Expression of Smad2 and Smad4 in cervical cancer: absent nuclear Smad4 expression correlates with poor survival

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Alterations in transforming growth factor- β signaling, due to a decrease in Smad2 and especially Smad4 expression, has primarily been reported in pancreatic and colorectal cancers, although loss of the chromosomal region 18q21.1, containing the loci of *Smad2* and *Smad4*, is among the most frequent molecular alterations in cervical cancer. The aim of our study was to investigate whether decreased Smad2 and Smad4 protein expression in primary cervical cancers is associated with molecular alterations at 18q21.1, mutations in the functional domains of *Smad2* and *Smad4* or hypermethylation, and to assess the biological relevance of decreased Smad2 and Smad4 expression. Subsequently, Smad2, Smad4 and p21 protein expression was determined by immunohistochemistry in 117 primary cervical carcinomas, assembled in a tissue array. Smad signaling was shown to be associated with p21 mRNA expression. All the tumors expressed Smad2 or Smad4. Weak cytoplasmic Smad2 or weak cytoplasmic Smad4 expression could not be attributed to loss of heterozygosity at 18q21.1. Despite weak/moderate Smad2 expression and absent nuclear Smad4 expression, the coding regions of the functional MH1 and MH2 domains of *Smad2* and *Smad4* were unchanged, as assessed by sequence analysis. The *Smad4* promoter region was unmethylated in tumor samples with weak/moderate cytoplasmic Smad4 expression. Remarkably, both weak cytoplasmic Smad4 expression and absent nuclear Smad4 expression significantly correlated with poor disease-free ($P=0.003$ and $P=0.003$, respectively) and overall 5-year survival ($P=0.003$ and $P=0.010$, respectively). Our findings support the hypothesis that Smad4 is a target molecule for functional inactivation in cervical cancer.

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Cervical cancer is caused by persistent infection with high-risk human papillomavirus (HPV). In addition to infection with HPV, molecular alterations of tumor suppressor genes and/or oncogenes are imperative for the development of cervical cancer. A characteristic molecular alteration associated with malignant progression is the loss of responsiveness to transforming growth factor- β 1 (TGF- β 1)-induced cell growth inhibition.^{1,2} TGF- β 1 acts as a double-edged sword; early in tumorigenesis, it operates as a tumor suppressor by inhibiting epithelial cell proliferation and, later in tumorigenesis, it operates as an angiogenic factor and suppresses an efficient anti-tumor immune res-

ponse. Loss of TGF- β 1 responsiveness is thought to be due to alterations in the TGF- β 1 signal transduction route.³ A mechanism by which loss of the tumor suppressor function of TGF- β can occur is through inactivation of *Smad* genes. As transcription factors, Smads are important for inducing the expression of cell cycle inhibitors, such as p15, p21 and p27, thereby facilitating TGF- β -mediated inhibition of cell growth.⁴

Loss of heterozygosity (LOH) at 18q, the locus of *Smad2* and *Smad4*, is a common mechanism for inactivation of *Smad4* in pancreatic tumors.⁵ In cervical carcinomas, LOH at 18q was observed in 10–37% of cases and has been associated with poor prognosis.^{6,7} In addition to LOH, mutations in *Smad4* in pancreatic cancer and colorectal cancer, resulting in loss of expression or function, have been frequently reported.^{5,8} Also, *Smad2* mutations have been found in colorectal cancer.⁹ For cervical cancer, *Smad4* mutations were reported in cervical cancer

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cell lines¹⁰ and a *Smad2* mutation in a cervical tumor has been described.¹¹ In addition to LOH and gene mutations, other mechanisms such as CpG methylation of the *Smad* promoter may play a role in downregulation of Smad expression. Recently, *Smad4* promoter methylation was reported in Barrett's esophagus and gastric carcinoma as a mechanism for decreased Smad4 expression.^{12,13}

The aim of our study was to investigate whether decreased Smad2 and Smad4 protein expression in primary cervical carcinomas was associated with molecular alterations at 18q21.1, mutations in the functional domains of *Smad2* and *Smad4* or hypermethylation, and to assess the biological relevance of decreased Smad2 and Smad4 expression.

For this purpose, we determined protein expression of Smad2, P-Smad2, Smad4 and p21 by immunohistochemistry on a tissue array of 117 primary cervical cancers. Subsequently, the association between Smad2 and Smad4 protein expression and molecular alterations was investigated by determining LOH status at 18q21.1 (*Smad2* and *Smad4* loci), the occurrence of mutations in the functional domains of *Smad2* and *Smad4* and CpG methylation of the *Smad4* promoter. Furthermore, we assessed the biological relevance of our findings, we correlated the experimental results with clinicopathological parameters.

Materials and methods

Patient Material

From 117 patients with cervical squamous carcinoma who underwent a radical hysterectomy combined with pelvic lymph adenectomy between 1985 and 2000, formalin-fixed paraffin-embedded tissue blocks were retrieved from the Department of Pathology, Leiden University Medical Center. None of the patients had received radio- or chemotherapy before surgery. The 117 primary cervical carcinomas consisted of 90 squamous cell carcinomas, 19 adenocarcinomas and 8 adenosquamous carcinomas. The use of clinical material was approved by the institutional review board, according to the guidelines of the Dutch Federation of Medical Research Associations. The clinical records of the women, all treated by the same three gynecologic oncologists of the Department of Gynecology, Leiden University Medical Center, with a radical hysterectomy type III for carcinoma of the uterine cervix between 1985 and 2000, were reviewed. The slides of all tumors were examined, using conventional histological sections stained with hematoxylin and eosin, by a trained pathologist.

Immunohistochemistry

Immunohistochemistry on a tissue array was performed on 4 μ m sections using aminopropylethoxysilane-coated slides. A representative tumor area was

selected by an experienced pathologist. Subsequently, three cores of each tumor were included in the tissue array. Standard immunohistochemical staining was performed as described previously.¹⁴ In the case of immunohistochemistry with the Smad2, P-Smad2 and Smad4 antibodies, antigen retrieval was performed with 0.01 M citrate buffer. mAb directed against Smad4 (clone B8; dilution 1:200; Santa Cruz, Santa Cruz, CA, USA),¹⁵ P-Smad2 (clone 138D4, dilution 1:50; Cell Signaling Technology, Beverly, CA, USA)¹⁶ and p21 (clone EA10, dilution 1:200; Calbiochem, San Diego, CA, USA)¹⁷ and a polyclonal antibody against Smad2 (dilution 1:200; Santa Cruz)¹⁸ were used. The cellular infiltrate provided an internal positive control (for Smad2 and Smad4). Immunohistochemistry was scored as described previously.¹⁹ The intensity of cytoplasmic staining was scored as weak, moderate or strong at low magnification ($\times 100$). In the case of nuclear staining, the percentage of positive tumor cells was determined and divided into three groups: nuclear staining in 0% of tumor cells (absent), nuclear staining in $\geq 0\%$ of tumor cells and $\leq 50\%$ of tumor cells (low) or nuclear staining in $\geq 50\%$ of tumor cells (high). Expression was scored by two independent researchers without knowing the clinical outcome of patients.

Genomic DNA Isolation from Flow-Sorted Tumor Cell Subpopulations

Formalin-fixed, paraffin-embedded cervical carcinomas were trimmed if necessary to remove normal epithelium and cervical intraepithelial neoplasia. The remaining tumor tissue was cut into 50 μ m sections, dewaxed and pretreated with 10 mM sodium citrate buffer (pH 6.4) as antigen retrieval. Dissociation of tissue was performed in a mixture of 0.1% collagenase I-a and 0.1% dispase in RPMI medium without FCS and antibiotics for 30–120 min at 37°C.²⁰ The cells were stained for keratin, vimentin and DNA as described previously.²⁰ Anti-vimentin antibodies applied were 3B4 (2 μ g/ml; DAKOCytomation, Glostrup, Denmark) and V9-2b (supernatant diluted 1:5). Monoclonal antibodies used against keratins included MNF116 (2 μ g/ml; DAKOCytomation) and Pan-keratin AE1/AE3 (5 μ g/ml; Chemicon International Inc., Temecula, CA, USA). The vimentin-negative keratin-positive (V–K+) fraction, which represents the epithelial cell subpopulation, and vimentin-positive keratin-negative (V+K–) fraction, consisting of normal cells, were flow-sorted using a FACS Vantage (BD Biosciences, San Jose, CA, USA). The number of sorted cells varied from 50 000 to 1 000 000 between samples. DNA was extracted as described previously.²¹

Microsatellite Analysis

Seven Hex-labeled primer pairs of microsatellite markers comprising D18s877, D18s65, D18s460,

D18s1137, D18s1118, D18s1109 and D18s844 (Genome Database, <http://www.gdb.org>) at chromosome 18q were used. The nearest marker for Smad2 is D18S1137 at 0.2 Mb distance and for Smad4, D18S1118 at 2.6 Mb. DNA, isolated from sorted tumor cell subpopulations, was used as source material.²⁰ PCR, electrophoresis and analysis were performed as described previously.²² A reduction of 50% of one allele, equating to a ratio of ≥ 2 or ≤ 0.5 , was considered as LOH. A reduction of 25% or more (1.5–2 and 0.75–0.5) of one allele was considered as allelic imbalance.

Mutation Analysis

Primers designed for amplification of the complete coding region of MH1 and MH2 domains of *Smad2* and *Smad4*, applicable for paraffin material as template, are listed in Supplementary Tables 1 and 2. The following PCR conditions were used: 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 53°C and 30 s extension at 72°C. Sequencing was performed by the Leiden Genome Technology Center (Leiden, The Netherlands). The obtained sequences were compared with DNA sequences of autologous normal material and with the corresponding sequences from the Genome Database (<http://www.gdb.org>).

Methylation Assay

For the detection of CpG methylation in cell cultures, cervical cancer cell lines and sorted tumor cell subpopulations, the bisulfite method was applied for DNA methylation analysis using the EZ DNA Methylation Kit (ZYMO Research, Orange, CA, USA) according to the manufacturer's protocol. Before conversion with the bisulfite method, DNA from sorted samples was purified and concentrated with Wizard DNA clean-up (Promega, Madison, WI, USA). DNA input for the conversion reaction was 500 ng/sample. Primers designed for PCR and sequencing of bisulfite-converted DNA, encompassing the promoter region and exon 1 of *Smad4*, were as follows: 5'-GGTTGGGTAAATAGTTATTATA (FWD) and CTCTAAATCTAAACCTAAACCTA (REV), 5'-TAGGTTTATAGTTTATAGATTAGAG (FWD) and AATTTAACCCCTTTTCCCCC (REV), 5'-GGGGGAA AAGGGTTAAATT (FWD) and CTAAACACCTAAC TCCCCTC (REV).

Statistical Analysis

Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Data were processed using the Chi-squared test. Kaplan–Meier survival curves were generated to assess differences in disease-free survival (defined as the time in months from surgery to relapse of the disease) or cumulative overall survival (defined as the time in months from surgery to death due to cervical cancer). A Cox regression was used for univariate and multivariate survival analyses. $P < 0.05$ was considered statistically significant.

Results

Smad2, P-Smad2, Smad4 and P21 Protein Expression in Primary Cervical Carcinomas

Protein expression of Smad2, P-Smad2, Smad4 and p21 was evaluated by immunohistochemistry in a tissue array containing 117 primary cervical carcinomas. Because of its nuclear localization p21 was chosen as the target product for TGF- β signaling.

All tumors showed a homogeneous cytoplasmic staining pattern for Smad2 and Smad4 that varied in intensity (Figure 1 and Table 1). For the analysis of cytoplasmic staining results, the three categories from Table 1 were combined into two categories (weak vs moderate/strong). Weak cytoplasmic Smad2 staining intensity correlated significantly with weak cytoplasmic Smad4 staining intensity ($P = 0.050$). All tumors showed nuclear staining for P-Smad2 (Table 1). Fifteen out of 111 tumors lacked nuclear staining for Smad4 (14%) and 15 out of 114 tumors lacked nuclear staining for p21 (13%). P-Smad2 consisted of two groups: a group with less than 50% of positive tumor cells (low) and a group with more than 50% of positive tumor cells (high). To analyze the results of the nuclear staining pattern, nuclear Smad4 and p21 were divided into a group without nuclear staining (absent) and a group with nuclear staining. The P-Smad2 nuclear staining pattern (1–50%) showed a significant correlation with the absence of p21 staining ($P = 0.011$). Although not significant, association between absence of nuclear Smad4 and absence of p21 ($P = 0.051$) was observed. In addition, a significant association between weak cytoplasmic Smad4 staining intensity and absent Smad4 nuclear staining was observed ($P = 0.001$).

Figure 1 Expression of Smad2, P-Smad2, Smad4 and p21 proteins in primary cervical carcinomas. A tissue microarray of 117 cervical tumors was stained using immunohistochemistry as described in Materials and methods ($\times 125$ magnification). (a) Smad2, strong cytoplasmic staining, (b) Smad2, moderate cytoplasmic staining, (c) Smad2, weak cytoplasmic staining, (d) positive nuclear staining of P-Smad2 in $\geq 50\%$ of tumor cells, (d1) panel d in detail ($\times 400$ magnification), (e) P-Smad2 nuclear staining in $\leq 50\%$ of tumor cells, (e1) panel e in detail ($\times 400$ magnification), (f) Smad4, positive nuclear staining in $\geq 50\%$ of cells, (f1) panel f in detail ($\times 400$ magnification), (g) Smad4, strong cytoplasmic intensity and absent nuclear staining, (g1) panel g in detail ($\times 400$ magnification), (h) Smad4, moderate cytoplasmic intensity, (i) Smad4, weak cytoplasmic intensity, (j) p21 nuclear staining in $\geq 50\%$ of tumor cells, (j1) panel j in detail ($\times 400$ magnification), (k) p21 nuclear staining in ≥ 0 and $\leq 50\%$ of tumor cells, (k1) panel k in detail ($\times 400$ magnification), (l) absent p21 nuclear staining and (l1) panel l in detail ($\times 400$ magnification).

Table 1 Differential expression of Smad2, P-Smad2, Smad4 and p21 proteins in cervical cancers

Nuclear	Percentage of positive tumor cells		
	0 (absent) N	1–50 (low) N	51–100 (high) N
P-Smad2	0 (0)	23 (20)	93 (80)
Smad4	15 (13)	71 (64)	25 (23)
p21	15 (15)	94 (90)	5 (5)
Cytoplasmic	Intensity ^a		
	Weak N	Moderate N	Strong N
Smad2	37 (33)	58 (52)	17 (15)
Smad4	40 (36)	53 (48)	18 (16)

Expression of Smad2, P-Smad2, Smad4 and p21 was measured by immunohistochemistry as described in Materials and methods. N, number of tumors. (%), percentage of tumors displaying either a nuclear or a cytoplasmic staining pattern for P-Smad2, Smad2, Smad4 or p21.

^aStaining intensity (weak, moderate or strong).

LOH of the *Smad2* and *Smad4* Loci at 18q21.1

To establish the basis for weak Smad2 and weak Smad4 protein expression, we investigated whether this was due to haplo-insufficiency of the *Smad2* or *Smad4* genes caused by LOH. For LOH analysis, DNA was used from a flow-sorted tumor cell subpopulation (keratin positive, vimentin negative; K+V-) to prevent contamination by either stromal or inflammatory cells. LOH at 18q21.1 was determined in 46 tumors with low, moderate and strong expression of either Smad2 or cytoplasmic Smad4 (Figure 2b). High allelic imbalance ratios were frequently observed, often ranging from 2 to 10, representing complete LOH. Out of the 46 cases, 19 cases (41%) showed loss at markers flanking the *Smad2* and *Smad4* loci (allelic imbalance ≥ 1.5). Loss of the complete *Smad2* and *Smad4* loci was noticed in 14 cases (30%). Eleven out of these 14 cases seemed to have lost the complete chromosome arm. No significant association between Smad2 or Smad4 protein expression and LOH at 18q21.1 was apparent.

Mutation Analysis of the Functional MH1 and MH2 Domains of *Smad2* and *Smad4*

Subsequently, we investigated whether mutations in the function-associated MH1 and MH2 domains of *Smad2* or *Smad4* were the underlying cause for their reduced expression. For this purpose, five tumors with a weak and five tumors with a moderate cytoplasmic Smad2 staining pattern were selected (all the tumors selected displayed less than 50% of P-Smad2-positive tumor cells). For Smad4, eight tumors with a weak and two tumors with a moderate cytoplasmic Smad4 staining pattern were selected

(without nuclear Smad4 staining). PCR amplification and sequence analysis of DNA from the flow-sorted tumor cell subpopulation (V-K+) as well as DNA from the flow-sorted stromal cell/inflammatory cell subpopulation (V+K-) as a control were performed. No mutations in the MH1 and MH2 domains of *Smad2* and *Smad4* were observed (data not shown).

Methylation of the Promoter Region and Exon 1 of *Smad4*

Because of the lack of a significant association between Smad2 or Smad4 expression and LOH at 18q21.1, and the absence of mutations in the MH1 and MH2 domains of *Smad2* and *Smad4*, we investigated whether methylation of the promoter region could explain the reduced protein expression. We focused on *Smad4* promoter methylation, as it has been previously described as a tumor suppressor mechanism.^{12,13} Four cervical cancer cell lines (HeLa, CaSki, CC-8 and CC-11) and four normal primary cervical epithelial cell cultures, both expressing the complete coding region of *Smad4* (as determined by RT-PCR), were sequenced after bisulfite conversion to determine the CpG methylation status. Also, DNA from five flow-sorted tumor cell populations with weak and five flow-sorted tumor cell populations with moderate Smad4 cytoplasmic staining was analyzed for CpG methylation status. The region up to 300 bp upstream from the transcription start point, containing dense CpG islands, and 125 bp downstream from the transcription start, which includes exon 1, was sequenced (Supplementary Figure 1). None of the CpG sites investigated was methylated (data not shown).

Association between Smad2, Smad4 and P21 Expression and Clinico-pathological Parameters

Despite the unknown nature of decreased Smad2 and cytoplasmic Smad4 expression, we investigated whether the expression pattern of Smad2, P-Smad2, cytoplasmic Smad4, nuclear Smad4 and p21 correlated with clinico-pathological parameters (Table 2).

Weak cytoplasmic Smad4 staining intensity strongly correlated with the presence of positive lymph nodes ($P=0.002$) and recurrent disease ($P=0.009$). Absent nuclear Smad4 staining correlated with tumors ≥ 40 mm ($P=0.020$), infiltration depth ≥ 15 mm ($P=0.036$) and the presence of HPV18 ($P=0.020$). Both weak Smad2 expression and P-Smad2 expression ($<50\%$ of tumor cells) were not significantly associated with any of the clinico-pathological parameters tested.

Because of the significant correlation between a weak cytoplasmic Smad4 staining intensity and lymph node metastases and recurrent disease, we also assessed the association between the Smad protein expression patterns and disease-free and

Figure 2 LOH at 18q21.1 in primary cervical carcinomas. **(a)** Microsatellite markers surrounding *Smad2* and *Smad4* genes at chromosome arm 18q. **(b)** LOH at 18q of 46 samples was measured using seven microsatellite markers as described in Materials and methods. For each tumor, *Smad2* and cytoplasmic *Smad4* staining intensity is depicted as w: weak, m: moderate and s: strong. A.I., allelic imbalance.

Table 2 Significant association between weak cytoplasmic Smad4, absent nuclear Smad4 expression and clinico-pathological parameters

Clinico-pathological characteristics	Smad4					
	Cytoplasmic		P	Nuclear		P
	Weak N	Moderate/strong		Absent N	Present	
<i>Lymph node</i>	108			109		
Positive	17 (61)	11 (39)	0.002	6 (21)	22 (79)	0.115
Negative	22 (28)	58 (72)		8 (10)	73 (90)	
<i>Recurrent disease</i>	100			100		
Yes	15 (40)	23 (60)	0.009	6 (24)	19 (76)	0.059
No	23 (31)	52 (69)		7 (9)	68 (91)	
<i>Tumor size</i>	103			104		
<40 mm	18 (29)	45 (71)	0.051	5 (8)	58 (92)	0.020
≥40 mm	19 (48)	21 (52)		10 (24)	31 (76)	
<i>Depth of infiltration</i>	109			110		
<15 mm	19 (30)	45 (70)	0.070	5 (8)	59 (92)	0.036
≥15 mm	21 (47)	24 (53)		10 (22)	36 (78)	
<i>HPV type</i>	80 ^a			80		
16	20 (36)	36 (64)	0.614	5 (9)	51 (91)	0.020
18	10 (42)	14 (58)		7 (29)	17 (71)	

N, number of tumors displaying either a cytoplasmic Smad4 or nuclear Smad4 staining pattern for the clinico-pathological parameters provided.

^aOnly data for cervical carcinoma samples positive for HPV16 and HPV18 were included.

Bold values were considered statistically significant.

overall 5-year survival. Indeed, weak cytoplasmic Smad4 staining intensity correlated with both poor disease-free survival ($P=0.003$; Figure 3a) and poor overall survival ($P=0.003$; Figure 3b). Also the absence of nuclear Smad4 staining correlated with poor disease-free survival ($P=0.010$; Figure 3c) and poor overall survival ($P=0.003$; Figure 3d).

Subsequently, we used the Cox proportional hazards model to calculate the predictive value for a shorter disease-free and overall 5-year survival of each of these parameters. Both weak cytoplasmic Smad4 expression and absent nuclear Smad4 expression were demonstrated to be prognostic factors for disease-free and overall 5-year survival (Table 3).

Furthermore, in a multivariate Cox analysis that included the four strongest prognostic factors according to univariate Cox analysis (lymph node status, tumor size, vaso-invasion and depth of infiltration),²³ in addition to lymph node status, absent nuclear Smad4 staining was found to be the only independent prognostic factor for both disease-free (hazard ratio of 2.91) and overall 5-year survival (hazard ratio of 2.74).

Discussion

Loss of responsiveness to TGF- β -induced cell growth inhibition during cancer development is a feature of many tumors, including cervical cancer.¹

Although a number of studies have investigated the expression status and alterations in the canonical TGF- β signaling (Smad) pathway, the role of Smad signaling in the progression of cervical cancer remains unclear.^{3,10,11,24} In this study, we investigated whether decreased Smad2 and Smad4 protein expression in primary cervical cancers was associated with molecular alterations at 18q21.1, mutations in the functional domains of *Smad2* and *Smad4* or hypermethylation, and we assessed the biological relevance of decreased Smad2 and Smad4 expression.

Previously, Smad4 deficiency has been reported in 4 out of 13 cervical cancer cell lines.¹⁰ It should be noted that of these cell lines, one cell line was derived from a lymph node metastases (HT-3) and two other cell lines (C4-I and C4-II) were derived from the same patient. The origin of the fourth cell line was not specified. In contrast to Baldus *et al*,¹⁰ who reported a Smad4 deficiency in 10 out of 40 invasive cervical carcinomas, we did not observe tumors negative for Smad4 staining. There is no clear explanation for this difference. Baldus *et al* did not report the FIGO stage of the investigated tumors, whereas in our study only FIGO stage IB and IIA cervical carcinomas were investigated. Conceivably, differences in Smad4 expression may occur as late events during tumor progression. The expression of P-Smad2, nuclear Smad4 or p21 in the majority of the cervical carcinomas investigated suggests that

Figure 3 Association between cytoplasmic and nuclear Smad4 expression and survival in primary cervical carcinomas. Kaplan–Meier survival curves were generated to assess differences in disease-free survival (defined as the time in months from surgery to relapse of the disease) or cumulative overall survival (defined as the time in months from surgery to death due to cervical cancer) as described in Materials and methods. **(a)** Disease-free 5-year survival in patients with weak cytoplasmic Smad4 staining intensity (N=38) and moderate/strong intensity (N=63). **(b)** Overall survival in patients with weak cytoplasmic Smad4 intensity (N=40) and moderate/strong intensity (N=70) in primary cervical carcinomas. **(c)** Disease-free survival in patients with absent (N=13) and present (N=88) Smad4 nuclear staining. **(d)** Overall survival in patients with absent (N=15) and present (N=96) Smad4 nuclear staining.

the canonical TGF- β signal pathway is operative. However, in cases with a limited amount of cytoplasmic Smad4, the amount of Smad4 may be rate limiting.

Loss or decrease in Smad protein expression can be due to genetic alterations such as LOH and inactivating mutations.⁵ We found a relatively high frequency (41%) of LOH at 18q21.1, the locus of *Smad2* and *Smad4* genes, compared to other studies (10–37%).^{6,7} The close proximity of the markers to

the *Smad2* and *Smad4* loci in combination with the purity of the tumor samples may explain this increased frequency. For LOH analysis, we used DNA samples from flow-sorted cancer samples. The advantage of flow sorting over other techniques, such as laser microdissection, is the lack of contaminating stromal cells or cells from the immune system in the tumor sample. As LOH at 18q21.1 did not associate with weak Smad2 or weak Smad4 expression, genetic haplo-insufficiency of

Table 3 Prognostic value of weak cytoplasmic Smad4 and absent nuclear Smad4

Parameter	Limits	DFS			Overall survival		
		HR	95% CI	P	HR	95% CI	P
Univariate Cox analysis							
Lymph nodes	Positive vs negative	7.16	3.16–16.25	0.001	5.69	2.53–12.82	0.001
Infiltration depth	<15 vs ≥15 mm	3.64	1.57–8.44	0.003	3.96	1.64–9.55	0.002
Tumor size	<40 vs ≥40 mm	4.24	1.79–10.03	0.001	7.90	2.92–21.37	0.001
Vaso-invasion	Positive vs negative	3.41	1.51–7.70	0.003	2.76	1.24–6.17	0.013
Smad4 cytoplasmic	Weak vs moderate/strong	3.23	1.41–7.38	0.006	3.45	1.44–8.23	0.005
Smad4 nuclear	Absent vs present	3.15	1.25–7.96	0.015	3.61	1.48–8.88	0.005
Multivariate Cox analysis							
Lymph nodes	Positive vs negative	4.00	1.40–11.45	0.010	4.40	1.55–12.51	0.006
Tumor size	<40 vs ≥40 mm	2.37	0.91–6.15	0.078	5.31	1.88–14.98	0.002
Vaso-invasion	Positive vs negative	1.50	0.59–3.81	0.399	0.65	0.54–3.37	0.520
Infiltration depth	<15 vs ≥15 mm	1.80	0.59–5.53	0.305	4.15	0.64–5.32	0.273
Smad4 nuclear	Absent vs present	2.91	1.08–7.89	0.035	2.74	1.07–6.78	0.035

CI, confidence interval; DFS, disease-free survival; HR, hazard ratio.
Bold values were considered statistically significant.

Smad2 and Smad4 as reported by Baldus *et al*¹⁰ is not supported by our data.

Only two studies have reported on mutations in the functional domains (MH1 and MH2) of *Smad2* and *Smad4* in cervical carcinoma.^{10,11} Malieka *et al*¹¹ identified a missense mutation in the MH1 domain of *Smad2* in a cervical tumor and reported lack of mRNA expression of the C-terminal part of the MH2 domains in six tumors. We did not detect mutations in the MH1 and MH2 domains of *Smad2* in the tumor samples; therefore, it remains uncertain whether cervical tumors contain inactivating mutations in the MH2 domain. Nevertheless, we cannot exclude the presence of less frequently occurring mutations outside the MH1 and MH2 domains of *Smad2* and *Smad4*.

Baldus *et al*¹⁰ reported deletions in part of the function-associated MH1 and MH2 domains of *Smad4* in four cervical cancer cell lines. In agreement with Malieka *et al*, in our study we did not observe mutations in MH1 and MH2 domains of *Smad4* in cervical tumors. However, our approach does not exclude the presence of possible missense mutations. Our data suggest that genetic inactivation of *Smad2* and *Smad4* plays a minor role in cervical cancer.

Another mechanism by which Smad expression could be downregulated is promoter methylation. In our study, similar to the study of Wang *et al*¹³ in gastric carcinoma, we investigated both the promoter region and exon 1 of *Smad4*. No hypermethylation of the promoter region or exon 1 of *Smad4* was observed in normal samples, cervical cancer cell lines and tumor samples. Also, in colorectal cancers, no hypermethylation of *Smad4* was observed, whereas in Barrett's esophagus, a weak correlation between mRNA expression and promoter methylation was found.^{12,25,26} In gastric tumors, hypermethylation was rare. In this tumor type, a significant correlation was reported between hypermethylation and loss of protein expression.

Weak protein expression of Smad2 and Smad4 in cervical cancer could also be due to other regulatory processes, such as interaction of HPV16 E7 with Smad2, Smad3 and Smad4,²⁷ degradation by a proteasome¹³ or activation of specific ubiquitin-Smad E3 ligases.^{28,29}

In our series, weak cytoplasmic Smad4 staining and the absence of Smad4 nuclear staining was associated with poor survival in cervical cancer patients. In a previous study, our group found an association between LOH at 18q and survival.⁷ Here, we did not find this association. The differences in the results could be explained by much closer proximity of the chosen markers to the *Smad2* and *Smad4* loci in the present study. In our study, weak nuclear Smad4 expression was found to be an independent prognostic factor for both disease-free and overall survival. Loss of nuclear Smad4 expression in gastric cancer also correlated with poor survival.¹³ In pancreatic cancer, Smad4 protein expression was an independent predictor for longer survival.³⁰ In colorectal and breast cancers, loss of Smad4 protein expression correlated with negative outcome or the presence of lymph node metastasis.^{31,32} These data emphasize the importance of the integrity of the canonical TGF- β pathway in carcinogenesis. Our findings support the hypothesis that Smad4 is a target molecule for functional inactivation in cervical cancer.

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Disclosure/conflicts of interest

The authors state no conflicts of interest.

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