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ORIGINAL ARTICLE

Expression of Smad4, E-cadherin and beta-catenin in advanced colorectal cancer: a retrospective study

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Summary

Purpose: *To correlate the expression of E-cadherin and beta-catenin with alterations of expression of Smad4 in advanced colorectal cancer (CRC).*

Methods: *Tissue specimens from 75 colorectal cancer cases (Dukes stage C and D) were tested for Smad4, E-cadherin and beta-catenin by the Avidin-Biotin immunoperoxidase method. The results were correlated with patients' clinicopathological parameters.*

Results: *Smad4 expression was lost or reduced in roughly 1 out of every 3 Dukes C and D CRCs. Association of Smad4 expression with other clinicopathological parameters was not noted. Association of expression of E-cadherin with other clinicopathological parameters was not noted, apart from tumor location. Expression of beta-catenin was not associated with clinicopathological parameters. Lack of expression of Smad4 was associated with lack of expression of both E-cadherin ($p<0.000$) and beta-catenin ($p<0.000$). As regards the relation between E-cadherin and beta-catenin, the expression of each seemed to parallel the expression of the other ($p<0.000$). Beta-catenin was overexpressed in 68.5% of the specimens studied.*

Conclusion: *Clinically advanced CRC is associated with a reduced or complete lack of expression of Smad4. E-cadherin and beta-catenin are expressed in parallel with each other and also with Smad4. This tumor suppressor role of Smad4 by affecting both E-cadherin and beta-catenin may indicate a novel pathway for metastatic tumor via cellular reshaping. The precise underlined mechanism(s) and the clinical significance of these findings remain to be determined.*

Key words: beta-catenin, colorectal cancer, E-cadherin, Smad4 protein

Introduction

CRC is a common malignancy, potentially curable if detected early. Cell membrane proteins involved in cell adhesion (cadherins) together with proteins associated with linking to cytoskeleton (catenins) and intracellular proteins mediating signaling for extracellular factors (Smad4) are of great importance in determining the aggressiveness of malignant cells. Recent studies indicate that invasive and metastatic CRC displays an increased incidence of mutations of Smad4 compared to adenomas and intramucosal carcinomas [1,2]. Smad4 is a protein involved in cell signaling which modulates members of the TGFβ protein superfamily. It is also of importance in cross-talking in other signaling pathways. Apart from signaling, it also acts as a co-modulator of transcription by regulating expression of a variety of genes through interaction with other transcriptional co-factors.

The E-cadherin/beta-catenin complex plays a critical role in epithelial cell to cell adhesion. The disruption of the cadherin/beta-catenin complex is observed in carcinomas of many types. Transcriptional repression mechanisms have been implicated in this disruption but the intermediate events remain unknown. Reduced or absent expression of the E-cadherin/beta-catenin complex is associated with poor tumor differentiation, infiltrative growth and distant metastases [3,4].

We investigated the relation between expression of Smad4 and cellular disruption of E-cadherin/beta-catenin complex in advanced CRC and we correlated

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the results with clinicopathological parameters. Despite plenty of data on these factors in experimental models, analogous clinical studies inter-relating all of them together in humans are rather limited.

Methods

Patients

We studied 75 Greek patients with CRC who underwent surgical tumor resection from May 2004 to December 2009. Only cases with stage C and D according to Dukes classification were included. The clinical data of the 75 patients studied are depicted in Table 1. All patients were operated upon by the same surgeons; the pathology and histopathology work-up was conducted by the same team.

Clinicopathological parameters, namely age, gender, location, size, pattern shape, pathological stage at presentation, histological grade, number of infiltrated lymph nodes and distant metastases for each case were retrieved. Regarding age classification, the following age groups were formed: 47-59, 60-69, 70-79 and 80-89 years.

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed, paraffin wax embedded tissue with the streptavidin-biotin-peroxidase complex (ABC) method; Samples were pre-treated with HCl (2N) at room temperature for 10-20 min. We applied monoclonal anti Smad4 (DPC4) (Biocare Medical USA), monoclonal anti E-cadherin (MU390-UC) (BioGenex USA), and polyclonal anti beta-catenin (Ab-1) (NeoMarkers Lab Vision corp. USA). Sections were incubated with biotinylated antimouse IgG and avidin-biotin peroxidase (Vector Laboratories, Burl-image, CA) and visualized using diaminobenzidine tetra hydrochloride. Negative controls were also performed.

Table 1. Clinicopathological data of the studied CRC patients

Characteristics	*Number of patients*	*%*
Gender		
Male	35	46.3
Female	40	53.7
Average age (years)	69 (±SD 9.2)	
Tumor site		
Ascending	13	17.3
Transverse	7	9.3
Descending	28	37.3
Rectum	27	36
Growth pattern		
Ulcerating	39	46.3
Flat	18	22.2
Polypoid	18	24.1
Dukes stage		
C	21	28
D	54	72
Grade of differentiation		
High	15	20
Moderate	43	57.3
Low	17	22.7

CRC: colorectal cancer, SD: standard deviation

Evaluation and scoring

Adjacent normal mucosa and stromal cells obtained at a distance of more than 1 cm from the tumor were used as internal positive controls. The scale used by Reinacher-Schick and his group was applied for scoring [5]. The intensity of staining was expressed as follows: (i) for Smad4 and E-cadherin, positive staining: score 2, reduced staining: score 1 and no staining: score 0. (ii) For beta-catenin: positive staining: score 2, moderately altered staining: score 1 and strongly altered staining: score 0.

Statistical analysis

The SPSS (14.0) software was applied. Variables were considered as qualitative; frequencies and relative frequencies were calculated. The Spearman coefficient association test was applied to determine the association between group differences. Any p-value less than 0.05 was considered as significant.

Results

Smad4 immunohistochemical expression

Smad4 protein expression was analysed in the 75 cancer specimens. Adjacent to cancer cells, the normal mucosa expressed positive Smad4 staining (score 2).

Cancer cells showed no staining (score 0) in 24 out of 75 (32%) cases. Reduced staining (score 1) and positive/normal staining (score 2) were expressed in 32 (42.6%) and 19 (25.4%) out of the 75 cases, respectively. The results are depicted in Table 2.

Regarding CRC staging we found that in the 21 patients with Dukes stage C cancers Smad4 expression was lost in 7 (33.3%), reduced in 11 (52.4%) and positive in 3 (14.3%). In the 54 patients with Dukes stage D cancers Smad4 expression was also lost in 17 (31.5%), reduced in 21 (38.9%) and positive in 16 (29.6%) as shown in Table 2.

Table 2. Expression of Smad4, E-cadherin and beta-catenin in CRC cases

Expression	*Number of patients*	*%*
Smad4		
Score 0	24	32
Score 1	32	42.6
Score 2	19	25.4
E-cadherin		
Score 0	25	33.3
Score 1	21	28
Score 2	29	38.7
beta-catenin		
Score 0	14	18.6
Score 1	28	37.4
Score 2	33	44

CRC: colorectal cancer

Table 3. Correlation between Smad4, E-cadherin, beta-catenin and clinicopathological parameters

Variables	*p-value*
Smad4	
Sex	0.239
Age	0.605
Location	0.461
Growth pattern	0.403
Dukes stage	0.187
Grade	0.837
E-cadherin	0.000
beta-catenin	0.000
E-cadherin	
Sex	0.494
Age	0.060
Location	0.007
Growth pattern	0.015
Dukes stage	0.093
Grade	0.324
beta-catenin	0.000
beta-catenin	
Sex	0.519
Age	0.367
Location	0.089
Growth pattern	0.590
Dukes stage	0.356
Grade	0.570

No significant association was observed between Smad4 expression and the other clinicopathological parameters i.e. gender, age, tumor location, size and grade, and number of infiltrated lymph nodes (data not shown).

E-cadherin and beta-catenin immunohistochemical expression

The normal mucosal cells adjacent to cancer cells showed a positive staining. E-cadherin expression was lost in 25 (33.3%), reduced in 21 (28%) (Table 2), and positive in 29 (38.7%) of the 75 tumors. No significant association with other clinicopathological parameters was found, the only exception being tumor location (Table 3).

Immunohistochemical staining for beta-catenin in tumor cells membrane displayed absence of staining in 14 (18.6%), reduced staining in 28 (37.4%) (Table 2) and positive expression in 33 (44%) of the 75 resected tumors. No significant association between the expression of beta-catenin and other clinicopathological parameters was found.

The non expression of Smad4 was correlated with the non expression of both E-cadherin ($p<0.0001$) and beta-catenin ($p<0.0001$) (Table 4). Reduced and/or positive expression of Smad4 was associated with positive expression of both E-cadherin and beta-catenin (data not shown).

Table 4. Spearman coefficient correlation of Smad4, E-cadherin and beta-catenin

	Variables	*Spearman (p-value)*
Smad4	E-cadherin	0.63 (p=0.000)
Smad4	beta-catenin	0.62 (p=0.000)
E-cadherin	beta-catenin	0.40 (p=0.000)

Regarding the relationship between E-cadherin and beta-catenin expression, universal agreement was observed in that the non expression of E-cadherin was associated with non expression of beta-catenin and positive expression of E-cadherin with positive expression of beta-catenin ($p<0.0001$) (Table 4).

As regards nuclear expression, beta-catenin was overexpressed in the nuclei of 49 (65.3%) of the 75 specimens studied.

Discussion

Carcinogenesis encompasses multistep alterations of the genetic material, including activation of oncogenes and inactivation of tumor suppressor genes. Recent studies have highlighted the potential role of signaling and interaction between cell components regarding cellular activity in morphological changes and metastasis. In this context, E-cadherin is decreased in invasive CRC [6,7].

It has been reported that loss of E-cadherin in poorly differentiated cancer is more frequent but not to a statistically significant degree [7]. The present clinical study failed to support this association. *In vitro* models also did not support this conclusion [8]. Alteration of E-cadherin expression does not seem to be correlated with clinicopathological parameters apart for tumor location. Namely, E-cadherin expression increases across the way from rectum to ascending colon ($p=0.007$). This progressive alteration of expression may explain the worse prognosis of right sided colonic cancers [9].

Beta-catenin is separated into 3 different subcellular forms, namely membrane, cytosolic and nuclear [10]. Beta-catenin may be degraded or translocated to the nucleus [10,11]. Translocation of beta-catenin from cell membrane to the nucleus is the key trigger in the dysregulation of the signaling pathway which plays an important role in the natural history of CRC [12,13].

In our study we observed nuclear overexpression of beta-catenin in 65.3% of the specimens studied. Expression of E-cadherin and beta-catenin are parallel to each other. This finding is in agreement with the findings of Lugi et al. [14]

Accumulation of nuclear beta-catenin and loss of

membranous E-cadherin have been shown to be associated with tumor progression in CRC [6,7,15].

An association between nuclear beta-catenin and survival rate in CRC has been reported by several authors; nuclear beta-catenin expression was associated with a significantly higher mortality rate in CRC patients [16]. In our study, we noted no significant association between beta-catenin expression and other clinicopathological parameters.

There is some evidence that aberrant expression of nuclear beta-catenin is a marker of aberrant cell signaling [17-19]. However, it is uncertain whether increased nuclear beta-catenin signaling is necessary for developing CRC [10], as at least 20% of CRCs show no nuclear staining on immunohistochemical analysis [18-20].

Immunohistochemical studies of beta-catenin in CRC have shown contradictory results with respect to distribution of nuclear/cytoplasmic/membranous staining and clinical outcome [18,21].

Increased expression of nuclear beta-catenin and loss of membranous E-cadherin are independent adverse prognostic factors [14].

Inactivation of Smad4 by mutations has been found in many human cancers, especially in CRC; it is also observed in pancreatic, prostatic, gastric, biliary, lung and head and neck cancers.

Other workers reported that the frequency of Smad4 mutation increased from 0% in adenoma to 10% in carcinomas, and up to 35% in invasive carcinomas with metastases [1]. Also, 43% of Dukes C and D patients show no expression of Smad4. In the CRC group we studied the results were broadly in agreement with previous findings.

Loss of heterozygosity, promoter hypermethylation and post-transcriptional mechanisms are known to be associated with loss of Smad4 expression in gastrointestinal tumors. Similar phenomena may account for the reduced Smad4 expression in our cases [22].

Regarding Smad4 and beta-catenin, the clinicopathological parameters (gender, age, tumor location, tumor size, grade and number of positive lymph nodes) showed no statistically significant association with immunohistochemical expression.

In this study, we noted that loss of expression of Smad4 was associated with non expression of E-cadherin and beta-catenin, whereas reduced expression of Smad4 seemed to parallel reduced expression of E-cadherin/beta-catenin ($p<0.000$). In addition, positive expression of Smad4 was found to correlate with positive expression of E-cadherin/beta-catenin, which leads to the conclusion that expressional changes of Smad4 are associated with changes of expression of E-cadherin and beta-catenin in the same direction.

Our findings on Smad4 and E-cadherin expression are in agreement with observations on SW480 cell lines [23]. Muller et al. induced re-expression of Smad4 and observed a marked change of the cellular distribution (from mesenchymal to epithelial); they also observed increased expression of E-cadherin and also of P-cadherin, which leads to the expression of beta-catenin within the plasma membrane and to an increase of cell-cell adhesion. Consequently, Smad4 could be the factor activating E-cadherin and P-cadherin transcription, leading to restoration of beta-catenin to the cellular membrane and cell-cell adhesion [23]. Our results are not against this theory.

Other authors have however suggested that, in pancreatic cancer, the Smad4 and E-cadherin relationship is affected by TGF-β regulation, induced by factors Snail and Slug for E-cadherin expression [24].

It may be possible that pancreatic cancer and colorectal cancer may differ in advanced stages as far as Smad4 expression is concerned. Muller et al. did not examine the expression of factor Snail which inhibits the E-cadherin in cells [23].

In conclusion, this retrospective clinical study indicates that expression of Smad4, E-cadherin and beta-catenin is reduced in an important number of clinically advanced CRCs. However, we failed to show any further association of this reduced expression with other pathological or clinical parameters.

References

1. Salovaara R, Roth S, Loukola A et al. Frequent loss of SMAD4/DPC4 protein in colorectal cancers. Gut 2002; 51: 56-59.
2. Maitra A, Molberg K, Albores-Saavedra J, Lindberg G. Loss of Dpc4 expression in colonic adenocarcinomas correlates with the presence of metastatic disease. Am J Pathol 2000; 157: 1105-1111.
3. Kinsella AR, Green B, Lepts GC et al. The role of the cell-cell adhesion molecule E-cadherin in large bowel tumor cell invasion and metastasis. Br J Cancer 1993; 67: 904-909.
4. Hugh TJ, Dillon SA, Taylor BA et al. Cadherin-catenin expression in primary colorectal cancer: a survival analysis. Br J Cancer 1999; 80: 1046-1051.
5. Reinacher-Schick A, Baldus SE, Romdhana B et al. Loss of Smad4 correlates with loss of the invasion suppressor E-cadherin in advanced colorectal carcinomas. J Pathol 2004; 202: 412-420.
6. Roca F, Mauro LV, Morandi A et al. Prognostic value of E-cadherin, beta catenin, MMPs (7 and 9), and TIMPs (1 and 2) in patients with colorectal carcinoma. J Pathol 2006; 208: 91-99.
7. Bravou V, Klironomos G, Papadaki E et al. Over-expression in human colon cancer progression correlates with activation of beta-catenin, down-regulation of E-cadherin and activation of the Akt-FKHR pathway. J Pathol 2006; 208: 91-99.
8. Vleminckx K, Vakaet L Jr, Mareel M et al. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals

an invasion suppressor role. Cell 1991; 66: 107-119.
9. Benedix F, Kube R, Meyer F et al. Comparison of 17,641 patients with right- and left-sided colon cancer: differences in epidemiology, perioperative course, histology, and survival. Dis Colon Rectum 2010; 53: 57-64.
10. Wang NA, Pignatelli M. Beta-catenin-a linchpin in colorectal carcinogenesis? Am J Pathol 2002; 160: 389-401.
11. Kikuchi A. Regulation of beta-catenin signalling in Wnt pathway. Biochem Biophys Res Commun 2000; 268: 243-248.
12. Molenaar M, Van de, Weltering M et al. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell 1996; 86: 391-399.
13. Behrens J, von Kries JP, Kuhl M. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 1996; 382-642.
14. Lugi A, Zlobec I, Minoo P. Prognostic significance of the Wnt signalling pathway molecules APC, β-catenin and E-cadherin in colorectal cancer-a tissue microarray-based analysis. Histopathology 2007; 50: 453-464.
15. Wong SC, Lo ES, Chan AK et al. Nuclear beta-catenin as a potential prognostic marker in patients with colorectal cancer from Hong Kong. Mol Pathol 2003; 56: 347-352.
16. Cheah PY, Choo PH, Yao J, Eu KW, Seow-Choen F. A survival stratification model of human colorectal carcinomas with beta catenin and p27kip1. Cancer 2002; 95: 2479-2486.
17. Morin PJ. Beta-catenin and signalling and cancer. Bioassays 1999; 21: 1021-1030.
18. Kobayashi M, Honma T, Matsuda Y et al. Nuclear translocation of beta catenin in colorectal cancer. Br J Cancer 2000; 82: 1689-1693.
19. Novak A, Hsu SC, Leung-Hagesteijn C et al. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta catenin signalling pathways. Proc Natl Acad Sci USA 1998; 95: 4374-4379.
20. Hugh TJ, Dillon SA, O'Dowd G. Beta catenin expression in primary and metastatic colorectal carcinoma. Int J Cancer 1999; 82: 504-511.
21. Iwamoto M, Ahnen DJ, Franklin WA. Expression of beta-catenin and full-length APC protein in normal and neoplastic colonic tissues. Carcinogenesis 2000; 21: 1935-1940.
22. Wang LH, Kim SH, Lee JH. Inactivation of SMAD4 tumor suppressor gene during gastric carcinoma progression. Clin Cancer Res 2007; 13: 102-110.
23. Muller N, Reinacher-Schick A, Baldus S et al. Smad4 induces the tumor suppressor E-cadherin and P-cadherin in colon carcinoma cells. Oncogene 2002; 21: 6049-6058.
24. Mikami Y, Somei M, Takagi M. A New Synthetic Compound, SST-VEDI-1, Inhibits Osteoblast Differentiation with a Down-Regulation of the Osterix Expression. J Biochem 2009; 145: 239-247.