The bone morphogenetic protein pathway in colorectal cancer progression

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Citation

Voorneveld, P. W. (2020, September 24). *The bone morphogenetic protein pathway in colorectal cancer progression*. Retrieved from https://hdl.handle.net/1887/136915

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Title: The bone morphogenetic protein pathway in colorectal cancer progression
Issue Date: 2020-09-24

Chapter 6

A meta-analysis of SMAD4 immunohistochemistry as a prognostic marker in colorectal cancer

Transl Oncol. 2015 Feb;8(1):18-24

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Aim

SMAD4 immunohistochemistry is considered a valuable prognostic marker in colorectal cancer, but individual studies have often been small and the results variable. A meta-analysis could potentially clarify these findings.

Methods

In September 2014 a Pubmed and Google Scholar search was conducted to find publications that reported the prognostic value of SMAD4 expression. A meta-analysis was performed to clarify the association between SMAD4 expression and survival outcomes.

Results

137 studies were found of which 13 were considered eligible. The studies consisted of a total of 3800 patients. Three different endpoints were taken into account, namely overall survival (OS), disease free survival (DFS) and cancer specific survival (CSS). Also, the studies were divided into univariate and multivariate analyses. The pooled hazard ratios were as followed; univariate CSS: 1,75 (95%CI: 0,93–3,32; z=1,69; p=0,09); multivariate CSS: 2,17 (95%CI: 1,56–3,01; z=4,65; p=0,000); univariate DFS: 2,11 (95%CI: 1,36–3,28; z=3,32; p=0,001); multivariate DFS: 2,15 (95%CI: 1,56–3,01; z=4,65; p=0,000) univariate OS DFS 2,30 (95%CI: 1,41–3,73; z=3,36; p=0,001); univariate OS 2,28 (95%CI: 1,30–4,00; z=2,89; p=0,004).

Conclusion

The results of the presented meta-analyses indicate that SMAD4 expression status using immunohistochemistry is a prognostic marker for patient survival.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer death in men and women combined in the US[1]. Colorectal cancer is thought to result from the accumulation of genetic alterations, which give cells a survival advantage over surrounding cells. An important genetic change in CRC is mutation of *SMAD4* leading to loss of SMAD4 protein expression. SMAD4 protein expression is lost in approximately 30-40% of the CRCs[2-4] and is associated with metastasis formation and poor response to chemotherapy[5-8]. *SMAD4* is the common mediator of the Transforming Growth Factor-β (TGF- β) and Bone Morphogenetic Protein (BMP) pathways and is located on chromosome 18q21. SMAD4 immunohistochemistry correlates very well with the genetic status as is shown in Juvenile Polyposis[9]. SMAD4 immunohistochemistry is therefore frequently used to ascertain the SMAD4 status of a tumour. Although the use of SMAD4 immunohistochemistry as a molecular marker has been studied in multiple studies, this has mostly been performed in small cohorts and in different subgroups using different survival endpoints. Most studies report an association between SMAD4 loss and a poor prognosis, but this is not consistent. To our knowledge, there has never been a comprehensive study combining all these results to truly establish the predictive value of SMAD4 immunohistochemistry for clinical use. Therefore, we conducted a meta-analysis to correlate the SMAD4 immunohistochemistry expression with survival outcomes.

Methods

Publication selection

Pubmed and Google Scholar were used to search for potentially relevant literature. The search entry used was: ('colorectal OR large intestine' OR 'large bowel' OR 'colon' OR 'colonic' OR 'rectal' OR 'rectum') AND ('cancer' OR 'carcinoma' OR 'tumor' OR 'tumour' OR 'neoplasm' OR 'cancers') AND ('SMAD4' OR 'DPC4') AND ('marker' OR 'signature molecule' OR 'molecular marker' OR 'markers' OR 'biomarkers' OR 'biomarker' OR 'marker' OR 'prognosis' OR 'predictive' OR 'survival'). Additionally, reference lists of the studies found and of systematic

Study	Year	Country	Stage	# patients	Inclusion period	AB	Dilution
Alazzouzi[15]	2005	Finland	III	86	1993-1997	Santa Cruz	1:1000
Alhopuro[5]	2005	Finland	III	75	1994-1998	Santa Cruz	1:1000
Isaksson[18]	2006	Sweden	I-III	86	1987-1994	Santa Cruz	1:50
Bacman[17]	2007	Germany	II-III	305	1991-2001	Santa Cruz	1:50
Mesker[19]	2009	Netherlands	I-II	118	1980-2001	Santa Cruz	1:400
Gulubova[20]	2010	Bulgary	I-IV	138	1997-2006	Santa Cruz	1:50
Li[21]	2011	China	I-IV	147	2003-2004	Zhongshan Biotechnology	1:150
Baraniskin[22]	2011	Germany	IV	190	NA	Santa Cruz	1:100
Ahn[23]	2011	South Korea	I-IV	429	1991-2000	Santa Cruz	1:200
Isaksson[24]	2012	Sweden	I-IV	441	1995-2003	Santa Cruz	1:100
Lampropoulos[25]	2012	Greece	I-IV	195	2005-2006	Santa Cruz	1:100
Roth[26]	2012	Switzerland	II-III	1381	NA	Santa Cruz	0,2 mg/mL
Voorneveld[16]	2013	Netherlands	I-IV	209	1983-2004	Santa Cruz	1:400

Study	Site	Age (y)	Follow up	Outcome	Cytoplasm Nuclear	Preserved SMAD4 (%)	Adj. therapy
Alazzouzi[15]	Colon/rectum	mean 70,1	at least 6 years	DFS and OS	NA	26	No
Alhopuro[5]	Colon/rectum	mean 59	mean 8,7 years	DFS and OS	NA	86,7	Yes
Isaksson[18]	Colon/rectum	NA	NA	CSS	Nuclear	90,7	No
Bacman[17]	Colon	median 64	median 91 months	CSS	Nuclear	85,6	Yes
Mesker[19]	Colon	mean 68,2	up to 25 years	DFS and OS	Nuclear	76,5	No
Gulubova[20]	Colon/rectum	median 65	median 37,6 months	OS	Nuclear	88,4	No
Li[21]	Colon	NA	up to 5 years	DFS and OS	Nuclear	74,1	No
Baraniskin[22]	Colon/rectum	mean 64,4	NA	OS	Nuclear	65,8	Yes
Ahn[23]	Colon/rectum	mean 57	median 56 months	DFS	NA	47,3	Yes
Isaksson[24]	Colon/rectum	NA	NA	CSS	Nuclear	80,3	Yes
Lampropoulos[25]	Colon/rectum	mean 68,6	median 56 months	CSS	Both	61,5	No
Roth[26]	Colon	median 60	median 69 months	OS and DFS	NA	78,8	Yes
Voorneveld[16]	Colon	mean 68,9	median 65 months	CSS	Nuclear	60	No

reviews were also checked for potential articles. The search was performed in September 2014.

First, the abstracts were checked for relevance and full articles were retrieved when potentially eligible. To be included in the analysis studies had to have been performed in resected colorectal carcinomas and immunohistochemistry for SMAD4 had to have been performed. One or more of the following endpoints had to be described; overall survival (OS), disease-free survival (DFS) or cancer-specific survival (CSS). Both univariate and multivariate analyses were taken into account, although separately analysed. All selected studies were checked according to a 20-point quality control system developed previously (Table S1). [10-12]

Statistical analysis

Data was extracted by two independent researchers. When not reported, the logHazard Ratio (HR) and Confidence Interval (CI) were extracted using previously published methods. [13-14] The meta-analysis was performed in STATA12 using the *metan* package. A pooled HR and 95% CI was calculated for each endpoint (OS, DFS and CSS) and presented in a forest plot. An HR 1> implies a worse prognosis for SMAD4 negative CRCs. A p-value of <0.05 was considered significantly different. Heterogeneity was calculated and presented as X^2 and I^2. We chose to perform random effect models based on the fact that the studies differed in size, country and most importantly in the method of scoring and cut off values. The $\log_e$ standard error of the HR and $\log_e$HR of each study were plotted in a funnel plot to assess potential publication bias.

Results

Search results

The search resulted in 137 studies. On the basis of the abstracts 19 studies were considered eligible of which 6 were eventually excluded based on the fact the HR and CI could not be extracted. The 13 eligible studies consisted of a total of 3800 patients, ranging from 86-1381 per study. The main characteristics of the studies can be found in table 1. We included overall survival (OS), cancer-specific survival (CSS) and disease-free survival (DFS) in this study, but analysed the different endpoints separately. We also made a distinction between univariate and multivariate analyses. Five studies only included colon cancer, the other eight studies included both colon and rectal cancers. Six studies reported that a portion or all of the patients received adjuvant chemotherapy after resection. Three studies did not report the mean or median age and three studies did not report the mean or median follow-up period. Five studies reported CSS, five studies reported DFS and seven studies reported OS. Eight studies described specifically that only nuclear staining of SMAD4 was considered positive. One study considered both cytoplasmic and nuclear staining as positive and four studies did not describe their scoring methods. All studies used SMAD4 antibodies produced by Santa Cruz Biotechnology Inc. (Dallas, TX, USA) except for Li et al who used an antibody from Zhongshan Biotechnology Inc. All studies except Isaksson 2012 had dichotomised the scoring. For Isaksson 2012 we have compared the categories depicted as SMAD4^{high} versus SMAD4^{loss} and we have not considered the category

SMAD4$^{\text{moderate}}$. The mean and median positive SMAD4 score is 70,9% and 76,5% respectively with a range of 26-90,7%.

Cancer specific survival

Three studies included a univariate CSS with a total of 600 patients (range 86-305). The pooled HR is 1,75, which is not significantly different (95%CI: 0,93–3,32; *z*=1,69; *p*=0,09). Four studies included a multivariate CSS with a total of 931 patients (range 86-441). The pooled HR is 2,17, which is significantly different (95%CI: 1,56–3,01; *z*=4,65; *p*=0,000), indicating that SMAD4 loss is associated with a worse cancer specific survival in this pooled multivariate analysis.

Disease free survival

Six studies included a univariate DFS with a total of 2236 patients (range 75-1381) and three studies included a multivariate DFS with a total of 1646 patients (range 118-1381). The pooled HR for the univariate DFS is 2,11 (95%CI: 1,36–3,28; *z*=3,32; *p*=0,001) and the pooled HR for the multivariate DFS is 2,15 (95%CI: 1,56–3,01; *z*=4,65; *p*=0,000), which are both significantly different. The univariate and the multivariate DFS meta-analyses confirm that SMAD4 loss is associated with a poor disease free survival.

Overall survival

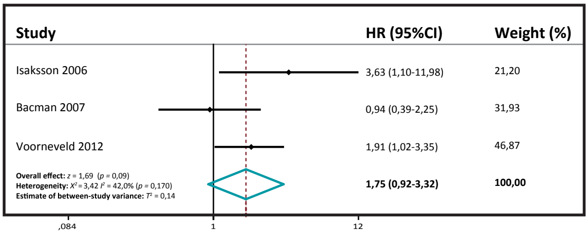
Seven studies included a univariate OS with a total of 2135 patients (range 75-1381) and four studies included a multivariate OS with a total of 1836 patients (range 118-1381). The pooled HR for the univariate OS is 2,30 (95%CI: 1,41–3,73; *z*=3,36; *p*=0,001) and the pooled HR for the multivariate OS is 2,28 (95%CI: 1,30–4,00; *z*=2,89; *p*=0,004). Both the univariate and the multivariate OS meta-analyses showed that SMAD4 loss is associated with a poor overall survival.

Publication bias

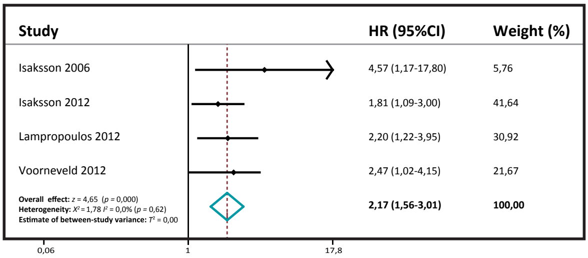
The funnel plots for the assessment of potential publication bias show one considerable outlier in the univariate and multivariate OS and the univariate DFS. Although in the univariate OS this does not result in a significant Egger test, we chose to exclude the outliers in all the meta-analyses and recalculate the pooled HRs. Exclusion of the outlier reduces the pooled HR of the unvariate OS to 1,673

Cancer specific survival

Univariate analyses

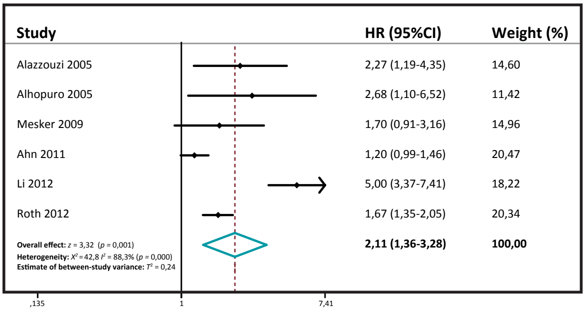


Multivariate analyses

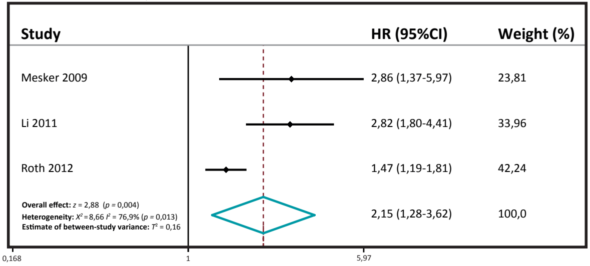


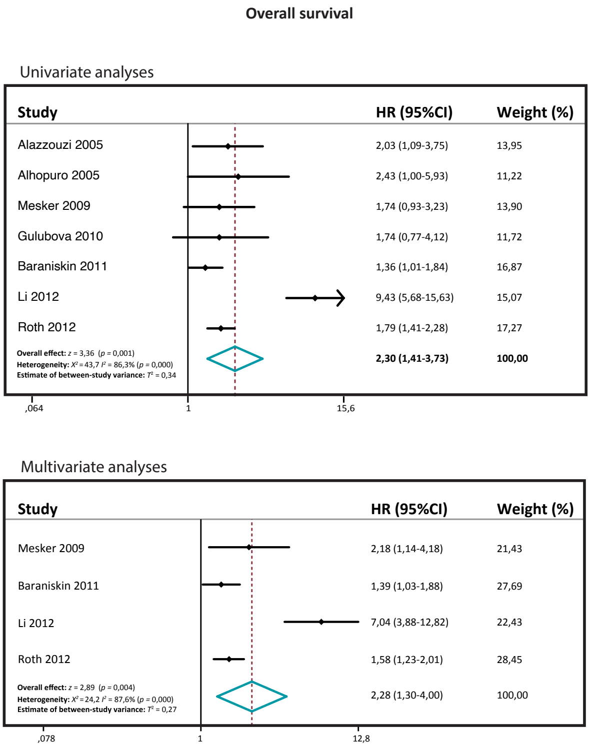
Disease free survival

Univariate analyses



Multivariate analyses





(95%CI: 1,42–1,96; z=6,08; p=0,000). The pooled HR of the multivariate OS is reduced to 1,55 (95%CI: 1,29–1,86; z=4,68; p=0,000) and the pooled HR of the univariate DFS is 1,6 (95%CI: 1,23–2,08; z=3,53; p=0,000).

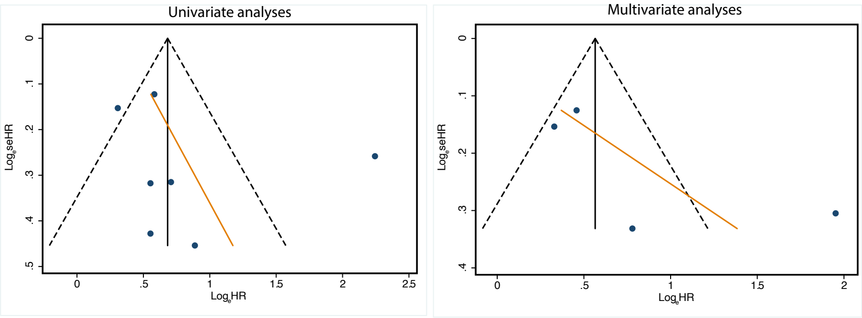
Discussion

Molecular profiling of individual tumours potentially allows a personalized approach to cancer treatment. Estimation of prognosis plays an important role in decisions about treatment, and this is currently almost entirely dependent on histopathological staging. *SMAD4*, located on chromosome 18q21, has frequently been reported to be a useful prognostic marker. Several studies have reported the prognostic value of SMAD4 expression using immunohistochemistry[15-16],

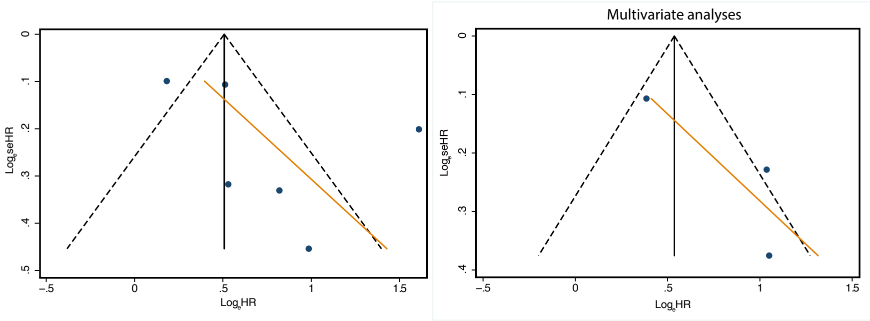
Funnel plots

Pseudo 95% confidence intervals
Pooled estimate
Egger regression line

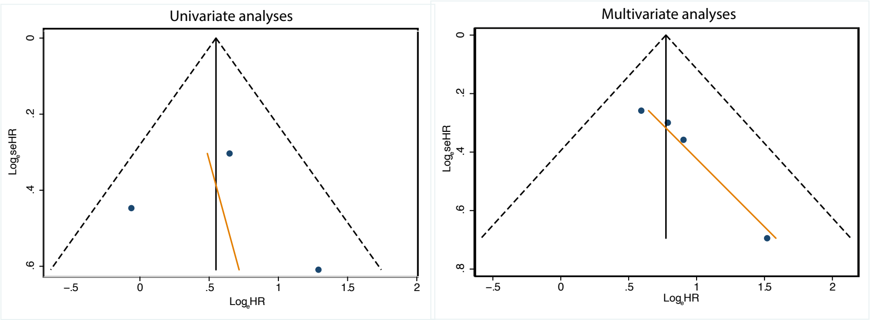
Overall survival



Disease-free survival



Cancer-specific survival



although this has not been entirely consistent[17]. To our knowledge, this is the first comprehensive meta-analysis of the predictive value of SMAD4 expression using immunohistochemistry.

We included three different endpoints, namely OS, CSS and DFS and both univariate and multivariate analyses. All the meta-analyses, except the univariate CSS, showed a significant difference in HR implying that loss of SMAD4 expression as measured by immunohistochemistry is associated with a poor prognosis. 13 studies have investigated the prognostic value of SMAD4 loss using different types of endpoints resulting in relative few studies per single endpoint. The analyses of the univariate OS and DFS included the largest numbers of studies (seven each) which makes these the most reliable results. To investigate the heterogeneity, I^2 was calculated which was more than 50% in most of the cases, except for the univariate and multivariate analysis of the CSS. Conventionally, when the I^2 is more than 50% and the distribution is significantly heterogeneous ($p<0{,}05$) the fixed model cannot be used and the random effects model has to be applied, but prior to conducting the analysis we had already decided to use the random effects model for all the meta-analyses because of the variation in the methodology of the studies included. One aspect that varies between the studies is the percentage of SMAD4 preservation, which is dependent on the staining and scoring method used. SMAD4 is the common mediator of the BMP/TGFβ signalling pathways and complexes with phosphorylated R-SMADs, which then enter the nucleus to modulate gene transcription. Only nuclear localisation shows active functional SMAD4. Not all studies used only nuclear staining and four studies did not report what they considered as positive. Another source of heterogeneity is the population that was used in each of the studies. Seven studies included all the stages and the other six included only one or two stages. Six studies included patients that had received chemotherapy, while in seven studies no adjuvant therapy was applied. These differences can affect the outcome of the individual studies and can increase the heterogeneity in the meta-analysis.

Despite the fact that the pooled studies of univariate cancer specific survival did not show a significant difference, we conclude, based on the other 5 meta-analyses all showing statistically significant associations, that immunohistochemical analysis of SMAD4 expression is a useful prognostic marker in colorectal cancer.

Immunohistochemistry is a relative easy technique to perform and is readily available in most hospital pathology departments. International recommendations to standardise SMAD4 scoring methodology are required.

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	Isaksson 2006	Bacman 2007	Li 2011	Isaksson 2012	Lampropoulos 2012	Roth 2012	Voorneveld 2013	Alazzouzi 2005	Alhopuro 2005	Baraniskin 20‘	Mesker 2009	Gulubova 2010	Ahn 2009
study group													
population described													
Gender/age	1	1	1	1	1	1	1	1	1	1	1	1	1
Histology	1	1	1	1	1	1	1	1	1	1	1	1	1
period of recruitment	1	1	1	1	1	0	1	1	1	0	1	1	1
Inclusion/exclusion	1	1	0	1	1	0	1	1	1	1	1	1	1
Study attrition													
>90% of identified included in final analysis	0	1	1	1	1	1	1	1	1	0	1	0	1
Reasons for loss to follow up given	1	1	0	1	1	1	1	1	1	1	1	0	0
Peri-operative mortality details	1	1	0	0	1	0	0	0	0	1	1	0	0
Scientific methodology													
IHC methodology outlined													
Details of 1ste/2nd Abs used	1	1	1	1	1	1	1	1	1	1	1	1	1
Concentration	1	1	1	1	1	1	1	1	1	1	1	1	1
Positive negative control	1	1	0	1	1	0	1	0	0	0	1	1	1
Description scoring system													
>1 independent scorer	1	0	1	1	1	1	1	1	1	1	0	1	1
Blind	0	0	1	1	1	1	1	1	1	1	0	0	0
Criteria for positive outlined													
Distribution (membr vs nuclear)	0	0	1	1	0	0	1	0	0	1	1	1	0
% pos for classification stated	1	0	0	1	1	0	1	0	0	1	1	1	1
Confounding factors considered													
Adjuvant therapy details provided	1	1	0	0	1	1	1	1	1	1	1	1	1
Histology breakdown according to IHC staining	1	1	1	1	1	1	1	1	1	0	0	0	1
Statistical analysis													
HR (CI) provided	1	1	1	1	1	1	1	0	0	0	0	0	0
Exact p-value	1	1	1	1	1	1	1	1	1	1	1	1	1
number at risk for Kaplan Meier	1	1	1	1	0	1	1	1	1	1	1	1	1
Number of censored cases	1	0	0	0	0	1	1	1	1	0	0	0	1
total	17	15	13	17	17	14	19	15	15	14	15	13	15