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Loss of expression, and mutations of *Smad 2* and *Smad 4* in human cervical cancer

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Mutations in Smads, intermediates of transforming growth factor-β signaling, are known to contribute to the loss of sensitivity to transforming growth factor-β, a common feature of many neoplastic cells. However, not much information is available on *Smad* alterations in cervical cancer and so we probed, for the first time, for alterations in *Smad 2* and *Smad 4* genes using human cervical cancer cell lines and human cervical tissue samples. Using PCR/reverse transcription–PCR, single-stranded conformation polymorphism analysis and DNA sequencing, we observed a deletion of 'G' in the L3 loop (crucial in Smad–receptor interaction) in C-33A cells, and an insertion of 'A' in codon 122 (loss of MH2 domain) from a cervical tumor sample, both of which caused frame shift and pretermination in *Smad 2*. In addition, a G/A transition at 31 bp upstream-nontranslated regions of exon 8 of *Smad 4* was found in Bu 25TK cells. *Smad 2* expression was less in some of the cervical tumor samples than that of nonmalignant samples and six cancer samples showed C-terminal deletions that abolish Smad 2 phosphorylation sites. The loss of expression of *Smad 4* found in some cervical tumor samples was due to transcription loss rather than deletion of the gene. Our results highlight an important role for Smad 2 and Smad 4 in human cervical tumors.
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Introduction

Cervical cancer is an important public health problem for adult women in developing countries, where it is the most or second most common cancer among women. Cervical cancer causes about 250 000 deaths annually worldwide, with women in the developing countries accounting for 80% of these deaths (Schoell *et al*., 1999; Cain and Howett, 2000). Although HPV infection is the major risk factor for cervical cancer, molecular alterations of tumor-suppressor genes and/or oncogenes either associated with HPV infection or independent of it are necessary for the cervical cancer progression (zur Hausen, 1996). Compatible with this hypothesis, frequent loss of heterozygosity (LOH) has been found at different chromosomal segments in cervical cancer (Mullokandov *et al*., 1996; Dellas *et al*., 1999). LOH at the 18q chromosomal region (37%) correlated with poor prognosis of cervical cancer and 18q is known to harbor two tumor-suppressor genes, *Smad 2* and *Smad 4*, that mediate transforming growth factor-β (TGF-β) signal transduction (Dellas *et al*., 1999).

TGF-βs are multifunctional polypeptide growth factors with diverse biological effects, including inhibition of epithelial cell proliferation both *in vitro* and *in vivo* (Attisano and Wrana, 2002). The biological effects of TGF-β are primarily mediated by a complex of two transmembrane serine/threonine kinases, the type I (TβRI) and type II (TβRII) receptors (Attisano and Wrana, 2002). TGF-β signaling cascade is activated when TGF-β1 binds to TβRII, then TβRI joins the complex and gets phosphorylated in the glycine-rich region by TβRII (Heldin *et al*., 1997). The recruitment of Smad 2 and Smad 3 to TβRI in the TGF-β receptor complex is controlled by a membrane-associated protein termed Smad anchor for receptor activation (SARA) (Tsukazaki *et al*., 1998). Upon ligand activation, phosphorylation of Smads 2 and 3 at the SSXS motif in the extreme C-terminal tail relieves the autoinhibitory interactions of the two Smad domains, MH1 and MH2, and allows heteromerization with Smad 4 (Jayaraman and Massague, 2000). The MH1 domains of Smads 3 and 4 of the heterotrimer have the intrinsic property of binding to specific DNA sequences of target genes, and additional protein–protein interactions occur which are essential for efficient transcriptional activation (Attisano and Wrana, 2002).

Loss of TGF-β sensitivity is observed in a wide variety of cancers including cervical cancer (de Caestecker *et al*., 2000). Mutations of *Smad 2* and *Smad 4* were reported in several cancers (de Caestecker *et al*., 2000), including pancreatic cancer (Costentin *et al*., 2002), colorectal cancer (Miyaki *et al*., 1999) and juvenile polyposis (Howe *et al*., 1998). However, except for a single report of *Smad 4* alteration in SiHa cells (Lee *et al*., 2001), no information is available on *Smad* alterations in cervical cancer. Hence, we investigated for the first time whether

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there are any molecular alterations in *Smad 2* and *Smad 4* genes using human cervical cancer cell lines and tumor samples. Using a combination of PCR/RT–PCR, single-stranded conformation polymorphism (SSCP), DNA sequencing and semiquantitative RT–PCR (SQRT–PCR) methods, we report a novel *Smad 2* mutation in a human cervical tumor sample and the *Smad 2* and *Smad 4* mutations in two of the six human cervical cancer cell lines and loss of expression of *Smad 2* and *Smad 4* in some of the cervical tumor samples analysed.

Materials and methods

Tissue samples and cell lines

Fresh cervical tissues (malignant or nonmalignant) were collected from patients undergoing hysterectomy from Sri Avittam Thirunal Hospital for women, Thiruvananthapuram. Written consent was obtained from patients and bystanders before sample collection. Samples were snap-frozen in liquid nitrogen, and used immediately for RNA preparation or stored at −70°C for DNA preparation and protein extraction and adjacent nonmalignant regions of each tumor sample were also used for analysis wherever possible. A pathologist, following Bethesda classification, carried out histopathological grading of the samples. SiHa cells (human cervical cancer cell line) were obtained from Dr Sudhir Krishna, National Center for Biological Sciences, Bangalore, India. Other cervical cancer cell lines used in this study were procured from the National Center for Cell Science, Pune, India. The cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (Life Technologies Inc., USA) containing 10% FBS (Sigma, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C.

PCR analysis

Genomic DNA was isolated from frozen fresh tissue samples or cell lines by the standardized phenol/chloroform extraction method. Genomic DNA (100 ng) was used for the PCR amplification in a 50 μl reaction mix containing 2.5 U Taq DNA polymerase (Promega) in 1 × reaction buffer, 1.5 mM $MgCl_2$, 150 μM dNTP mix and 25 pmol of sense and antisense primers (Life Technologies Inc., Genosys, Sigma). The PCR conditions included initial denaturation at 94°C for 3 min, 30 cycles of denaturation for 30 s and annealing at 55°C for 1 min followed by extension at 72°C for 1.5 min and final extension of PCR products was carried out at 72°C for 7 min. The annealing temp of C-amplification was 50°C, G-amplification was 60°C and that of full-length amplification was 48°C.

RT–PCR analysis

Total RNA from fresh tissue samples or cell lines was isolated using Trizole reagent (Life Technologies Inc.), following the manufacturer's protocol. Total RNA (10 μg) was reverse transcribed to cDNA in a 25 μl reaction mix containing 200 U of MMLV reverse transcriptase (Life Technologies Inc.) in 1 × reaction buffer with 2 μg of random hexamer (New England BioLabs), 6 U of RNA guard (Amersham) and 100 μM of dNTP mix. The reverse transcription was performed at 37°C for 1 h and heating at 90°C for 4 min followed by quick chilling inactivated the enzyme and 2 μl of the cDNA was used for PCR amplification.

SQRT–PCR analysis

SQRT–PCR was performed as described earlier (Barabas *et al*., 2001) and the cDNA synthesis was similar to RT–PCR, except that 5 μg of RNA was used and 1.5 μl of cDNA from the same RT mix was used for each gene amplification, taking β2-microglobulin as control. The PCR reaction mix for each gene was 100 μl and the reaction was divided into equal parts, and amplified for different cycles, and amplification conditions were similar to PCR conditions for a particular region.

Single-strand conformation polymorphism (SSCP)

The amplified products (50 ng) were denatured in SSCP dye containing 90% formamide and 10 mM NaOH at 95°C for 7 min and quick chilled. The separated single strands were resolved in a nondenaturing 10% PAGE containing 5% glycerol buffered with 0.5 × TBE, for 14 h at 20°C and the bands were visualized by silver staining (Lohmann *et al*., 1996).

DNA sequencing

The amplified products were used for sequencing with an ABI PRISM big dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). The same primer sets used for the amplification were used for sequencing using an automated sequencer (ABI PRISM 310, Perkin-Elmer) and the analysis was performed using sequencing analysis software version 3.4.

Western blotting

Total cell lysates from fresh frozen tissue samples were prepared with RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate and 1% SDS) on ice for 1 h and proteins were quantitated by Bradford's method according to the manufacturer's protocol (Bio-Rad). Samples (50 μg protein) were resolved on 10% SDS–PAGE and transferred to a nitrocellulose membrane (hybond C-pure, Amersham) using a Bio-Rad Mini PROTEAN III apparatus. Smad 2 was detected with a polyclonal antibody that detects the N-terminal of the molecule (*sc-6200*, Santa Cruz Biotechnology) and β-actin was detected using a monoclonal antibody (A-5441, Sigma). Alkaline phosphatase-conjugated secondary antibodies were used and the color development was performed with bromo-chloroindolyl phosphate/nitroblue tetrazolium substrate–dye mixture (Amersham).

Results

Altered SSCP patterns of MH1 and MH2 regions of Smad 2 and MH2 region of Smad 4 are found in human cervical cancer

Human cervical cancer cell lines and cervical tissue samples were used in the present study for the mutational analysis of *Smad 2* and *Smad 4*. Suitable PCR primers were designed for *Smad 4* to amplify the exon regions of both the functional domains along with the 3′ and 5′ intron regions that are important for splicing (Woodford-Richens *et al*., 2001). RT–PCR primers were designed to cover the important functional regions of *Smad 2* and variations in the expression of Smads were analysed by SQRT–PCR. *Smad 2* gene was amplified by RT–PCR (2R1, 2R4 and 2R3 regions) or

PCR (S2 region), which together covered the MH1 and MH2 domains as depicted in a schematic representation (Figure 1a) and a representative gel showing products of the expected sizes (Figure 1c). Similarly, the MH1 and MH2 domains of *Smad* 4 were also amplified by PCR (regions a–g) or RT–PCR (S4 region), as illustrated in a schematic diagram (Figure 1b), and the products obtained are shown in a representative gel (Figure 1d). The oligonucleotide sequences of the different primer sets used for the amplification of *Smad 2*, *Smad 4* and *β2-microglobulin* (control) are noted in Table 1. When the amplified products were analysed by SSCP, altered patterns in 2R3 and 2R1 regions of *Smad 2* were observed in C-33A, a human cervical cancer cell line (Figure 2a), and 10T, a cervical tumor sample (Figure 2b), respectively. For other *Smad 2* regions, no alteration was noted for 20 tissue samples analysed by RT–PCR SSCP (2R3 and 2R4) and 25 tissue samples analysed for S2 by PCR-SSCP (data not shown). Although we used 25 tissue samples for PCR-SSCP analysis, we could use only 20 samples for RT–PCR SSCP analysis due to the degradation of RNA in five samples. In Bu 25TK cells, there was an alteration in SSCP pattern for the E region of *Smad 4* (Figure 2c) and for other *Smad 4* regions, there was no alteration in all the six cervical cancer cell lines and 25 tissue samples that were analysed by PCR SSCP or 20 tissue samples analysed by RT–PCR SSCP (data not shown). Thus, we could detect two SSCP alterations of *Smad 2*, one in C-33A, a human cervical cancer cell line (2R3 region of MH2 domain), and another from a cervical tumor sample (2R1 region of MH1 domain). In addition, an altered SSCP band was found in the MH2 domain ('E' region) of *Smad 4* in Bu 25TK cells.

DNA sequencing of altered SSCP bands reveals novel mutations of Smad 2 and Smad 4

The altered bands from C-33A (Figure 2a) and the human cervical tumor (10 T) sample (Figure 2b) were gel-eluted, reamplified and then used for sequencing with sense strand primers for 2R3 and antisense strand

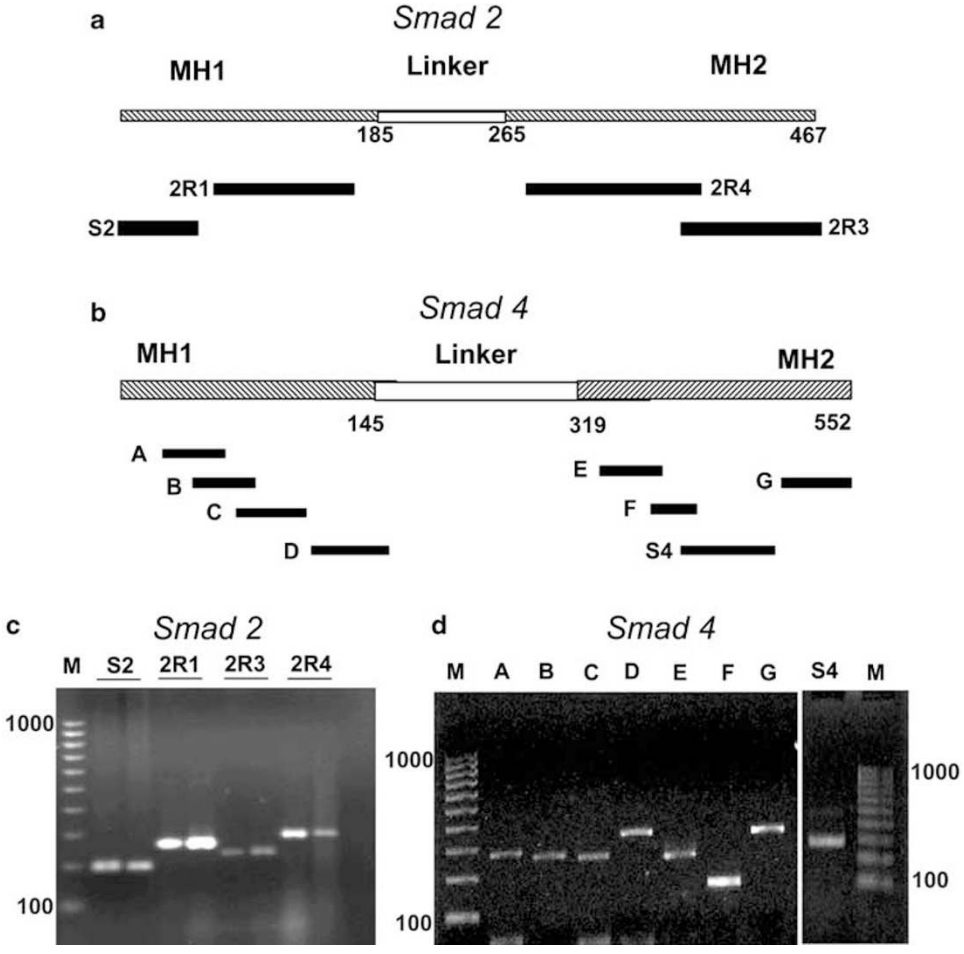

Figure 1 Schematic representation of *Smad 2* and *Smad 4* regions and separation of products amplified. From the total RNA extracted from cervical tissue samples or cell lines, RT–PCR was used to amplify 2R1, 2R4 and 2R3 regions, whereas the DNA extracted was used for PCR to amplify the S2 region (**a**). S4 region of *Smad 4* was amplified by RT–PCR and other regions were amplified by PCR (**b**). Lanes representing the amplified products from S2, 2R1, 2R3 and 2R4 regions of *Smad 2* are shown and M represents the 100 bp ladder (**c**). The amplified products of *Smad 4* from different regions (A–G and S4) are shown and M denotes the 100 bp ladder (**d**)

Table 1 Different primers used for the amplification

Gene	*Name*	*Region*	*Sequence*	*Size of the product (bp)*
Smad 2	S2 S	S2	5′-GTCCATCTTGCCATTCACG-3′	192
	S2 AS		5′-TGGTGATGGCTTTCTCAAGC-3′	
	2R1 S	2R1	5′-AGATCAGTGGGATACAACAGG-3′	264
	2R1 AS		5-GGCACTAATACTGGAGGCAA-3′	
	2R3 S	2R3	5′-CAGGGTTTTGAAGCCGTCTAT-3′	230
	2R3 AS		5′-CATGGGACTTGATTGGTGAA-3′	
	2R4 S	2R4	5′-TGTTAACCGAAATGCCACGG-3′	294
	2R4 AS		5′-TCTTATGGTGCACATTCTAGT-3′	
	FL S	FL	5′-GAATTCGGGAGGTTCGATACAAGAGG-3′	1528
	FL AS		5′-ATGGGCCCACACACAATGCTATGACAGAAG-3′	
Smad 4	A	A	5′-AATGGGGATTGTAATACTGAGTTGG-3′	265
	A′		5′-AGGTGATACAACTCGTTCGTAG-3′	
	B	B	5′-TCCTCATGTGATCTATGCCCG-3′	253
	B′		5′-CGGGCTATCTTCCAA-3′	
	C	C	5′-ACTACGAACGAG TTG TAT CAC C-3′	242
	C′		5′-GGC AAA ATA TCA ACT ACA ATA CTC GGT-3′	
	D	D	5′- GAT AGC GTT TAT GCT ACT TCT G-3′	329
	D′		5′-GCC CCT AAC CTC AAA ATC TAC-3′	
	E	E	5′-TGT TTT GGG TGC ATT ACA TTT C-3′	229
	E′		5′-ATC AAC AGT AAC AAT AGG GCA G-3′	
	F	F	5′-GCT GCC CTA TTG TTA CTG TTG-3′	147
	F′		5′-CAA TTT TTT AAA GTA ACT ATC TGA-3′	
	G	G	5′-GGA AGA GAT CAC CCT GTC CC-3′	319
	G′		5′-ATT GTA TTT TGT AGT CCA CC	
	S4 S	S4	5′-ACCTGGAGATGCTGTTCA-3′	285
	S4 AS		5′-TGTCTTGGGTAATCCGGTC-3′	
β2-microglobulin	B2 S		5′-ACCCCCACTGAAAAAAGATGA-3′	114
	B2 AS		5′-GCATCTTCAAACCTCCATGAT-3′	

primers for 2R1. Sequencing of the 2R3 region of C-33A revealed a heterozygous condition with one normal allele and the other allele showing a 'G' deletion at codon 428 that caused a frame shift (Figure 3a) and also introduced a termination codon after codon 430 (Figure 3b). When the altered band from the tissue sample (10T) was sequenced, we could detect an insertion of 'A' in codon 122, which causes a frame shift (Figure 3c) and a pretermination after codon 135 (Figure 3d). Similarly, the altered band from Bu 25TK cells, after sequencing, was found to be heterozygous with one normal allele and another showing a G/A transition at 31 bp upstream-nontranslated regions of exon 8 of *Smad 4* (Figure 3e). These results report two novel *Smad 2* sequence alterations, one in a human cervical cancer cell line, C-33A (a 'G' deletion at codon 428), and another from a cervical tumor sample (an insertion of 'A' in codon 122), both of which caused frame shift and pretermination. In addition, a G/A transition at 31 bp upstream-nontranslated regions of exon 8 of *Smad 4* in Bu 25TK cells was also noticed in the present work.

Expression of Smad 2 is reduced in human cervical tumor samples

Using the Bethesda system of classification (Nguyen and Nordqvist, 1999), human cervical tissue samples were graded as nonmalignant, low-grade squamous intraepithelial lesion (LSIL/CIN-I), high-grade squamous intraepithelial lesion (HSIL/CIN-II/CIN-III) and squamous cell carcinoma (SCC). A total of 20 tissue samples – six nonmalignant, 10 SCC and four premalignant (LSILs and HSILs) samples – were analysed for *Smad 2* expression by SQRT–PCR using primers for the 2R3 region and the results of SQRT–PCR are summarized in Table 2. The amplified products of four nonmalignant, six SCC and three premalignant samples are shown in Figure 4a. All the nonmalignant samples gave high or very high expression (3+ or 4+) and, in contrast, none of the premalignant or SCC tissue samples showed high expression of *Smad 2* (Figure 4a and Table 2). There was loss of *Smad 2* expression in one out of four premalignant samples (25%), whereas it was six out of 10 SCC samples (60%) (Table 2). It appears that *Smad 2* expression is reduced in SCCs and SILs of human cervical cancer; however, further analysis with a large number of samples is necessary to correlate these results with different stages of cervical cancer progression.

Loss of Smad 4 expression is noticeable in some of the human cervical cancer samples

In all, 20 human cervical tissue samples used above were also analysed for *Smad 4* expression by amplifying the S4 region. Figure 4b shows the amplified products of four nonmalignant, six SCC and three premalignant cervical tissue samples, but the overall results (20 samples) are summarized in Table 2. All nonmalignant samples showed high expression of *Smad 4*, and in

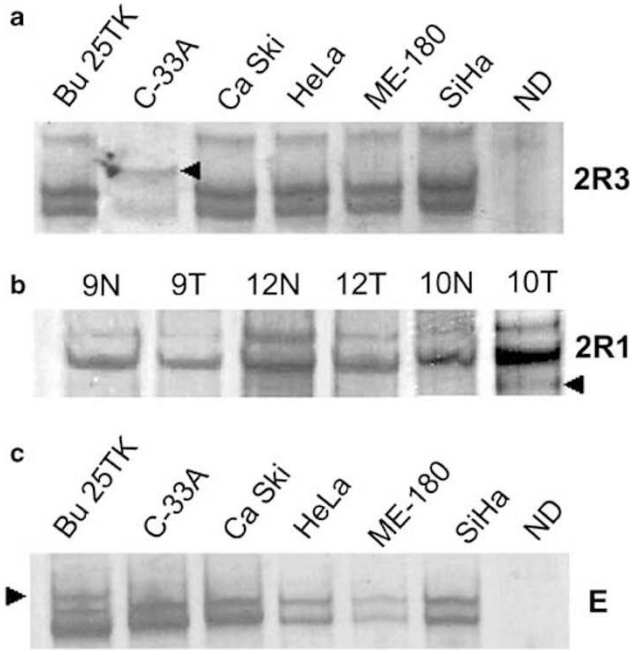

Figure 2 Screening for mutations of *Smads*. Total RNA was extracted from the human cervical cancer cell lines and 2R3 region of *Smad* 2 was then amplified by RT–PCR and analysed by SSCP as described in Materials and methods and the nondenatured control (ND) and an altered band (arrowhead) are shown (**a**). Nonmalignant (N) and malignant (T) regions from the same human cervical tissue samples were used for SSCP analysis of the 2R1 region of *Smad 2* and the arrowhead indicates alteration in SSCP pattern (**b**). E region of *Smad 4* from the human cervical cancer cell lines was analysed by SSCP and the nondenatured control (ND) and the additional band (arrowhead) in SSCP are shown (**c**)

premalignant stages there was no considerable change in the expression level (Table 2). However, in SCC samples, 30% showed loss of expression of *Smad 4*, another 30% showed low expression and yet another 30% showed moderate expression (Table 2). These results, with a limited number of samples, indicate that loss of expression of *Smad 4* tends to occur in SCC of human cervical cancer, although a firm conclusion warrants statistical evaluation with more samples.

C-terminal of Smad 2 is deleted in human cervical cancer

In SQRT–PCR analysis, six SCC samples and one premalignant sample showed the absence of *Smad 2* expression (Table 2), and in these seven samples, 2R1, 2R3 and 2R4 regions were PCR-amplified (Figure 5a). It was not possible to amplify the 2R3 region by PCR; however, 2R1 and 2R4 regions of all the samples could be PCR-amplified (although the intensity of amplification in one sample was low), suggesting a deletion. Since the 2R4 region is amplified in all samples, the 2R3S region should be intact, since its sequence overlaps with the N-terminal of the 2R4 region. To check whether the 2R3AS region is altered by any point mutation or deletion covering that region, a full-length amplification of *Smad 2* was performed (FL S, FL AS, Table 1), the

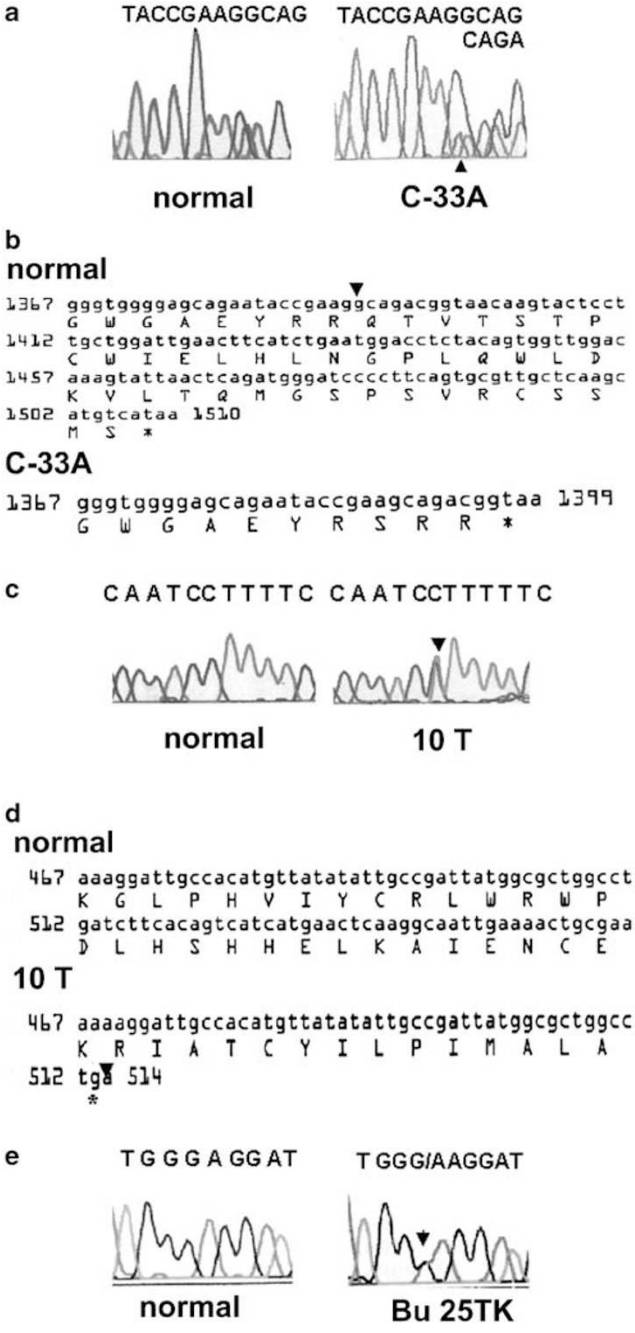

Figure 3 Sequence alterations of *Smads* in cervical cancer. Partial DNA sequence from the 2R3 region of C-33A is shown along with that of a representative nonmalignant tissue sample that matched with the published sequence of *Smad 2* (normal), when sequenced with sense strand primers (**a**). After the 'G' deletion (indicated with the arrowhead), the frame is shifted and the mutant sequence obtained from C-33A shows the heterozygous condition (**b**). The 2R1 region of a human cervical cancer tissue sample (10T) and the adjacent normal region were sequenced with antisense strand primers and the arrowhead indicates an additional 'T' indicating an 'A' insertion (**c**, **d**). E region of *Smad 4* in Bu 25TK was sequenced, which shows the G/A transition as indicated with an arrowhead, when compared with the normal sequence (**e**). The translation of *Smad 2* with the mutant 2R3 allele of C-33A or mutant 2R1 from the tumor sample (10T) was determined along with the normal sequence (accession number AF027964) using the online software ORF finder (GeneBank). The asterisk represents termination codon

Table 2 Expression of Smads in cervical tissue samples

Nature of samples	*No. of samples*	*Smad 2*					*Smad 4*				
		Neg	*1+*	*2+*	*3+*	*4+*	*Neg*	*1+*	*2+*	*3+*	*4+*
Nonmalignant	6				5	1				6	
Premalignant	4	1	2	1					1	2	1
Squamous cell carcinoma	10	6	3	1			3	3	3	1	

Scoring of expression was as follows: Neg – no amplification, 1+ – amplification in only one cycle (37 cycles), 2+ – amplification in two cycles (33 and 37 cycles), 3+ – amplification in three cycles (29, 33 and 37 cycles), 4+ – amplification in four cycles (25, 29, 33 and 37 cycles)

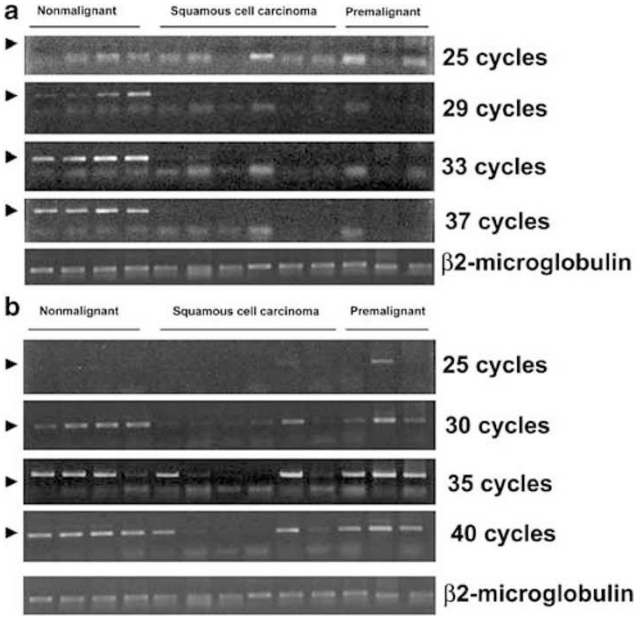

Figure 4 SQRT–PCR analysis of *Smads* in human cervical tissue samples. (**a**) 2R3 region of *Smad 2* was amplified for different cycles as described in Materials and methods. The position of products obtained from nonmalignant, SCC or premalignant samples is indicated with an arrowhead. *β2-microglobulin* amplification for 25 cycles was used as a control and the amplified products were run in an agarose gel (1.5%). (**b**) S4 region of *Smad 4* was amplified for different cycles along with *β2-microglobulin* control as described in Materials and methods

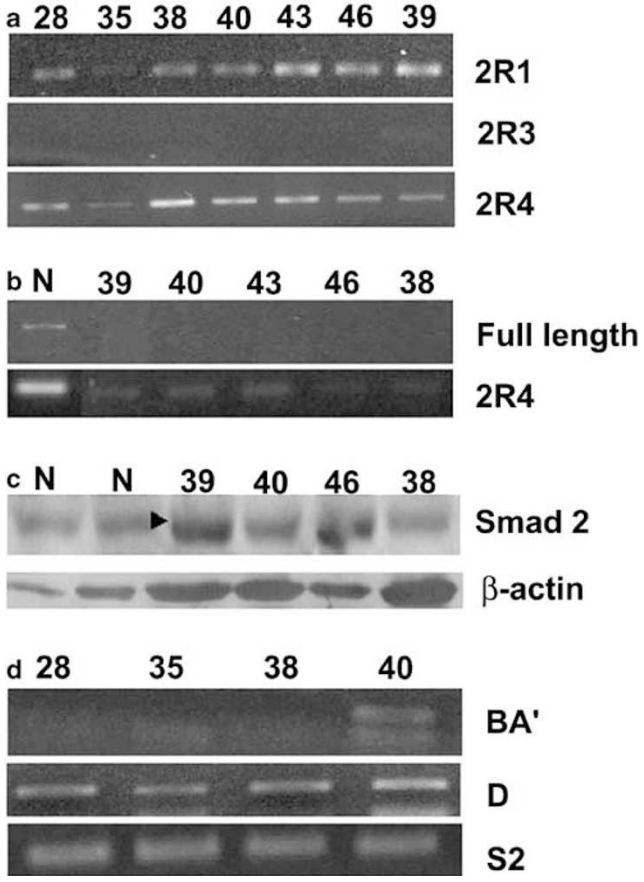

Figure 5 (**a**) SCC samples (28, 35, 38, 40, 43 and 46) and a premalignant sample (39), which showed no amplification in SQRT–PCR of the 2R3 region of *Smad 2* in Figure 4, were amplified for 2R1 and 2R4 regions of the gene, as described before, using the same cDNA mix. Three independent amplifications of all the three regions from three different cDNA mixes were used to confirm these results. (**b**) Full-length amplification of *Smad 2* was performed as described in Materials and methods. Taking the full-length product as the template, 2R4 region was amplified (lower panel) and N represents a nonmalignant sample. (**c**) Western blot of Smad 2 was carried out with the tissue samples taking *β*-actin as control and N represents the nonmalignant sample. (**d**) MH1 region of *Smad 4* was amplified by RT–PCR using B and A′ primers for the three samples that showed absence of S4 amplification in SQRT–PCR (28, 35 and 38), along with a sample that showed high expression (40). D region was amplified by PCR using genomic DNA. PCR amplification of S2 was carried out as a positive control

antisense primer being 44 bp downstream of the stop codon. Except for the nonmalignant sample, bands of expected size (1528 bp) were not observed in all the other samples (Figure 5b). Even though some low molecular weight bands below 700 bp were observed in the samples (Figure 5b lower panel), they were found to be nonspecific bands by reamplification of the internal region and sequencing with 2R4S primers (data not shown). Thus, the absence of full-length products in human cervical tumor tissue samples suggested that in those samples, the region covering 2R3AS (the C-terminal region) is deleted. However, the 5′ end of the deletion could be anywhere after 2R4AS (codon 415), and the deletion length may also vary (from codon 415 to codon 467) among samples. To see whether there are changes at the protein level, Western blot was carried out, and in three tissue samples (39, 40 and 46), a slight

shift in the position of Smad 2 (a slightly low molecular weight band) was observed (Figure 5c). These results support the possibility of the formation of a truncated protein product (maximum difference in the molecular weight expected is 5.7 kDa), predicted by the C-terminal deletion. However, in one of the samples (38), no change in molecular weight was visible; instead, the protein level was low (Figure 5c), probably due to increased protein degradation rather than loss of transcription (Figure 5a). These results suggest that the C-terminal of Smad 2 is deleted in human cervical cancer.

Absence of Smad 4 expression is due to loss of transcription of the gene

The three SCC samples that showed loss of expression of the S4 region from the MH2 domain of *Smad 4* (Table 2) were used to amplify the MH1 domain by RT–PCR using B and A′ primers (Table 1). The MH1 domain also did not amplify in samples in which there was no amplification in SQRT—PCR, whereas the sample that gave 3+ amplification (40) yielded a fragment of 147 bp in BA′ amplification (Figure 5d). Using genomic DNA, the D region of *Smad 4* was amplified along with a control amplification of S2 (*Smad 2*), and these two amplified in all the samples (Figure 5d). Although we screened 25 tissue samples and six cervical cancer cell lines for mutational status of MH1 and MH2 domains of *Smad 4* using eight different primer sets, we could not detect any sequence alteration in the coding region of the gene. Thus, the expression level of *Smad 4* was not altered in nonmalignant and premalignant (LSIL or HSIL) samples when analysed by SQRT–PCR, but in SCC, there was a decrease in the expression level of *Smad 4*. The absence of expression of MH1 and MH2 domains ruled out the possibility of intragenic deletions, and PCR amplification using genomic DNA indicated that there is no deletion of the *Smad 4* alleles. So, the absence of expression in SQRT–PCR is due to transcription loss rather than deletion of the gene.

Taken together, our results reveal two novel *Smad 2* sequence alterations, one in a human cervical cancer cell line, C-33A (a 'G' deletion at codon 428), and another from a cervical tumor sample (an insertion of 'A' in codon 122), both of which cause frame shift and pretermination. *Smad 2* expression decreased in SCC of human cervical cancer and interestingly, out of the 10 SCC samples analysed, alteration of *Smad 2* was found in seven of them, including the 10T sample showing novel mutation and six with C-terminal deletions. In addition, a G/A transition in the nontranslated regions of exon 8 of *Smad 4* in Bu 25TK cells was also observed, and the loss of expression of *Smad 4* found in some of the tumor samples is due to transcription loss rather than deletion of the gene.

Discussion

TGF-β is well known for its antiproliferative effects, and the neoplastic cells often lose their sensitivity to TGF-β (de Caestecker *et al*., 2000) and mutations of *Smads* also contribute to the TGF-β resistance in esophageal, colorectal and pancreatic cancers (Eppert *et al*., 1996; Hilgers *et al*., 2000; Tanaka *et al*., 2000; Costentin *et al*., 2002; Salovaara *et al*., 2002). The present study reveals a frame shift mutation (Arg428Ser) of *Smad 2* in C-33A as a result of deletion of 'G' in the L3 loop that determines the specificity of Smad–receptor interaction. This mutation can potentially abolish Smad 2 binding to TβRI, since Arg428 is one of the six conserved residues of the L3 loop (Lo *et al*., 1998). Furthermore, the pretermination caused by this mutation predictably results in a truncated protein, which lacks the residues for phosphorylation by TβRI (Ser465 and Ser467 of Smad 2), a key step in TGF-β signal transduction (Abdollah *et al*., 1997). The C-terminal deletion noted in this study is important, since the region harbors the site of phosphorylation that is essential not only for the function of Smad 2, but also for the nuclear accumulation of active Smads (Liu *et al*., 1997). In C-33A, there could be alterations of other components of the TGF-β pathway such as the mutation of TβRII (Kang *et al*., 1998; Chu *et al*., 1999). Presumably, another frame shift mutation detected in the present study in a cervical cancer squamous cell carcinoma sample (10T) also results in a truncated product without the MH2 domain of Smad 2 that is involved in receptor binding, phosphorylation, heteromeric complex formation and in the interaction with other transcription factors (ten Dijke *et al*., 2000). Interestingly, mutations of TβRI or TβRII alone could not explain the loss of sensitivity to TGF-β reported in human cervical cancer cell lines, suggesting that inactivation of signal mediators downstream of the receptors such as Smads could be the reason for TGF-β resistance (Kang *et al*., 1998; Chu *et al*., 1999). Consistent with the low expression of *Smad 2* in all of the tissue samples (premalignant and SCC) observed by us in human cervical cancer, loss of expression of Smad 2 was reported in head and neck squamous cell carcinoma by immunohistochemical analysis (Muro-Cacho *et al*., 2001). Homozygous deletions of *Smad 4* were reported in pancreatic cancer (Hahn *et al*., 1996) and a 9 bp deletion of *Smad 2* was reported in a human lung cancer cell line (Uchida *et al*., 1996), but in other cancers and other Smads usually the mutations reported are point mutations (MacGrogan *et al*., 1997; Miyaki *et al*., 1999; Woodford-Richens *et al*., 2001).

We have identified a sequence change in the 3′ splice acceptor site of intron 7 of *Smad 4* in Bu 25TK and splice site selection is mediated through the recognition of conserved sequences at the 5′ and 3′ splice sites, the branch point and the polypyrimidine tract (in the splice acceptor arm) by a number of proteins and U-rich small ribonucleoprotein particles (Bindereif and Green, 1987). However, we could not show that the mutation we detected could alter the splicing, as we did not observe any alternate spliced products of Smad 4 in the Western blot (data not shown). In a few cancers, *Smad 4* alterations are not involved in carcinogenesis (Kim *et al*., 1996; Schutte *et al*., 1996; Bevan *et al*., 1999; Osawa *et al*., 2000; Patel *et al*., 2002). The expression of

Smad 4 was absent or reduced in colorectal cancer (Salovaara *et al*., 2002) and correlated with the progression of colonic adenocarcinoma (Maitra *et al*., 2000) when analysed with immunohistochemistry. RT–PCR analysis showed no considerable expression variation in pancreatic cancer (Barbera *et al*., 2000) and myelogenous leukemia (Kaneko *et al*., 1998), but there was expression loss in neuroblastoma (Kong *et al*., 1997). With a tissue microarray study, Xie *et al*. (2002) have shown that loss of *Smad 4* is inversely correlated with the presence of axillary lymph node metastasis in breast cancer. Present data showing loss of *Smad 4* expression in some of the SCC samples of human cervical cancer are consistent with the inactivation of *Smad 4* associated with later stages in breast cancer (Xie *et al*., 2002), pancreatic cancer (Barbera *et al*., 2000), colorectal cancer (Salovaara *et al*., 2002) and colonic adenocarcinoma (Maitra *et al*., 2000). It is possible that alteration of *Smad* genes found in the present work may, at least in part, explain the LOH at 18q reported in cervical cancer with distant metastasis (Dellas *et al*., 1999). Further studies (with more number of samples), especially on the expression of Smads in correlation with different histological grades and clinical stages of cervical cancer, may give a better picture on the role of Smads during cervical tumor progression. Nevertheless, our results do highlight an important role for Smads 2 and 4 in human cervical tumors.

Abbreviations

TGF-β, transforming growth factor-β; TβRI, transforming growth factor receptor type I; TβRII, transforming growth factor receptor type II; RT–PCR, reverse transcription–PCR; SQRT–PCR, semiquantitative reverse transcription–PCR; EMSA, electrophoretic mobility shift assay; SSCP, single-strand conformation polymorphism; CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma.

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