CHAPTER FOUR

EVALUATION OF THE PROGNOSTIC VALUE OF pSMAD IMMUNOHISTOCHEMISTRY IN COLORECTAL CANCER

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ABSTRACT

SMAD4 mutations and recent Genome-Wide Association Studies (GWAS) show the importance of bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) signaling in the development of colorectal cancer (CRC). Loss of SMAD4 has been implicated as a predictive marker in CRC. As activation of the BMP and TGF-β pathways leads to phosphorylation of SMAD1,5,8 and SMAD2,3, respectively, and both need SMAD4 for translocation to the nucleus, we aimed to investigate whether nuclear staining of pSMAD1,5,8 and pSMAD2, 3 can be used as predictive markers in CRC. A Tissue Microarray (TMA) was constructed of tissue from 209 patients diagnosed with CRC. TMA was stained and scored for the nuclear presence of SMAD4, pSMAD2,3 and pSMAD1,5,8. Loss of SMAD4, pSMAD2,3 and pSMAD1,5,8 was observed in 40, 38 and 73% of the cases, respectively. The incidence of SMAD4 loss was significantly higher in the advanced stages. There was a correlation between loss of SMAD4 and loss of pSMAD1,5,8, but not between loss of SMAD4 and loss of pSMAD2,3. Loss of SMAD4 correlated with a poorer survival. Loss of one of the pSMADs did not correlate with a poorer outcome. Combining different SMAD stainings did not improve the prediction. SMAD4 expression is a prognostic marker in CRC. Nuclear expressions of pSMAD1,5,8 and pSMAD2,3 are not useful prognostic markers in CRC.

INTRODUCTION

Survival and disease recurrence of colorectal cancer (CRC) have improved significantly over the last decade mainly due to early diagnosis and new treatment options. Despite this progress CRC remains one of the leading causes of cancer-related deaths in the western world.[1] Improved molecular understanding of colorectal cancer has made detailed molecular profiling of individual tumors possible to potentially allow personalized therapy. Essential to decision making is estimation of the prognosis currently based almost entirely dependent on histopathological staging. In the search for simple molecular prognostic markers *KRAS* and *p53* mutations and loss of 18q have been the most intensively investigated possible prognostic factors.[2, 3] SMAD4, a key signal transduction element of the TGF-β and BMP tumor suppressor pathways, is located in chromosome 18q21 and is believed to be targeted by the deletion of 18q in CRC.[4] Moreover, frequent germ line mutations in *SMAD4* in patients with Juvenile Polyposis and somatic alterations in the *SMAD4* in CRC underscore the importance of SMAD4 in CRC.[5] Patients with Dukes C CRCs expressing high SMAD4 levels by immunohistochemical (IHC) analysis have significantly better survival.[5, 6] Recent Genome Wide Association Studies in CRC have identified genetic alterations within multiple members of the BMP pathway as being associated with an increased risk of CRC, namely BMP2, BMP4, SMAD4, BMPR1a ,Gremlin1 and Smad7.[7, 8] Therefore, both, the results of prognostic studies and GWAS studies indicate the importance of the BMP/TGF-β signaling pathways in the development of CRC.

Activation of TGF-β signaling results in phosphorylation of SMAD2 or 3 (pSMAD2,3) while activation of BMP signaling leads to phosphorylation of SMAD1,5 or 8 (pSMAD1,5,8). Both pSMAD2,3 and pSMAD1,5,8 complex with SMAD4 to translocate to nucleus for gene transcription. Therefore nuclear localization of pSMAD2,3 or pSMAD1,5,8 should indicate activation of the TGF-β or BMP pathways respectively. We have previously shown loss of nuclear pSMAD1,5,8 expression in 70% of patients with CRC indicating loss of BMP pathway activity.[6, 7] In this study we set out to investigate whether nuclear pSMAD2,3 or pSMAD1,5,8 expression, biomarkers for the activity of the BMP and TGF- β pathways, can be used as a prognostic markers in CRC and to compare this with the prognostic value of SMAD4 which has been shown to have prognostic significance in a cohort of Dukes C CRCs.[8-10]

PATIENTS AND METHODS

Formalin-fixed, paraffin-embedded tissues from 209 CRC cases between the years of 1983 and 2004 were used for the compilation of the TMA. Blocks were selected from the archives of the Pathology Department at the Academic Medical Centre, Amsterdam and Leiden

University Medical Center, Leiden. The study was approved by the investigator's institutional review boards. Our study included 115 men (55%) and 94 women (45%), their ages ranging from 30 to 91 years, with a mean (±SD) of 68,9 years and median 70 years. Dukes stage was known for all tumors and our study includes 6 patients with Dukes A (3%), 136 patients with Dukes B (65%), 55 patients with Dukes C (26%) and 12 patients with Dukes C (6%). Patient's characteristics are provided in table 1. Three cores of tissue from each cancer specimen were taken plus one normal core as a control. The TMA was sectioned and stained for SMAD4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pSMAD1,5,8 and pSMAD2,3 (both Cell Signaling, Boston, MA, USA) according to previously described methods [10]. Stainings were scored blindly and independently by two researchers based on the number of cells stained and the intensity of the staining as described in table 2, counting only the cells with a nuclear staining. A mean score of 0 or 1 point was considered negative and a mean score 2 or 3 points was considered positive. Figure 1 shows examples of positive and negative stainings of the SMADs. Statistical analyses were performed with the Statistical Package of Social Science (SPSS) version 17.0.2 for Windows (SPSS Inc, Cary, NC, USA). Kaplan-Meier graphs were used to visualize patients' survival and Log rank-test was performed to calculate the p-value for the difference in survival. $P<0.05$ was considered a significant difference. Fisher's exact test and chi-square tests were used as appropriate.

Table 1

Scoring System for SMAD4, p-SMAD1,5,8 and p-SMAD2,3

	Percentage of cells stained			
Intensity	**<10%**	**10-30%**	**30-50%**	**>50%**
No staining	0	0	0	0
Weak staining	0	0	1	1
Moderate staining	0	1	2	3
Strong staining	1	2	3	3

Table 2

		Dukes				
	total	**A**	**B**	**C**	**D**	**p**
SMAD4						
negative	84 (40%)	2 (33%)	48 (35%)	29 (53%)	5 (42%)	0.17
positive	125 (60%)	4 (67%)	88 (65%)	26 (47%)	7 (58%)	
pSMAD1,5,8						
negative	151 (72%)	4 (67%)	102 (75%)	38 (69%)	7 (58%)	0.56
positive	58 (28%)	2 (33%)	34 (25%)	17 (31%)	5 (42%)	
pSMAD2,3						
negative	79 (38%)	2 (33%)	55 (40%)	14 (25%)	8 (67%)	0.04
positive	130 (62%)	4 (67%)	81 (60%)	41 (75%)	4 (33%)	

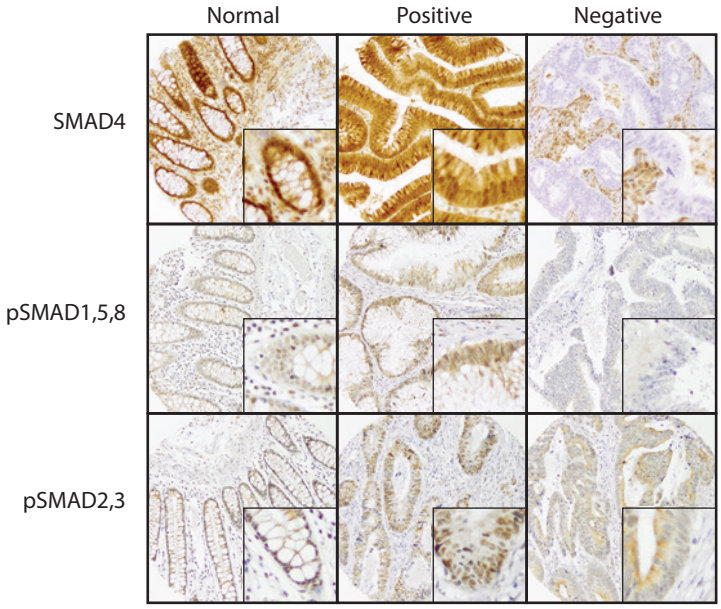

Figure 1: Examples of positive and negative SMAD4, pSMAD1,5,8 and pSMAD2,3 stainings on the basis of the scoring system. Normal tissue cores from the same patients were compared with tumor tissue cores. Original magnification, x 200.

RESULTS

To investigate the association between nuclear SMAD4, pSMAD1,5,8 and pSMAD2,3 expression in tumors and patient survival, we divided patients into two groups dependent on the intensity of the stainings as described in the methods section. In our study 84 patients (40%) show negative nuclear SMAD4 expression, 151 patient (72%) exhibit negative expression of nuclear pSMAD1,5,8 and 79 patient (38%) scored negative for nuclear pSMAD2,3 in the tumor tissue (table 3). We observe significantly more negative SMAD4 staining in more advanced CRCs with Dukes C+D stages (51% negative) versus early Dukes stages A+B (35% negative)($p=0.04$). The distribution of the pSMAD2,3 staining scores in the different Dukes stages was also significantly different ($p<0.04$), showing relatively more negative stainings in the Dukes D stage (67% negative) compared with Dukes A-C (33%, 40% and 25% negative respectively) (table 3). We do not see significant differences in pSMAD1,5,8 stainings between cancers with different Dukes stages.

As SMAD4 is considered necessary for the nuclear translocation of the phosphorylated R-SMAD complexes (pSMAD1,5,8 or pSMAD2,3) one should expect an high correlation between absence of nuclear pSMAD1,5,8 or SMAD2,3 stainings and negative SMAD4 staining. Indeed, we see a significant correlation between SMAD4 and SMAD1,5,8 ($p=0.01$), although 18% of SMAD4 negative CRCs still exhibit positive nuclear pSMAD1,5,8 staining. However, there is no correlation between SMAD4 and pSMAD2,3, and a majority of

SMAD4 negative cancers (64%), show positive staining for pSMAD2,3 (table 4). Interestingly, 66% of SMAD4 positive CRC show negative expression of pSMAD1,5,8 and 39% of SMAD4 positive CRC are negative for pSMAD2,3 (table 4). This could be due to mutations at the receptor level or methylation of R-SMADs as alternative mechanisms leading to inactivation of BMP or TGF-β pathway.[9, 12]

Finally, we analyzed the effect of the expression status of SMAD4, SMAD1,5,8 and SMAD2,3 on the patients survival. In our study population loss of SMAD4 correlates with a poorer survival (p=0.04) confirming that SMAD4 is a valuable prognostic marker for CRC not only in patients with Dukes C, but in unselected group with different Dukes stages. (figure 2A). In contrast, pSMAD1,5,8 (p=0.95) or pSMAD2,3 (p=0.67) immunohistochemical status does not predict a poorer survival (figure 2B-C). Since SMAD4 loss equates to inactivation of both pathways simultaneously, we then investigated whether simultaneous pSMAD1,5,8 and pSMAD2,3 loss which should also equate to loss of both signaling pathways simultaneously, was predictive of survival. We found no significant association between coexistent pSMAD1,5,8 and pSMAD2,3 loss and survival. We next investigated whether there was any possible added value of combining pSMAD 1,5,8 or pSMAD2,3 stainings to the prognostic value of SMAD4 status. We found no improvement over the prognostic value of SMAD4 staining (data not shown).

Table 3

	SMAD4		
	negative *n=84* (%)	**positive *n=125 (%)***	***p***
pSMAD1,5,8			
positive	15 (18)	43 (34)	0.01
negative	69 (82)	82 (66)	
pSMAD2,3			
positive	54 (64)	76 (61)	0.66
negative	30 (36)	49 (39)	

Table 4. Univariate and multivariate analysis

	Univariate			Multivariate		
	HR	**95% CI (low-high)**	**p-value**	**HR**	**95% CI (low-high)**	**p-value**
Age	1,017	(0,990-1,045)	0.222	-	-	-
Gender (male/female)	1,024	(0,547-1,917)	0.941	-	-	-
Tumour grade	1,554	(0,920-2,624)	0.099	2,468	(1,343-4,535)	0.004
Dukes stage	4,735	(3,096-7,241)	<0.0001	4,703	(2,839-7,790)	<0.0001
SMAD4	1,905	(1,022-3,553)	0.043	2,468	(1,023-4,148)	0.047
pSMAD1,5,8	1,022	(0,511-2,046)	0.951	2,048	(0,908-4,766)	0.084
pSMAD2,3	1,148	(0,559-2,199)	0.678	0,869	(0,430-1,756)	0.696

HR = Hazard Ratio, CI = Confidence Interval

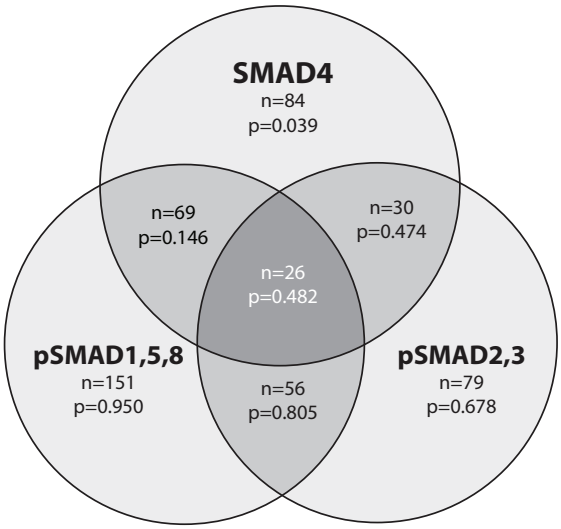

Figure 2: A Venn diagram showing the log-rank survival prediction of SMAD expression patterns; n, number of cancers that have a negative expression for the indicated SMADs.

DISCUSSION

In this study we see a significant correlation between nuclear SMAD4 staining and poorer survival of patients with CRC. These data confirm the significance of SMAD4 loss. We observed loss of SMAD4 in 40% of cases which is in concordance with previous studies proving the reproducibility of the SMAD4 staining.[6, 10, 11]

Nuclear expression of pSMAD1,5,8 and pSMAD2,3 can be used to monitor the activity of the BMP and TGF-β pathways respectively.[11] We therefore evaluated whether the nuclear expression of pSMAD1,5,8 and pSMAD2,3 can be used to predict CRC outcome and if it could be combined with SMAD4 expression. Although loss of BMP signaling, judged by nuclear pSMAD1,5,8 expression, was seen in 72% of the cases, this does not lead to a worse survival. The same applies for nuclear pSMAD2,3, which is lost in 38% of the cases. These results are perhaps surprising as the importance of BMP and TGF-β signaling and their role as tumor suppressors in CRC is well documented in the literature.[14, 15] From this it might be expected that loss of BMP or TGF-β pathway activity would be associated with worse patient outcome. We see that only SMAD4 loss and not pSMAD1,5,8 or pSMAD2,3 loss predicts a poorer outcome. The possible explanation could be that loss of SMAD4, which is a key signal transduction molecule of all pathways of TGF-β family, leads to simultaneous inactivation of BMP, TGF-β and Activin pathways, while loss of one of the arms on the level of R-SMADs (either pSMAD1,5,8 or pSMAD2,3) is not sufficient and loss of both is needed for a significant difference. Moreover, loss of SMAD4 does not necessarily lead to loss of nuclear pSMAD2,3 expression as is shown in table 4. pSMAD2,3 is able to shuttle

between nucleus and cytoplasm without SMAD4 and this could be the reason for the inability to use pSMAD2,3 as a marker. Currently it is believed that pSMAD1,5,8 can only translocate to the nucleus in complex with SMAD4.[12] While we see a concordance between SMAD4 loss and nuclear pSMAD1,5,8 loss, as we have shown previously[9], we do see nuclear pSMAD1,5,8 in SMAD4 negative cancers which suggests that pSMAD1,5,8 may be able to translocate to the nucleus without SMAD4 contrary to current dogma. It is possible that despite the care we have taken in developing reliable immunohistochemistry protocols for the antibodies we have used, our results are affected by the reliability and reproducibility of immunohistochemistry using phoshospecific antibodies which are more sensitive to tissue processing and fixation than other antibodies.[13]

To our knowledge this is the first study where the prognostic value of pSMAD1,5,8 has been evaluated, while two studies have shown pSMAD2,3 expression to have prognostic value[14, 15]. These two studies showed a 6,2% and 28,7% loss of pSMAD2, respectively compared to our 38%. The differences may be due to the use of different antibodies and scoring systems. In summary our study shows that SMAD4 expression is a prognostic marker in CRC. Conversely, nuclear expression of pSMAD1,5,8 and pSMAD2,3 are not useful prognostic markers in CRC.

REFERENCE LIST

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
2. Pritchard CC, Grady WM. Colorectal cancer molecular biology moves into clinical practice. Gut 2011;60:116-129.
3. Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer 2009;9:489-499.
4. Thiagalingam S, Lengauer C, Leach FS, Schutte M, Hahn SA, Overhauser J, Willson JK, Markowitz S, 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.
5. Howe JR, Bair JL, Sayed MG, Anderson ME, Mitros FA, Petersen GM, Velculescu VE, Traverso G, Vogelstein B. Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat Genet 2001;28:184-187.
6. Kodach LL, Wiercinska E, de Miranda NF, Bleuming SA, Musler AR, Peppelenbosch MP, Dekker E, van den Brink GR, van Noesel CJ, Morreau H, Hommes DW, Ten DP, Offerhaus GJ, Hardwick JC. The bone morphogenetic protein pathway is inactivated in the majority of sporadic colorectal cancers. Gastroenterology 2008;134:1332-1341.
7. Tomlinson IP, Carvajal-Carmona LG, Dobbins SE, Tenesa A, Jones AM, Howarth K, Palles C, Broderick P, Jaeger EE, Farrington S, Lewis A, Prendergast JG, Pittman AM, Theodoratou E, Olver B, Walker M, Penegar S, Barclay E, Whiffin N, Martin L, Ballereau S, Lloyd A, Gorman M, Lubbe S, Howie B, Marchini J, Ruiz-Ponte C, Fernandez-Rozadilla C, Castells A, Carracedo A, Castellvi-Bel S, Duggan D, Conti D, Cazier JB, Campbell H, Sieber O, Lipton L, Gibbs P, Martin NG, Montgomery GW, Young J, Baird PN, Gallinger S, Newcomb P, Hopper J, Jenkins MA, Aaltonen LA, Kerr DJ, Cheadle J, Pharoah P, Casey G, Houlston RS, Dunlop MG. Multiple common susceptibility variants near BMP pathway loci GREM1, BMP4, and BMP2 explain part of the missing heritability of colorectal cancer. PLoS Genet 2011;7:e1002105.
8. Alazzouzi H, Alhopuro P, Salovaara R, Sammalkorpi H, Jarvinen H, Mecklin JP, Hemminki A, Schwartz S Jr, Aaltonen LA, Arango D. SMAD4 as a prognostic marker in colorectal cancer. Clin Cancer Res 2005;11:2606-2611.
9. Kouvidou C, Latoufis C, Lianou E, Kouvatseas G, Kakouri E, Anagnostakis D, Vrettou-Aravani V, Betsi E, Karatapanis S. Expression of Smad4 and TGF-beta2 in colorectal carcinoma. Anticancer Res 2006;26:2901-2907.
10. Isaksson-Mettavainio M, Palmqvist R, Forssell J, Stenling R, Oberg A. SMAD4/DPC4 expression and prognosis in human colorectal cancer. Anticancer Res 2006;26:507-510.
11. Itoh S, Itoh F, Goumans MJ, Ten DP. Signaling of transforming growth factor-beta family members through Smad proteins. Eur J Biochem 2000;267:6954-6967.
12. Ten DP, Goumans MJ, Itoh F, Itoh S. Regulation of cell proliferation by Smad proteins. J Cell Physiol 2002;191:1-16.
13. Pinhel IF, Macneill FA, Hills MJ, Salter J, Detre S, A'hern R, Nerurkar A, Osin P, Smith IE, Dowsett M. Extreme loss of immunoreactive p-Akt and p-Erk1/2 during routine fixation of primary breast cancer. Breast Cancer Res 2010;12:R76.
14. Lampropoulos P, Zizi-Sermpetzoglou A, Rizos S, Kostakis A, Nikiteas N, Papavassiliou AG. Prognostic significance of transforming growth factor beta (TGF-beta) signaling axis molecules and E-cadherin in colorectal cancer. Tumour Biol 2012.
15. Xie W, Rimm DL, Lin Y, Shih WJ, Reiss M. Loss of Smad signaling in human colorectal cancer is associated with advanced disease and poor prognosis. Cancer J 2003;9:302-312.