SMAD4/DPC4 Expression and Prognosis in Human Colorectal Cancer

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Abstract. *Background: Colorectal cancer (CRC) is one of the most common malignancies in Western countries. One major event during CRC development is loss of genetic material on chromosome 18, on which the smad4/dpc4 gene is located. SMAD4 is an important mediator of intracellular signaling in the TGF-β pathway. The functional inactivation of SMAD4 has been reported to occur in CRC. Materials and Method: The protein expression of SMAD4 was evaluated immunohistochemically in 86 formalin-fixed and paraffin-embedded CRC samples. The results were related to clinicopathological variables including survival. Results: The loss of nuclear SMAD4 protein expression was observed in 9.3% of the investigated CRCs and was correlated to poor prognosis in univariate Kaplan-Meier (p=0.034) as well as in multivariate Cox-regression (p=0.028) analyses. Conclusion: The loss of nuclear SMAD4 protein expression occurs in a subset of CRC and is associated with poor prognosis.*

Colorectal cancer (CRC) is one of the most common malignancies in Western countries. Approximately 50% of CRC patients die from their disease, a figure that has not changed substantially over the last 50 years (1). This emphasizes the need for a greater understanding of the molecular mechanisms contributing to CRC development and progression. CRC is the result of a long process and the progression model from normal colonic mucosa through hyperplastic polyps and adenomas to neoplasia is characterized by a number of genetic events (2). Multiple genetic hits must occur in the same cell or its descendants and one of the major events appears to be the loss of genetic material on chromosome 18q, typically found in late adenomas and carcinomas (2).

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Recently, the *dpc4* gene, also known as the *SMAD4* gene and a member of the *SMAD* gene family, was identified on chromosome 18q21 (3). *SMAD* genes encode intracellular mediators of the TGF-β signaling pathway. Activation of the TGF-β receptor by ligand binding results in the phosphorylation of the receptor-activated SMADs (*i.e.,* SMAD2 and 3) and promotes their interaction with co-SMADs (SMAD4). Interaction of the receptor-activated SMADs with SMAD4 is required for the heteromeric SMAD complex to enter the nucleus and affect signaling, indicating that SMAD4 is a critical TGF-β mediator and a potential rate-limiting factor in the signaling cascade (4).

The TGF-β pathway is involved in the regulation of a variety of cellular processes, such as proliferation, differentiation, motility and apoptosis and generally functions as a negative regulator of cell growth (5). The loss of TGF-β-induced growth inhibition is often observed in tumor cells (6) and reduced responsiveness to TGF-β is an important event in colorectal carcinogenesis (7). The resistance to TGF-β-mediated growth inhibition in CRC is associated with mutations in either the TGF-β receptor, or in the signal transducers, the *SMAD* gene family (8). The functional inactivation of SMAD4 in CRC has been suggested to occur at late stages when tumors acquire invasive and metastatic potential (9).

Given the importance of the TGF-β signaling pathway in colon carcinogenesis as well as the critical role of SMAD4 as a TGF-β mediator, an immunohistochemical approach was used to investigate the prognostic impact of nuclear SMAD4 expression in clinical material of CRC.

Materials and Methods

Patients and tissue specimens. Included in this study were 107 cancer specimens, originally collected for standard pathological diagnostics from patients undergoing potentially curative surgery of CRC at the Department of Surgery, Umeå University Hospital in Umeå, Sweden. Twenty-one out of the 107 specimens were excluded due to repetitive tissue loss during the antigen retrieval procedure or lack of positive staining in internal control stromal

cells, leaving 86 cases for analysis. The tumor samples used in this study were collected between 1987 and 1994, before adjuvant chemotherapy was established as routine treatment.

Immunohistochemical staining procedure. The specimens were fixed in 4% formaldehyde and embedded in paraffin, according to routine procedures at the Department of Clinical Pathology, Umeå University Hospital. One 4-μm section from each specimen was cut, dried, dewaxed and rehydrated before microwave treatment in citrate buffer (pH 6.0) for 3 x 5 min. A semi-automatic staining machine (Ventana ES; Ventana Inc., Tucson, AZ, USA) was used for the immunohistochemical procedures. A primary anti-SMAD4 monoclonal antibody (SMAD4 B-8; sc-7966; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was applied at a concentration of 1:50. Bound antibody visualization was performed according to the Ventana program, using a secondary anti-mouse antibody and amino-ethylcarbazole (AEC) as the chromogen. The slides were counterstained with hematoxylin.

Evaluation of immunohistochemical staining. The immunohistochemically-labelled slides were interpreted using conventional light microscopy and the fraction of cells staining positively in their nucleus was semi-quantitatively evaluated using a 4-grade scale: (–) <5%, (+) 5-10%, (++) 11-35%, (+++) >35% (10). For cells to be classified as SMAD4-negative, they had to show no immunoreactivity in their nuclei. In order to have an internal positive control, the stromal cells in the proximity had to show a clearly positive immunoreaction and cases lacking staining in the stromal internal control cells were excluded.

The slides were independently interpreted twice and intra-observer disagreements were reviewed a third time followed by a conclusive judgment. The same observer performed all the evaluations.

Statistics. In a cross tabulation, the expression of SMAD4 in relation to clinicopathological variables was analyzed using the Fisher's exact test. The Kaplan-Meier survival analysis was used to estimate the cancer-specific survival and comparisons between groups were performed using the log-rank test. SPSS statistical software version 12.0.1. was used for all statistical analyses and *p*-values <0.05 were considered statistically significant.

Results

A total of 86 clinical specimens were stained for SMAD4 and the fraction of positively-stained tumor nuclei was estimated over the whole section using a 4-grade scale spanning from negative (–) to strongly positive (+++). To control for potential unspecific staining variability between specimens, SMAD4 staining in tumor cells was compared to the SMAD4 staining observed in adjacent stromal cells. Eight (9.3%) tumors were classified as SMAD4-negative (–), 21 (24.4%) as weakly-positive (+), 48 (55.6%) as moderately-positive (++) and 9 (10.5%) as strongly-positive (+++). SMAD4 expression was not significantly related to any of the clinico-pathological variables (Table I).

The presence of nuclear SMAD4 is indicative of active TGF-β signaling, whereas a loss of SMAD4 staining reflects genetic alterations in the *SMAD4* gene or in genes required for SMAD4 expression and/or function (11). Thus, loss of SMAD4 staining would ultimately be expected to result in a blunted TGF-β response. Therefore, the prognosis of SMAD4-negative cases was compared with that of all SMAD4-positive cases, regardless of the staining indices. As depicted in Figure 1, SMAD4-negative cases had a poor prognosis compared to patients in the remaining 3 categories of SMAD4 immunoreactivity ($p=0.034$). Seven out of the 8 SMAD4-negative cases were women. Although not statistically significant, the men had a worse outcome than the women (Table II).

Table I. *Expression of SMAD 4 in relation to clinicopathological characteristics.*

Variable	SMAD4 expression				*p* value
	–	+	++	+++	
Gender					
Male	1	11	26	4	0.18
Female	7	10	22	5	
Age, years					
≤59	0	7	3	2	0.60*
60-69	2	2	17	1	
70-79	4	4	17	5	
80-	2	8	11	1	
Localization					
Right colon	3	8	21	3	0.92
Left colon	3	8	15	2	
Rectum	2	5	12	4	
Stage					
Dukes' A	0	3	7	1	0.079
Dukes' B	2	9	31	4	
Dukes' C	6	9	10	4	
Differentiation					
Well	0	2	2	0	0.46
Moderate	7	18	39	8	
Poor	1	0	1	0	
Tumor type					
Mucinous	3	3	11	2	0.61
Non-mucinous 5	17	30	6		
Growth pattern					
Pushing	3	11	28	4	0.36
Infiltrating	5	8	13	4	
Lymphocytic reaction tumor front					
Low	5	11	19	3	0.68
High	3	9	23	5	

* The linear-by-linear association test.

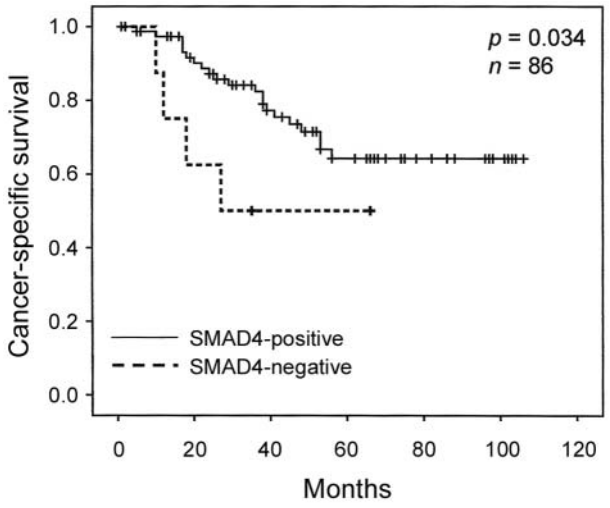

Figure 1. *Kaplan-Meier cancer-specific survival curves for 86 patients with colorectal cancer, grouped according to their SMAD4 expression.*

Table II. *Results of the Cox proportional hazard model in colorectal cancer.*

Variable	Relative risk (e^{β})	95% Confidence interval	*p* value
Age*	0.99	0.96-1.04	0.97
Gender			
Male	1.00		
Female	0.39	0.15-1.01	0.053
Localization			
Right colon	1.00		
Left colon	0.97	0.33-2.84	0.95
Rectum	2.61	0.99-6.92	0.054
Dukes' stage			
A	1.00		
B	1.18	0.25-5.56	0.83
C	2.14	0.46-9.98	0.34
SMAD4			
Positive	1.00		
Negative	4.57	1.17-17.8	0.028

*Continuous variable

In multivariate Cox regression analysis, SMAD4 was again significantly associated with poor patient survival ($p=0.028$) (Table II), also when including the Dukes' stage in the model.

Discussion

The TGF-β signaling pathway plays a complex role in tumorigenesis as it can both promote and inhibit cancer development and progression. While originally described for its transforming capability, TGF-β is also a growth inhibitor in epithelial tissues (12). Reduced responsiveness to TGF-β is an important event in colorectal carcinogenesis (7) and several studies have implicated SMAD4 in this process (13-15). In the present study, immunohistochemistry was used to examine the expression of nuclear SMAD4 proteins in primary CRC. Of the 86 primary tumors assessed, 8 were classified as SMAD4-negative (–), whereas 78 demonstrated nuclear SMAD4 staining ranging from weak to strong.

Normally, activation of the TGF-β-signaling pathway stimulates the nuclear localization of SMAD4. Defective SMAD4 signaling results in insensitivity to TGF-β growth-inhibitive signaling (16). In the present study, the absence of SMAD4 was significantly associated with poor prognosis in both univariate and multivariate survival analyses.

Among the SMAD4-negative cases in our study, the vast majority (75%) were Dukes' C, whereas none were Dukes' A. In contrast, only 29% of the SMAD4 reactive cases were Dukes' C and 14% were Dukes' A. In addition, 62% of the SMAD4-negative cases had an infiltrating growth pattern, compared to 32% in the SMAD4-positive group. Thus, loss of SMAD4 expression appears to coincide with progression towards more advanced Dukes' stages and an infiltrative growth pattern. This is consistent with previous reports where defective SMAD4 signaling has been suggested to occur when tumors acquire invasive and metastatic potential (9).

Results from Wilentz *et al.* suggested that *dpc4*-expression in pancreatic adenoma assessed by immunohistochemistry reflects to a high degree *dpc4*-gene alterations (17). In line with these results, the allelic loss in 18q has previously been linked to poor prognosis in Dukes' B patients (18, 19). However, in Dukes' C patients allelic loss has not been associated with prognosis (18-23). The reason for these discrepancies remains unclear, but it is interesting that Alazzouzi *et al.* recently demonstrated that low SMAD4 protein levels were a strong prognostic factor, while allelic imbalance at 18q in the same patient material did not correlate with prognosis (24).

The loss of SMAD4 immunostaining has different causes, including mutations, epigenetic changes and increased protein degradation and, thus, the immunohistochemical evaluation of nuclear SMAD4 is not restricted to genetic changes, but can account for all possible causes of SMAD4 deficiency. Therefore, the immunohistochemical evaluation of nuclear SMAD4 might be a valuable tool to identify a subset of CRC patients with poor prognosis. In addition, immunohistochemical analysis is technically less complicated and more widely available in routine clinical use than genetic analysis.

In conclusion, our results indicated that the absence of SMAD4 in CRC is associated with a poor prognosis in both univariate and multivariate analyses and thus confirms the recent findings by Alazzouzi *et al.* (24). These results are interesting from a clinical point of view, as loss of SMAD4 expression might be used to identify a subset of CRC patients with a poor prognosis. To verify these findings, further studies on more clinical materials are needed.

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References

1 Tomlinson I, Ilyas M and Novelli M: Molecular genetics of colon cancer. Cancer Metastasis Rev *16*: 67-79, 1997.

2 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM and Bos JL: Genetic alterations during colorectal-tumor development. N Engl J Med *319*: 525-532, 1988.

3 Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH and Kern SE: DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science *271*: 350-353, 1996.

4 Derynck R and Feng XH: TGF-beta receptor signaling. Biochim Biophys Acta *1333*: F105-150, 1997.

5 Engel ME, Datta PK and Moses HL: Signal transduction by transforming growth factor-beta: a cooperative paradigm with extensive negative regulation. J Cell Biochem Suppl *30-31*: 111-122, 1998.

6 Markowitz SD and Roberts AB: Tumor suppressor activity of the TGF-beta pathway in human cancers. Cytokine Growth Factor Rev *7*: 93-102, 1996.

7 Manning AM, Williams AC, Game SM and Paraskeva C: Differential sensitivity of human colonic adenoma and carcinoma cells to transforming growth factor beta (TGF-beta): conversion of an adenoma cell line to a tumorigenic phenotype is accompanied by a reduced response to the inhibitory effects of TGF-beta. Oncogene *6*: 1471-1476, 1991.

8 Matsushita M, Matsuzaki K, Date M, Watanabe T, Shibano K, Nakagawa T, Yanagitani S, Amoh Y, Takemoto H, Ogata N, Yamamoto C, Kubota Y, Seki T, Inokuchi H, Nishizawa M, Takada H, Sawamura T, Okamura A and Inoue K: Down-regulation of TGF-beta receptors in human colorectal cancer: implications for cancer development. Br J Cancer *80*: 194-205, 1999.

9 Miyaki M, Iijima T, Konishi M, Sakai K, Ishii A, Yasuno M, Hishima T, Koike M, Shitara N, Iwama T, Utsunomiya J, Kuroki T and Mori T: Higher frequency of *Smad4* gene mutation in human colorectal cancer with distant metastasis. Oncogene *18*: 3098-3103, 1999.

10 Salovaara R, Roth S, Loukola A, Launonen V, Sistonen P, Avizienyte E, Kristo P, Jarvinen H, Souchelnytskyi S, Sarlomo-Rikala M and Aaltonen LA: Frequent loss of SMAD4/DPC4 protein in colorectal cancers. Gut *51*: 56-59, 2002.

11 Calonge MJ and Massague J: Smad4/DPC4 silencing and hyperactive Ras jointly disrupt transforming growth factor-beta antiproliferative responses in colon cancer cells. J Biol Chem *274*: 33637-33643, 1999.

12 Moses HL, Coffey RJ Jr, Leof EB, Lyons RM and Keski-Oja J: Transforming growth factor beta regulation of cell proliferation. J Cell Physiol Suppl *5*: 1-7, 1987.

13 Howe JR, Roth S, Ringold JC, Summers RW, Jarvinen HJ, Sistonen P, Tomlinson IP, Houlston RS, Bevan S, Mitros FA, Stone EM and Aaltonen LA: Mutations in the *SMAD4/DPC4* gene in juvenile polyposis. Science *280*: 1086-1088, 1998.

14 Thiagalingam S, Lengauer C, Leach FS, Schutte M, Hahn SA, Overhauser J, Willson JK, Markowitz S, Hamilton SR, Kern SE, Kinzler KW and Vogelstein B: Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nat Genet *13*: 343-346, 1996.

15 Takagi Y, Kohmura H, Futamura M, Kida H, Tanemura H, Shimokawa K and Saji S: Somatic alterations of the DPC4 gene in human colorectal cancers *in vivo*. Gastroenterology *111*: 1369-1372, 1996.

16 Fink SP, Swinler SE, Lutterbaugh JD, Massague J, Thiagalingam S, Kinzler KW, Vogelstein B, Willson JK and Markowitz S: Transforming growth factor-beta-induced growth inhibition in a Smad4 mutant colon adenoma cell line. Cancer Res *61*: 256-260, 2001.

17 Wilentz RE, Su GH, Dai JL, Sparks AB, Argani P, Sohn TA, Yeo CJ, Kern SE and Hruban RH: Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas: a new marker of DPC4 inactivation. Am J Pathol *156*: 37-43, 2000.

18 Ogunbiyi OA, Goodfellow PJ, Herfarth K, Gagliardi G, Swanson PE, Birnbaum EH, Read TE, Fleshman JW, Kodner IJ and Moley JF: Confirmation that chromosome 18q allelic loss in colon cancer is a prognostic indicator. J Clin Oncol *16*: 427-433, 1998.

19 Lanza G, Matteuzzi M, Gafa R, Orvieto E, Maestri I, Santini A and del Senno L: Chromosome 18q allelic loss and prognosis in stage II and III colon cancer. Int J Cancer *79*: 390-395, 1998.

20 Diep CB, Thorstensen L, Meling GI, Skovlund E, Rognum TO and Lothe RA: Genetic tumor markers with prognostic impact in Dukes' stages B and C colorectal cancer patients. J Clin Oncol *21*: 820-829, 2003.

21 Cohn KH, Ornstein DL, Wang F, LaPaix FD, Phipps K, Edelsberg C, Zuna R, Mott LA and Dunn JL: The significance of allelic deletions and aneuploidy in colorectal carcinoma. Results of a 5-year follow-up study. Cancer *79*: 233-244, 1997.

22 Jen J, Kim H, Piantadosi S, Liu ZF, Levitt RC, Sistonen P, Kinzler KW, Vogelstein B and Hamilton SR: Allelic loss of chromosome 18q and prognosis in colorectal cancer. N Engl J Med *331*: 213-221, 1994.

23 Laurent-Puig P, Olschwang S, Delattre O, Remvikos Y, Asselain B, Melot T, Validire P, Muleris M, Girodet J, Salmon RJ *et al*: Survival and acquired genetic alterations in colorectal cancer. Gastroenterology *102*: 1136-1141, 1992.

24 Alazzouzi H, Alhopuro P, Salovaara R, Sammalkorpi H, Jarvinen H, Mecklin JP, Hemminki A, Schwartz S Jr, Aaltonen LA and Arango D: SMAD4 as a prognostic marker in colorectal cancer. Clin Cancer Res *11*: 2606-2611, 2005.

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