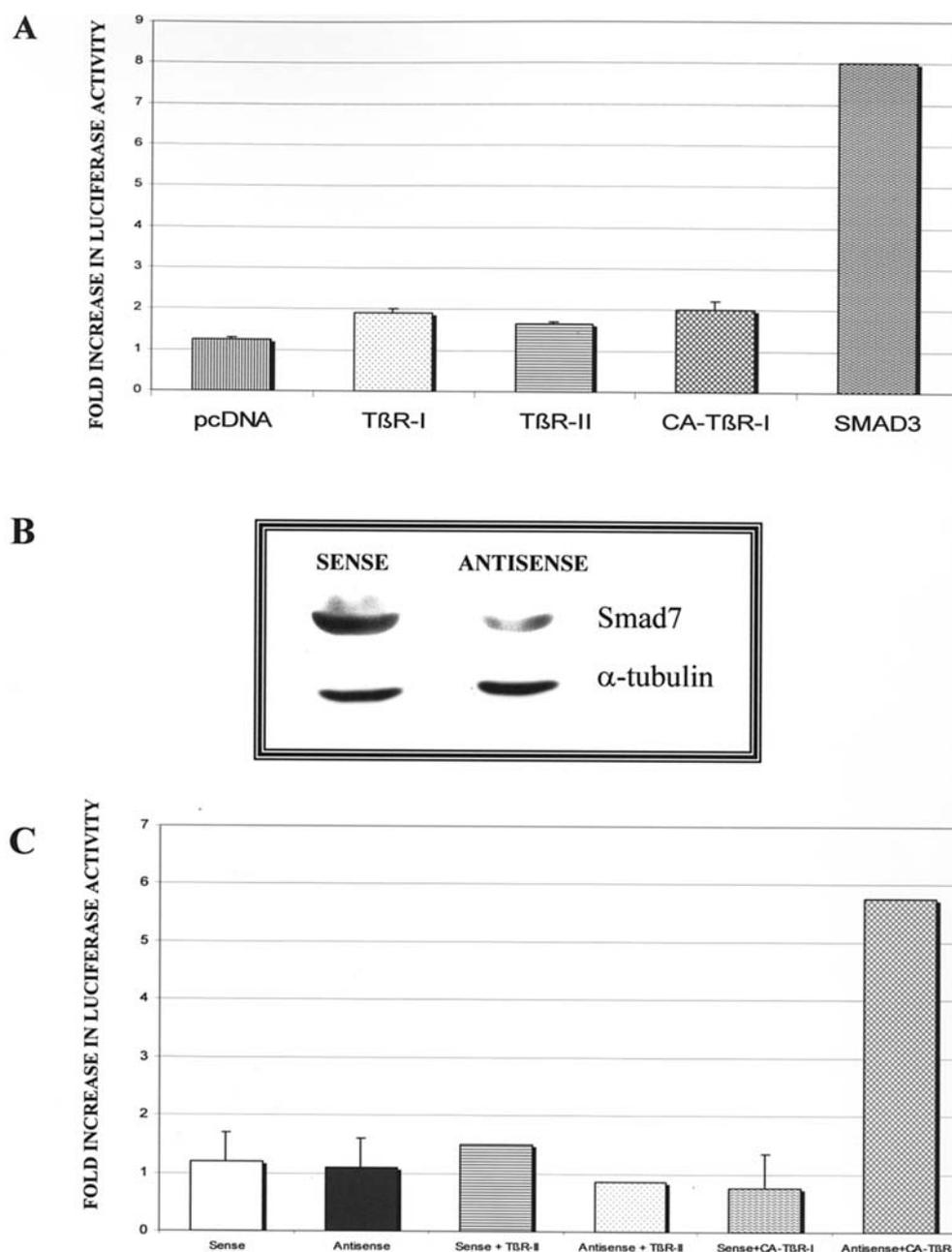
Figure 6. Ectopic expression of TGF-ß signalling components in BICR19. (A) Transcriptional activation of SBE-luc following transient transfection of the pcDNA3 vector, TßR-I, TßR-II, Smad3 or CA-TßR-I. Cells were transfected (2 µg/well) with wild-type TßR-I, TßR-II or pcDNA3 24 h prior to luciferase assays. The cells were then treated with 1 ng/ml TGF-ß1 6 h after transfection of the luciferase reporter and harvested 20 h later. Data are presented as a fold increase in luciferase activity following application of TGF-ß1. To examine CA-TßR-I, cells were transfected with either a mutant TßR-I incapable of binding/activating Smads (TßR-ImL45TD, not shown) or with the CA-TßR-I construct. Baseline transcriptional activity was compared between the two constructs and is presented as a fold increase in luciferase activity. To examine the effect of ectopic expression of Smad3, cells were transfected with either wild-type Smad3 or the empty pcDNA3 vector and baseline transcriptional activity compared between the two constructs. Data are presented as a fold increase in luciferase activity. Data are representative of three separate experiments (± standard deviations). (B) Cells were transfected with sense or antisense Smad7 oligonucleotides and Smad7 protein levels examined by Western blotting. (C) Transcriptional activation of SBE-luc in BICR19 following transient transfection of Smad7 sense and antisense oligonucleotides with or without TßR-II and CA-TßR-I.

activation through expression of constitutively active TßR-I (ALK5-TD). By-passing the receptor complex through the ectopic expression of Smad3, however, increased the base-line activation of the SBE-luc reporter. BICR19 cells appeared to overexpress Smad7 and transfection of anti-sense Smad7, together with constitutively active TßR-I, restores Smad3/4-dependent signalling. The use of anti-sense Smad7 oligo-nucleotides alone was not sufficient to restore responsiveness most probably because the reduction of Smad7 protein levels

was not sufficient to allow R-Smad phosphorylation following ligand-induced receptor activation. Taken together, the results strongly suggest that loss of TGF-ß1-induced Smad signalling in BICR19 occurs through overexpression of Smad7, but the possibility of a dual Smad7-TGF-ß receptor defect cannot be excluded.

H314, BICR31 and H357 expressed relatively low levels of Smad7 protein. It could be argued that the loss of Smad7 protein is predictable in the Smad4-deficient cell lines (H314,

BICR31) because the Smad7 promoter contains the palindromic SBE and its activation is dependent on the presence of functional Smad3/4 complexes (34). However, the low levels of Smad7 expression in H357, a cell line with functional Smad4, is intriguing. H357 cells exhibited hyper-responsive Smad signalling, with TGF-ß1-induced activation of the SBE-luc reporter being >20-fold higher than HaCaT cells. H357 cells contain oncogenic Ras (35) and Erk activation, as can arise from the presence of oncogenic Ras, can negatively regulate Smad7 transcription in certain cell types (36). Further, we have shown recently that H357 cells are capable of undergoing EMT in response to TGF-ß1, a phenomenon that is associated with elevated ligand-induced Smad-dependent transcription (37). It seems unlikely, however, that the presence of oncogenic Ras alone would be sufficient to account for the degree of hyper-responsiveness seen in H357 cells. Reduced Smad7 expression in H357 may be a critical factor in this context and lead to unrestrained TGF-ß signalling. Furthermore, we have preliminary data indicating increased expression of the R-Smads in H357 cells relative to other HNSCC cell lines (unpublished data). These observations parallel the condition of Scleroderma where reduced expression of Smad7, together with increased expression of Smad3, lead to an unchecked hyper-responsive autocrine signalling pathway that results in vascular dysfunction, excessive ECM synthesis and fibrosis (38). It seems likely, therefore, that the hyper-responsive Smad signalling in H357 cells is due to a combination of the presence of oncogenic Ras and an imbalance between R-Smads and Inhibitory Smads. As such, it is tempting to suggest that the relationship between R-Smads and I-Smads may be critical for epithelial cells to adopt a fibroblastic phenotype in EMT.

In summary, this study has shown that deregulation of TGF-ß-induced Smad signalling in HNSCC cell lines is common and can occur as a consequence of a variety of defects within the TGF-ß signal transduction pathway. A loss of Smad signalling can occur as a result of defects of TGF-ß receptors, the loss of Smad4 and via elevated expression of Smad7. Enhanced Smad-dependent signalling in cells containing oncogenic Ras can occur due to the loss of Smad7. The consequences of these different abnormalities on the transcriptome and with regard to tumour cell behaviour remain to be determined.

Acknowledgements

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