Published in final edited form as:

Cancer Lett. 2007 January 8; 245(1-2): 163–170.

Disruption of Transforming Growth Factor β -Smad Signaling Pathway in Head and Neck Squamous Cell Carcinoma as Evidenced by Mutations of *SMAD2* and *SMAD4*¹

Wanglong Qiu¹, Frank Schönleben¹, Xiaojun Li¹, and Gloria H. Su^{1,2}

¹The Department of Otolaryngology and Head and Neck Surgery, Columbia University College of Physicians and Surgeons, New York, NY.

²The Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY.

Abstract

The role of the TGF- β -Smad signaling pathway in the carcinogenesis of head and neck cancer has not been fully evaluated genetically. In this study, we screened for mutation in the five main members of the TGF- β -Smad signaling pathway, TGF- β type I receptor (*TGFBRI*), TGF- β type II receptor (*TGFBRII*), *SMAD2*, *SMAD3* and *SMAD4*, in eight human head and neck squamous cell carcinoma (HNSCC) cell lines. Two mutations with presumed loss of heterozygosity (LOH) were identified. A novel missense mutation of *SMAD2*, located in exon 8 at codon 276 TCG (ser) $\rightarrow$ TTG (leu), was identified in cell line SCC-15. This is the first report of a biallelic mutation of the *SMAD2* gene in HNSCC. A nonsense mutation of the *SMAD4* gene in exon 5 codon 245 CAG (glut) $\rightarrow$ TAG (stop) was found in cell line CAL27. Western blotting verified that this nonsense mutation gives rise to the complete loss of the Smad4 protein in the cells. While the down-regulation and loss of expressions of the TGF- β -Smad signaling pathway have been described frequently in HNSCC, here we offer further genetic evidence that the pathway is directly targeted for mutation during the HNSCC tumorigenesis.

Request for reprints: Gloria Su, Department of Otolaryngology/Head and Neck Surgery, Columbia University, 1130 St. Nicholas Ave., ICRC 10-04, New York, NY 10032. Phone: 212-851-4624, Fax: 212-851-4627, E-mail: gs2157@columbia.edu..

Abbreviations:

TGFβ	Transforming growth factor- β
HNSCC	head and neck squamous cell carcinoma
TGFBRI	TGF- β type I receptor
TGFBRII	TGF- β type II receptor
LOH	loss of heterozygosity
ACVRIB	activin receptor type IB
ACVR2	activin receptor type II

Keywords

SMAD2; SMAD4; HNSCC; genetic mutation; TGF β receptors

1. Introduction

TGF- β is a potent natural inhibitor of normal epithelial cell replication that also regulates migration, adhesion, differentiation, and death [1]. It is now generally accepted that all of the components of the TGF- β signal transduction, namely, the ligand, the receptors, the signal transducers and their transcriptional targets constitute an important tumor-suppressive pathway. Many of the genes that encode the components of this signaling pathway have been reported to have inactivating mutations in several tumor types [2-5]. There is considerable evidence that TGF- β signaling also plays an important role in the development of squamous cell carcinomas. For example, most human squamous carcinoma cell lines also appear to be refractory to TGF- β -mediated cell cycle arrest [6,7].

The underlying mechanism that may be responsible for this common refractory response to TGF- β in HNSCC is not yet fully understood. The frequency of the documented genetic inactivation of the *TGFBR* and *SMAD* genes in tumors have been relatively low and do not seem to account for all the loss of TGF- β responsiveness observed in tumor cells. While mutations of *TGFBRI*, *TGFBRII*, and *SMAD4* have been reported in human head and neck cancer [8-12], biallelic inactivations of *SMAD2* and *SMAD3* have not been reported in HNSCC up-to-date [3]. In this study, we screened for mutations in five members of the TGF- β -Smad signaling pathway in eight human HNSCC cell lines in a comprehensive manner.

2. Materials and Methods

2.1. Cell culture.

HNSCC cell lines RPMI 2650, A253, SW579, Detroit 562, FADU, CAL27, SCC-15 and SCC-25 were purchased from American Type Culture Collection (Rockville, MD).

2.2. PCR amplification and PCR product directly sequencing.

All encoding exons and flanking intronic sequences of *TGFBRI*, *TGFBRII*, *SMAD2*, *SMAD3* and *SMAD4* were analyzed by sequencing. Genomic DNAs from each cell line (40 ng per reaction) were amplified with primers designed for amplification of all encoding exons and at least 50 base pairs of flanking intronic sequences of each target gene. Amplified genomic DNA fragments were directly sequenced utilizing the same forward or reverse primers used in the original PCR amplification. All sequencing was performed on the ABI's 3100 capillary automated sequencers at the DNA facility of Columbia University Medical Center, New York, NY. All samples found to have a mutation in the target gene were confirmed by subsequent sequencing in the reverse direction. The mutation was then further verified by sequencing of a second PCR product derived independently from the original template. All primers are listed in the Table 1 or as previously reported [13].

2.3. Western blotting.

Cells were washed twice with ice-cold PBS and scraped into cell lysis buffer (2.5% NP-40, 50mM Tris-base, 150mM NaCl, and 5mM EDTA), and a protease inhibitor cocktail tablet in every 10mL of the lysis buffer. 25 μ g of total protein was electrophoresed in 8%SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk TBST buffer (50mM Tris/HCl, PH 7.5, 0.15 M NaCl, 0.1% (v/v) Tween) at room temperature for 1 h, and blotted with a primary antibody in 5% BSA TBST buffer at 4 °C overnight.

Detection was performed using a horseradish peroxidase-conjugated secondary antibody in 5% milk TBST buffer at room temperature for 2 h. The dilutions of primary antibodies were at 1:1000 for mouse monoclonal anti-Smad4 (B-8, Santa Cruz Biotechnology, Santa Cruz, CA), and 1:5000 for mouse monoclonal anti- β -actin (Sigma-Aldrich, St. Louis, MO). The secondary antibody anti-mouse-IgG-HRP (Sigma-Aldrich) was added to give a final dilution of 1:5000.

2.4. Duplex-RT-PCR.

First strand cDNA was synthesized from 0.8 μ g of total RNA by reverse transcriptase Superscript II (Life Technologies, MD) using random hexamers as primers. Duplex-PCR was performed by using primers G3PDH-F (5'-TGAAGGTCGGAGTCAACGGATTGGT-3') and G3PDH-R (5'-CATGTCTCGCCATGAGGTCCACCAC-3') for *G3PDH* as internal control, and primers SMAD4-F (5'-CTGCCAACTTTCCCAACATT-3') and SMAD4-R (5'-TGACCCAAACATCACCTTCA-3') for *SMAD4*.

3. Results

3.1. A novel missense mutation of SMAD2 identified in a HNSCC cell line.

The human *SMAD2* gene is composed of 12 exons, including two exon 1 (exon1a and 1b). A missense mutation of *SMAD2* gene was identified in a human oral squamous cell carcinoma cell line SCC15 (Fig. 1A). The mutation changed codon 276 from TCG to TTG, thus resulting in a serine being replaced by a leucine at the codon (Table 2). This *SMAD2* mutation has not been described before. This is the first reported *SMAD2* mutation in human head and neck cancer.

Interestingly, all eight HNSCC cell lines displayed heterozygous deletions in the polypyrimidine tract (T18) located in the junction of the intron 3 and exon 4 at position -39 to -56 (Table 2). Mutations in this region have been reported previously with no obvious effects to the splicing of the *SMAD2* gene [14,15].

3.2. A nonsense mutation of the SMAD4 gene resulted in the loss of its protein expression in a HNSCC cell line.

All 11 encoding exons and the flanking intronic sequences of *SMAD4* were amplified for sequencing. We were unable to amplify any exon of the *SMAD4* gene in the FaDu cell line, thus confirming the homozygous deletion of the gene as previously described [16]. Direct genomic sequencing was performed on the remaining seven cell lines. We uncovered a nonsense mutation in exon 5 at codon 245 CAG→TAG (glutamine) (Fig. 1B) in the oral SCC cell line CAL27 (Table 2). It was noted that there is clearly no overlapping peak at the mutation site (Fig. 1B). It is presumed that the *SMAD4* gene has been completely inactivated by a nonsense mutation on one allele and LOH on the other allele in this cell line due to the high frequency of LOH (>50%) often observed at this chromosomal region [11,16,17]. Western blotting further confirmed that the Smad4 protein was entirely lost in the cell lines CAL27 and FaDu (Fig. 2A). A miniscule level of *SMAD4* RNA transcripts was detected by semi-quantitative duplex RT-PCR in CAL27 (Fig. 2B).

A heterozygous alteration in the polypyrimidine tract (T16) at position +30 to +35 of intron 6 was found in seven cell lines (Table 2). Since a two-bp deletion in this T16 tract has been reported previously in esophageal squamous cell carcinomas as a polymorphism [18], therefore we conclude that the alterations that we observed here are likely due to polymorphism as well.

3.3. Mutational analyses of SMAD3, TGFBR1, and TGFBR2 uncovered novel polymorphic changes.

The human *SMAD3* consists of nine exons separated by introns ranging in size from 162 base pairs up to 98.5 kilo base pairs. A silent mutation at codon 103 CTG (Leu) → CTA (Leu) of exon 2 was found in five of the eight HNSCC cell lines (Table 2). This polymorphism has been previously reported in esophageal squamous cell carcinomas [18] and in 41.7% (15/36) of human ovarian cancers [19]. A previously reported polymorphism in intron 2 at +59 position (C→G) was observed in five cell lines [20]. Another novel intronic alteration was located in intron 7 at position +159 T→C (Table 2).

The entire encoding exons and flanking intronic sequences of *TGFBR1* gene were analyzed by the same method described above. No novel mutation was found the *TGFBR1* gene in these cell lines. A previously reported nine nucleotide polymorphic deletion [del(GCG)3] in exon 1 (GCG)9 microsatellite repeats of the *TGFBR1* gene was found heterozygous in two cell lines (Table 2). This mutation leads to the deletion of three alanines within the 9-alanine microsatellite region. The role of this Tgfr1 protein variant in HNSCC carcinogenesis remains unclear [21-23]. Another previously described polymorphic change at the +24 of intron 7 was also noted in three cell lines (Table 2) [21]. A five bp (CTTTT) deletion at the +86 nucleotide position of intron 8 was detected in three cell lines (Table 2). This alteration has not been reported before.

We also screened for mutations in the *TGFBR2* gene locus in these HNSCC cell lines except for cell lines A253, SCC-25 and FaDu, which had been studied previously [8,24]. Of the three, only cell line A253 harbors a missense mutation in *TGFBR2* [8] (Table 3). We were able to confirm this missense mutation in A253 cells- a transversion from nucleotide G to C at 1610, leading to the codon 537 arginine replaced by a proline. All seven exons and flanking intronic regions of *TGFBR2* were amplified and sequenced by using eight sets of unique primers; no inactivating mutation was found in the five remaining cell lines. Three intronic changes were observed in introns 2, 3 and 6 (Table 2). The A→G alteration in intron 2 at +7 nucleotide position is a previously reported polymorphism [25].

4. Discussion

In this study, we found two mutations in the Smad mediators and a combination of 13 polymorphisms (Table 2). Frequent LOH at chromosome 18q21 has been reported in HNSCC [17,26]. Both the *SMAD4* and *SMAD2* genes are mapped to 18q [2,3]. *SMAD2* is a known tumor suppressor gene. Mutations of *SMAD2* gene have been reported in various types of tumors including human colorectal, lung, hepatocellular, and cervical cancer [27-29], but not in head and neck cancer [3,17,30]. Here we identified a novel missense mutation of the *SMAD2* gene in human HNSCC. This is the first *SMAD2* mutation with presumed LOH reported in human head and neck cancer. Alteration of *SMAD2* in HNSCC probably is a low-frequency event, since no mutation has been found in previous efforts [3,17,30]. The sensitivities of the assays chosen for previous studies may have also contributed to the lack of positive findings-*in vitro* translation was used for one study [3], while PCR-single-strand conformational polymorphism (SSCP) was applied in another [30]. The novel mutation in exon 8 of *SMAD2* gene at codon 276 is located in a highly conserved C-terminal domain amongst the *SMAD* gene family, termed the MH2 domain. The MH2 domain is responsible for receptor interaction, homomeric and heteromeric formations, and transactivation function of Smad proteins [1]. There was no change of *SMAD2* at RNA and protein levels in SCC-15 cells (data not shown); therefore we speculate that this mutation probably aimed to disrupt the function of the Smad2 protein by affecting its homomeric and heteromeric protein-protein interactions or destabilizing the whole structure of the protein. Future study is needed to demonstrate the

effect of the mutation on Smad2 function. Our finding strongly suggests that the *SMAD2* gene may be involved in the carcinogenesis of HNSCC.

Mutations in the *SMAD4* tumor suppressor gene are seen in 55% of pancreatic carcinomas, 20% of colon carcinomas and 10% of lung cancers [2,31,32]. Kim *et al.* first reported a nonsense mutation of the *SMAD4* gene (GAA526TAA) in two cell lines derived from the same patient with HNSCC [11]. Reiss *et al.* found a homozygous deletion that encompasses the *SMAD4* gene locus in HNSCC cell line FaDu [16]. Here we identified the third case of *SMAD4* mutation in a HNSCC cell line. The result of the Western blotting analysis in our study verified that this nonsense mutation causes the complete loss of the Smad4 protein. This is consistent with previous observations that the majority of missense mutations outside of codons 330-370 inactivate Smad4 by protein degradation [33]. These data reiterate the importance of *SMAD4* in the carcinogenesis of HNSCC.

No mutations of *SMAD3*, *TGFBRI* and *TGFBRII* genes were found in the current study. The *SMAD3* mutation has not yet been reported in any cancer type thus far [3,18,19]. Our result on *SMAD3* is therefore consistent with previous observations. Although *TGFBRI* gene is frequently mutated in primary ovarian carcinomas, metastatic breast cancer, and cervical carcinomas [15,22,23,34], we didn't detect any biallelic inactivation of *TGFBRI* in the eight human HNSCC cell lines. The result is consistent with a study that no mutation of the *TGFBRI* gene was found in 30 head and neck carcinomas using "Cold" SSCP and direct sequencing [21]. However, Chen *et al.* reported that 4 of 21 head and neck cancer metastases were found carrying *TGFBRI* mutation [10]. This suggests that *TGFBRI* mutation may be only associated with metastatic head and neck cancers. Although we did not observe any novel mutation of *TGFBRII* in our study, we were able to confirm a previously reported missense mutation in A253 cell line [8]. Since TGF- β /activin signaling pathways share the same Smad mediators (Smad2, Smad3 and Smad4), we also screened for mutations in activin receptor type IB (*ALK4* or *ACVR1B*) and type II (*ACVR2*) by direct genomic sequencing. No mutation was found in all the encoding exons and flanking intronic sequences of the *ACVR1B* gene and the two 8-bp polyadenine [(A)₈] tracts of the *ACVR2* gene analyzed in these cell lines (data not shown). Mutation of *ACVR1B* and *ACVR2* could be a rare event in HNSCC; a larger sample set may be required to determine the importance of *ACVR1B* and *ACVR2* in head and neck cancer development.

In this comprehensive study of the eight human HNSCC cell lines, we uncovered a novel *SMAD2* mutation in SCC15 cell line and a nonsense mutation of *SMAD4* in CAL27 cell line. We also confirmed a homozygous deletion at the *SMAD4* locus and a missense mutation of the *TGFBRII* gene previously reported in cell lines FaDu and A253 respectively (Table 3) [8, 16]. Despite our small sample size, we were able to show that the TGF- β -Smad signal pathway is genetically mutated in half of these cell lines. These data strongly suggest that the TGF- β -Smad signal pathway is critically involved in the carcinogenesis of head and neck cancer and genetic mutation unequivocally contributes to the inactivation of the pathway.

Acknowledgements

This work was supported by the NCI Temin Award CA95434 and the NCI R01 CA109525.

References

1. Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 2000;19:1745–1754. [PubMed: 10775259]
2. Hahn SA, Schutte M, Hoque ATMS, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. DPC4, a candidate tumor-suppressor gene at 18q21.1. *Science* 1996;271:350–353. [PubMed: 8553070]

3. Riggins GJ, Kinzler KW, Vogelstein B, Thiagalingam S. Frequency of SMAD gene mutations in human cancer. *Cancer Res* 1997;57:2578–2581. [PubMed: 9205057]
4. Goggins M, Shekher M, Turnacioglu K, Yeo CJ, Hruban RH, Kern SE. Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas. *Cancer Res* 1998;58:5329–5332. [PubMed: 9850059]
5. Grady WM, Myeroff LL, Swinler SE, Rajput A, Thiagalingam S, Lutterbaugh JD, Neumann A, Brattain MG, Chang J, Kim SJ, Kinzler KW, Vogelstein B, Willson JK, Markowitz S. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999;59:320–324. [PubMed: 9927040]
6. Reiss M, Stash EB. High frequency of resistance of human squamous carcinoma cells to the anti-proliferative action of transforming growth factor beta. *Cancer Commun* 1990;2:363–369. [PubMed: 2173622]
7. Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit Rev Oncog* 1993;4:493–540. [PubMed: 8241322]
8. Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, Reiss M. Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. *Cancer Res* 1995;55:3982–3987. [PubMed: 7664267]
9. Wang D, Song H, Evans JA, Lang JC, Schuller DE, Weghorst CM. Mutation and downregulation of the transforming growth factor beta type II receptor gene in primary squamous cell carcinomas of the head and neck. *Carcinogenesis* 1997;18:2285–2290. [PubMed: 9395234]
10. Chen T, Yan W, Wells RG, Rimm DL, McNiff J, Leffell D, Reiss M. Novel inactivating mutations of transforming growth factor-beta type I receptor gene in head-and-neck cancer metastases. *Int J Cancer* 2001;93:653–661. [PubMed: 11477574]
11. Kim SK, Fan Y, Papadimitrakopoulou V, Clayman G, Hittelman WN, Hong WK, Lotan R, Mao L. DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. *Cancer Research* 1996;56:2519–2521. [PubMed: 8653689]
12. Yan W, Vellucci VF, Reiss M. Smad protein expression and activation in transforming growth factor-beta refractory human squamous cell carcinoma cells. *Oncol Res* 2000;12:157–167. [PubMed: 11216674]
13. Moskaluk CA, Hruban RH, Schutte M, Lietman AS, Smyrk T, Fusaro L, Fusaro R, Lynch J, Teo CJ, Jackson CE, Lynch HT, Kern SE. Genomic sequencing of DPC4 in the analysis of familial pancreatic carcinoma. *Diag Mol Pathol* 1997;6:85–90.
14. Takenoshita S, Tani M, Mogi A, Nagashima M, Nagamachi Y, Bennett WP, Hagiwara K, Harris CC, Yokota J. Mutation analysis of the Smad2 gene in human colon cancers using genomic DNA and intron primers. *Carcinogenesis* 1998;19:803–807. [PubMed: 9635866]
15. Wang D, Kanuma T, Mizunuma H, Takama F, Ibuki Y, Wake N, Mogi A, Shitara Y, Takenoshita S. Analysis of specific gene mutations in the transforming growth factor-beta signal transduction pathway in human ovarian cancer. *Cancer Res* 2000;60:4507–4512. [PubMed: 10969799]
16. Reiss M, Santoro V, de Jonge RR, Vellucci VF. Transfer of chromosome 18 into human head and neck squamous carcinoma cells: evidence for tumor suppression by Smad4/DPC4. *Cell Growth Differ* 1997;8:407–415. [PubMed: 9101086]
17. Papadimitrakopoulou VA, Oh Y, El-Naggar A, Izzo J, Clayman G, Mao L. Presence of multiple contiguous deleted regions at the long arm of chromosome 18 in head and neck cancer. *Clin Cancer Res* 1998;4:539–544. [PubMed: 9533520]
18. Osawa H, Shitara Y, Shoji H, Mogi A, Kuwano H, Hagiwara K, Takenoshita S. Mutation analysis of transforming growth factor beta type II receptor, Smad2, Smad3 and Smad4 in esophageal squamous cell carcinoma. *Int J Oncol* 2000;17:723–728. [PubMed: 10995883]
19. Wang D, Kanuma T, Takama F, Mizunuma H, Ibuki Y, Wake N, Mogi A, Shitara Y, Hagiwara K, Takenoshita S. Mutation analysis of the Smad3 gene in human ovarian cancers. *Int J Oncol* 1999;15:949–953. [PubMed: 10536178]
20. Arai T, Akiyama Y, Okabe S, Ando M, Endo M, Yuasa Y. Genomic structure of the human Smad3 gene and its infrequent alterations in colorectal cancers. *Cancer Lett* 1998;122:157–163. [PubMed: 9464505]

21. Knobloch TJ, Lynch MA, Song H, DeGross VL, Casto BC, Adams EM, Alam KY, Lang JC, Schuller DE, Weghorst CM. Analysis of TGF-beta type I receptor for mutations and polymorphisms in head and neck cancers. *Mutat Res* 2001;479:131–139. [PubMed: 11470488]
22. Chen T, Triplett J, Dehner B, Hurst B, Colligan B, Pemberton J, Graff JR, Carter JH. Transforming growth factor-beta receptor type I gene is frequently mutated in ovarian carcinomas. *Cancer Res* 2001;61:4679–4682. [PubMed: 11406536]
23. Chen T, Carter D, Garrigue-Antar L, Reiss M. Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* 1998;58:4805–4810. [PubMed: 9809982]
24. Vincent F, Nagashima M, Takenoshita S, Khan MA, Gemma A, Hagiwara K, Bennett WP. Mutation analysis of the transforming growth factor-beta type II receptor in human cell lines resistant to growth inhibition by transforming growth factor-beta. *Oncogene* 1997;15:117–122. [PubMed: 9233784]
25. Barlow J, Yandell D, Weaver D, Casey T, Plaut K. Higher stromal expression of transforming growth factor-beta type II receptors is associated with poorer prognosis breast tumors. *Breast Cancer Res Treat* 2003;79:149–159. [PubMed: 12825850]
26. Takebayashi S, Ogawa T, Jung KY, Muallem A, Mineta H, Fisher SG, Grenman R, Carey TE. Identification of new minimally lost regions on 18q in head and neck squamous cell carcinoma. *Cancer Res* 2000;60:3397–3403. [PubMed: 10910046]
27. Eppert K, Scherer SW, Ozcelik H, Pirone R, Hoodless P, Kim H, Tsui LC, Bapat B, Gallinger S, Andrulis IL, Thomsen GH, Wrana JL, Attisano L. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* 1996;86:543–552. [PubMed: 8752209]
28. Uchida K, Nagatake M, Osada H, Yatabe Y, Kondo M, Mitsudomi T, Masuda A, Takahashi T, Takahashi T. Somatic in vivo alterations of the JV18-1 gene at 18q21 in human lung cancers. *Cancer Research* 1996;56:5583–5585. [PubMed: 8971158]
29. Maliekal TT, Antony ML, Nair A, Paulmurugan R, Karunakaran D. Loss of expression, and mutations of Smad 2 and Smad 4 in human cervical cancer. *Oncogene* 2003;22:4889–4897. [PubMed: 12894231]
30. Muro-Cacho CA, Rosario-Ortiz K, Livingston S, Munoz-Antonia T. Defective transforming growth factor beta signaling pathway in head and neck squamous cell carcinoma as evidenced by the lack of expression of activated Smad2. *Clin Cancer Res* 2001;7:1618–1626. [PubMed: 11410498]
31. Thiagalingam S, Lengauer C, Leach FS, Schutte M, Hahn SA, Overhauser J, Willson JKV, Markowitz SD, Hamilton SR, Kern SE, Kinzler KW, Vogelstein B. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet* 1996;13:343–346. [PubMed: 8673134]
32. Nagatake M, Takagi Y, Osada N, Uchida K, Mitsudomi T, Saji S, Shimokata K, Takahashi T, Takahashi T. Somatic in vivo alterations of the DPC4 gene at 18q21 in human lung cancers. *Cancer Res* 1996;56:2718–2720. [PubMed: 8665501]
33. Iacobuzio-Donahue CA, Song J, Parmigiani G, Yeo CJ, Hruban RH, Kern SE. Missense mutations of MADH4: characterization of the mutational hot spot and functional consequences in human tumors. *Clin Cancer Res* 2004;10:1597–1604. [PubMed: 15014009]
34. Chen T, de Vries EG, Hollema H, Yegen HA, Vellucci VF, Strickler HD, Hildesheim A, Reiss M. Structural alterations of transforming growth factor-beta receptor genes in human cervical carcinoma. *Int J Cancer* 1999;82:43–51. [PubMed: 10360819]

Figure 1.

Mutations of the *SMAD2* and *SMAD4* genes in HNSCC cell lines. (A) SCC-15 cell line harbors a missense mutation in the exon 8 of the *SMAD2* gene at codon 276 from TCG to TTG, leading to a serine replacement by a leucine. Cell line SCC-25 represents the wild-type *SMAD2* gene. (B) CAL27 cell line displays a nonsense mutation in the exon 5 of the *SMAD4* at codon 245 from CAG to TAG, leading to an amino acid glutamine replacement by a stop codon. Cell line RPMI 2650 represents the wild-type *SMAD4* gene sequence.

Figure 2.

Smad4 expression in the HNSCC cell lines. (A) Western blotting was performed using the monoclonal anti-Smad4 antibody. The result showed that the expression of Smad4 protein was completely lost in cell lines FaDu and CAL27. (B) *SMAD4* mRNA expression was determined by duplex-RT-PCR using *G3PDH* as the internal control. CAL27 cell line showed very weak *SMAD4* mRNA transcription, while cell line FaDu entirely lacked the *SMAD4* mRNA expression.

Table 1.
Primers designed for the PCR amplification and sequencing

Name	nucleotide sequence	product size (bp)	annealing temperature(°C)
SMAD2E1F	ggtggcaggcgggtctac	564	53
SMAD2E1R	cgcaaacacttccctagctg		
SMAD2E2F	atcttgcttgcagtttgctt	469	56
SMAD2E2R	ggcaacttgaaaggaaacaaa		
SMAD2E3F	aacctgcttccatgttcac	251	58
SMAD2E3R	ttaaggaaacatcctaggcaaaa		
SMAD2E4F	gccatttgactgcactttt	354	56
SMAD2E4R	aaatttctctgggtcacagag		
SMAD2E5F	tcgagtaggtggaccctagc	342	60
SMAD2E5R	ccacaatttactaaaactggaatgc		
SMAD2E6F	gcagctgtgcttgattgtt	222	58
SMAD2E6R	ttggtatgcgtctcaactctc		
SMAD2E7F	ttgcaccttttatactggaacc	374	56
SMAD2E7R	ttcattaggatcccttctcg		
SMAD2E8F	cttcctgagcttttggccag	408	60
SMAD2E8R	acaccatgcaatgcctacat		
SMAD2E9/10F	ttccaagaaaatgcttccaaa	575	56
SMAD2E9/10R	cactgtggaaaatttaagaaccaa		
SMAD2E11F	gcggaataatcgtgtccaaa	346	56
SMAD2E11R	tccatagggaccacacacaaa		
SMAD3E1F	cgagagttgaggcggaagttt	437	62
SMAD3E1R	tccctctctcctctctcc		
SMAD3E2/3F	cagaaagcaagcacaatcca	621	58
SMAD3E2/3R	gggccacaggactgatgt		
SMAD3E4F	tgggtgtgcatgtgtgatgc	265	61
SMAD3E4R	tagaaggaggaggaggagag		
SMAD3E5F	ccaggccaagaatcttttgt	335	58
SMAD3E5R	cccaggggaaatacaaacct		
SMAD3E6F	tggaaatctctccagacacc	472	61
SMAD3E6R	ctgggggtgggatagagtg		
SMAD3E7F	tctgctgttctgcctcttt	414	60
SMAD3E7R	gaagtcaggggaaagcacag		
SMAD3E8F	agtgtggagtggtgtggcaaa	327	62
SMAD3E8R	tgatgtagcagcaccata		
SMAD3E9F	ctgtgttaacccctggaga	365	61
SMAD3E9R	aacatccacctctgggttg		
TGFBRIE1F	cctccgagcagttacaaagg	319	60
TGFBRIE1R	gcgcatgtttgagaagag		
TGFBRIE2F	gagcaacaacagtgcatagaaa	490	60
TGFBRIE2R	tgcctctaaccggaatgagc		
TGFBRIE3F	ggctctttggctaagtgtg	454	59
TGFBRIE3R	tcacattctagcaagttggctta		
TGFBRIE4F	tctccccagtgaataaattcc	462	58
TGFBRIE4R	tgtctcatctactttgatgggt		
TGFBRIE5F	ttgcagtggtgactcagga	271	60
TGFBRIE5R	cttgggtaccacaatctcca		
TGFBRIE6F	gtgggctgaaatgctttgat	367	58
TGFBRIE6R	tttaagctgagtttcagcaatga		
TGFBRIE7F	atgtgcaaacagtggtgat	409	59
TGFBRIE7R	tcggtgacatcctgtttcag		
TGFBRIE8F	gccttggcattagctgaataa	395	58
TGFBRIE8R	aaaggccactgcaaatgttc		
TGFBRIE9F	tccagaccaatgaaaatgg	352	59
TGFBRIE9R	cctgggtccaagaaatcct		
TGFBRIIE1F	ctgggggctcggctcatg	342	60
TGFBRIIE1R	cccttgcaactgaacttcc		
TGFBRIIE2F	gcctggcagttggataatca	338	59
TGFBRIIE2R	ggaaagggaatggaacagg		
TGFBRIIE3F	cctcgcttccaatgaatctc	322	58
TGFBRIIE3R	aatccaccacaggaggaaatg		
TGFBRIIE4F	catgaaccacttctctgaca	586	59
TGFBRIIE4R	gccagtattgtttcccaac		
TGFBRIIE4Fa	ataaggccaagctgaagcag	525	61
TGFBRIIE4Ra	aggccaggctcaaggtaaag		
TGFBRIIE5F	aaatgatggcctcactgtc	375	59
TGFBRIIE5R	atgctccaatgtggcttctc		
TGFBRIIE6F	ccagcgtaacacttagcaca	445	61
TGFBRIIE6R	tccagaattctctgccacct		
TGFBRIIE7F	caggcactcagtcagacat	488	62
TGFBRIIE7R	actccctgctgctgttttt		

Table 2.

Mutations and polymorphisms of the components of the TGF- β -Smad signaling pathway identified in the HNSCC cell lines.

Exonic mutations with presumed LOH						
Gene	Exon	Codon	Nucleotide change	Amino acid change	Presence of a wild-type allele	Cell line
SMAD2	8	276	TCG→TTG	Ser→Leu	No	SCC-15
SMAD4	5	245	CAG→TAG	Glu→Stop	No	CAL27
Polymorphic changes in exonic coding regions						
Gene	Exon	Codon	Nucleotide change	Presence of a wild-type allele (number of cell lines)		
SMAD3	2	103	CTG(Leu)→CTA(Leu)	No (2) Yes (3)		
TGFBRI	1	18-26	del (GCG)3	Yes (2)		
Polymorphic changes in intronic sequences						
Gene	Intron	Nucleotide position	Polymorphic change	Presence of a wild-type allele (number of cell lines)		
SMAD2	3	-39 to -56	(T18)del TT	Yes (5)		
	3	-39 to -56	(T18)del T	Yes (3)		
SMAD3	2	+59	C→G	No (3) Yes (2)		
	7	+159	T→C	Yes (1)		
SMAD4	6	+30	del T	Yes (6)		
	6	+30	ins T	Yes (1)		
TGFBRI	7	+24	G→A	No (2) Yes (1)		
	8	+86	del CTTTT	No (2) Yes (1)		
TGFBRII	2	+7	A→G	No (2)		
	3	-4	T→A	No (1)		
	6	-101	C→A	Yes (1)		

GenBank accession number: *SMAD2*: AF027964, U78727, U7872; *SMAD3*: AF025294, AF025298; *SMAD4*: U44378, AF045442; *TGFBRI*: AF054590, AF054596, AF054597; *TGFBRII*: AY675319.

Table 3.The genetic profile of the TGF- β /activin-Smad pathways in the 8 HNSCC cell lines

Cell lines	ACVR1B	TGFBRI	TGFBRII	SMAD2	SMAD3	SMAD4
RPMI 2650	-	-	-	-	-	-
A253	-	-	missense ¹	-	-	-
SW 579	-	-	-	-	-	-
Detroit 562	-	-	-	-	-	-
FaDu	-	-	-	-	-	HD ¹
CAL27	-	-	-	-	-	nonsense ²
SCC-15	-	-	-	missense ²	-	-
SCC-25	-	-	-	-	-	-

-, absence of biallelic inactivation; HD, homozygous deletion; missense, missense mutation with presumed LOH; nonsense, nonsense mutation with presumed LOH.

¹ as previously reported and confirmed in this study [8,16].

² mutations uncovered in the present study.