J Interdiscipl Histopathol 2014; 2(3): 135-144 *ISSN: 2146-8362*

Original Research

Association of Claudin-1 with E-Cadherin/Catenin Complex, Microvessel Density (MVD)-Related Markers, and Clinicopathological Features in Colorectal Carcinoma

Urania Skoufi[1], Dimitrios L Arvanitis[2], Litsa Lampri[3], Elli Ioachim[1], Jim Koutsogiannis[4], Christina Skoufi[5], Dimitris Tsironis[6] and Antigony Mitselou[7]

Departments of Pathology[1], Oncology[5], and Surgery[6], General Hospital "Hatzikostas" Ioannina, Greece
Department of Anatomy[2], University of Thessaly, Greece
Departments of Pathology[3], and Forensic Pathology[7] University Hospital of Ioannina, Greece
Department of Critical Care Medicine[4], University of Alberta, Canada

Received: April 05, 2014

Accepted: April 29, 2014

Published Online: May 27, 2014

DOI: 10.5455/JIHP.20140429121735

Corresponding Author:
Antigony Mitselou, M.D., Ph.D.
Assistant Professor
Department of Forensic Pathology and Toxicology
Medical School, University of Ioannina
Panepistimioupoli, 45110, Greece
E-mail: amitselo@cc.uoi.gr

Keywords: Colorectal cancer; claudin-1; E-cadherin; β-catenin; endoglin; CD31; immunohistochemistry

Abstract

Objectives: Intercellular adhesion mediated by claudin and cadherin/catenin complex is a prerequisite of epithelial integrity and differentiation and has been suggested to be frequently disturbed in cancers. Endoglin (CD105) has been shown to be a more useful marker to identify proliferating endothelium involved in angiogenesis than pan-endothelial markers such as CD31. The aim of this study was to assess the relationship between these markers and clinicopathological features of colorectal carcinomas.

Materials and Methods: Surgical specimens from 69 patients with colorectal cancer were immunostained for claudin-1, E-cadherin, β-catenin, endoglin and CD31.

Results: Forty-six (66.7%), 67 (97.1%), and 67 (97.1%) of the tumors, expressed immunostaining for claudin-1, E-cadherin and β-catenin, respectively. A significant association was seen between claudin-1 and E-cadherin expression ($p=0.002$), as well with β-catenin ($p=0.009$). High β-catenin expression appeared to reduce the risk of poor outcome. Endoglin vessel expression was correlated significantly with vessel invasion ($p<0.0001$), lymph node metastases ($p=0.039$), liver metastases ($p<0.0001$) and recurrence of the disease ($p<0.010$). Endoglin tumor cell expression was associated with E-cadherin and β-catenin expression ($p=0.001$ and $p=0.068$), but not with claudin-1 expression ($p=0.299$).

Conclusions: Loss of the expression of claudin-1, E-cadherin/β-catenin downregulation, and high CD105-MVD counts may play a significant role in the high incidence of angiolymphatic invasion, metastases and prognosis in colorectal-cancer patients, but further studies are needed to clearly define their role in colorectal carcinoma.

INTRODUCTION

The junctional complex is composed of two main structures: tight and adherens junctions. The former is responsible for the barrier function of the epithelia and keeping cell polarity. Tight junction controls two major features of epithelial tissues: play a crucial role in tissue differentiation and homeostasis [1]. Claudins are tight junction's main transmembrane proteins. Up to now, 24 different claudins have been identified in human cells. Epithelial cells present multiple claudins proteins, but some family members exhibit tissue-specific expression patterns [1, 2]. Claudins proteins seem to be important for tight junction formation and play an important role in ionic paracellular permeability control. Indeed, expression of claudin genes is enough to induce tight junction fibrils formation in fibroblast cells [3].

The cellular organization observed in normal differentiated tissues often lost in cancer, where tumor cells frequently exhibit decreased differentiation and cell polarity [4]. These features are important for an invasive phenotype development, and consequently for metastasis [5]. Colorectal cancer is one of the major causes of cancer deaths in the Western world [6]. About 80% of colorectal carcinomas are histologically characterized by adequate gland formation (epithelial polarity) and varying degrees of differentiation, from well-moderate-poorly differentiated [7]. Expression of claudin-1 has shown to be decreased in breast, pancreas and colorectal carcinomas [8-13]. However, there are studies which have demonstrated upregulation of claudin-1 by immunohistochemistry analysis in pancreatic, thyroid and colorectal carcinoma [14-20].

Adherens junction is responsible for calcium mediated hemophilic adhesion between adjacent cells and has cadherins as its major protein molecules [21]. Studies conducting the two past decades have revealed that abnormal expression of cadherins contributes to cancer progression, angiogenesis, cancer cell invasion, and metastasis, under normal conditions. E-cadherin/catenin complex provides cell-cell adhesion. The reduction in E-cadherin induces a positive feedback loop by liberation of β-catenin from the E-cadherin/catenin complex on the cell membrane. These complexes are typically found in the adherens junctions [22]. For a carcinoma to metastasize, cancer cells must first detach from their neighboring cells in the primary tumor. This process necessitates malfunction of the E-cadherin/catenin complex, and indeed, several studies have demonstrated reduced expression of E-cadherin and catenins [23-26] in a variety of carcinomas. All these studies indicate that E-cadherin/catenin-mediated cell adhesion is crucial in the development and progression of human carcinomas [27].

β-catenin, a key element in the Wnt- β-catenin-Tcf/Lef pathway, is found predominantly in 3 locations: at the plasma membrane in a complex with E-cadherin; in the nucleus, where it promotes transcription of target genes together with the Tcf/Lef DNA binding factors; and in the cytoplasm associated with a multiprotein complex formed by GXK3β axin, and APC among other proteins. In colon cancer, E-cadherin down-regulation is linked to enhanced β-catenin-mediated transcriptional activity [28].

Angiogenesis is an important step in the process of cancer growth. It promotes metastatic spread by providing the means of cells to detach from the primary tumor and to travel in the bloodstream to distant metastatic sites. Cancer is always accompanied by angiogenesis at its growths and invading the surrounding tissues, and angiogenesis is a complex of endothelial cell growth and stem cell differentiation brought about by interactions of growth factors and their ligands [29]. Endoglin (CD105) is a receptor for transforming growth factor TGF-β1 and TGF-β3, and it modulates TGF-β signaling by interacting with TGF-β receptor I (TGF-βRI) and/or TGF-β receptor II (TGF-βRII) [29]. CD105 is predominantly expressed on cellular lineages within the vascular system and is overexpressed on proliferating endothelial cells. Several studies indicate that CD105 is involved in the development of blood vessels and that it represents a powerful marker of neovascularization of various types of cancer, including colon cancer [30, 31]. In colorectal cancer (CRC), many reports indicate that endoglin assessed immunohistochemically correlates with MVD, but also with survival curves, and it has been identified as a valuable parameter for predicting increased risk of developing metastatic disease [32]. In other studies, the presence of endoglin also had a prognostic meaning, showing a positive correlation with the presence of angio-lymphatic invasion, lymph node metastases, tumor stage and hepatic metastases, reinforcing the premise that endoglin might be considered for further therapeutic trials as anti-angiogenic therapy [33].

As colorectal cancer is one of the most commonly occurring solid tumors worldwide, we tried to clarify in the present study the pattern of expression of claudin-1 in CRC, by immunohistochemistry method of surgical resected CRC samples. In addition we investigated the association between the expression of claudin-1 and adherens junction markers E-cadherin and β-catenin, as well with neovascularization markers likewise endoglin (CD105) and a conventional pan-endothelial marker (CD31). Furthermore, we also evaluated the relationship between the expression of these markers, and the clinicopathological parameters, as well the patients' prognosis and survival.

MATERIALS AND METHODS

Patients

Sixty-nine colorectal cancer cases, registered at General Hospital "Hatzikostas" Ioannina-Greece between 2003 and 2005, were enrolled in the study group. The study was approved by the local ethics committee. Pathological reports and paraffin blocks were obtained from the pathology archive. Histological diagnosis of tumors was performed according to World Health Organization Classification of Tumours (WHO) criteria [34]. Pathologic staging was performed according to Duke's stage [35]. Every patient underwent an operation. None of these patients received neo-adjuvant chemoradiation therapy before surgery. All tissue samples were formalin-fixed and paraffin-embedded. The corresponding hematoxylin and eosin slides were reviewed by two pathologists (A.M. and U.S.). Each case was classified according to

grade as well, moderately, and poorly differentiated carcinoma, mucinous differentiation, invasion depth, lymphatic invasion, vessels invasion, peritoneal infiltration, lymph node involvement and liver metastasis.

Immunohistochemistry

Immunostaining was performed with a DakoCytomation Autostainer Instrument (DakoCytomation, Denmark). Briefly, 4 μm thick tissue sections were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation with peroxidase-blocking solution (Dakocytomation) for 5 min. Antigen retrieval consisted of autoclave treatment for sections for 30 min in Target Retrieval Solution (pH 6.0, DakoCytomation). The primary antibodies employed were claudin-1 (polyclonal, dilution 1:100; Dako, Glostrup, Denmark); E-cadherin (clone NCH-38, dilution 1:100, Dako); β-catenin (clone 7C2, dilution 1:100; Leyca, Germany); CD31 (clone JC70A, dilution 1:40; Dako); and CD105 (clone SN6h, dilution 1:100, Dako, Denmark). Using an Envision Kit, the slides were incubated with horseradish peroxidase-labeled polymer conjugated with secondary antibody for 30 min and then with substrate chromogen (diaminobenzidine) solution, followed by light counterstaining with Mayer's hematoxylin. In each case normal mucosa was used as an internal positive control for claudin-1, E-cadherin and β-catenin. For negative controls, the primary antibodies were omitted.

Immunohistochemical evaluation

The immunostainig was assessed from numerically coded slides without any knowledge of survival or other clinical data. The slides were reviewed and scored in a blind test by two pathologists (A.M. and U.S.). Differences in interpretation were reconciled by re-review of slides separately or jointly at a double-headed microscope. Caludin-1 expression was evaluated in cell membrane of tumor epithelial cells (Figure 1a). Cytoplasmic or nuclear immunoreactivity for claudin-1 was ignored. The membranous and nuclear β-catenin expression was evaluated separately, and cytoplasmic staining was evaluated as negative. In case of E-cadherin only the membranous staining was considered as positive (Figure 1b). The intensity of claudin-1, E-cadherin and β-catenin was graded semiquantitatively into four groups: 0= negative (absence of staining); 1= weak; 2= moderate; and 3= strong reactivity. For CD31 and CD105 (Figure 1c and 1d), after scanning the immunostained section at low magnification (x40), three areas of tumor with the highest number of distinctly highlighted microvessels ("hot spots") were selected by the two observers at the same time. Then the two observers independently evaluated the slides of microvessel counting using x400 magnification [0.15 mm^2 field] without knowledge of patient's status. Any single cell or spot that stained by immunohistochemical markers was counted as vessel.

Statistical analysis

Comparison of the variables was performed using student's test, Fisher's exact test or Pearson's χ^2 test, depending of the nature of data. Correlation analysis was performed using Spearman's correlation analysis. Survival curves were estimated using the Kaplan-Meier product limit method, and the significance of differences between survival curves was determined using the rank test. Multivariate analysis was performed by Cox proportional hazards regression modeling. All statistical tests were two-sided, and statistical significance was accepted at the $p<0.05$ level. All analyses were performed using SPSS version 16.0.

RESULTS

Clinicopathological features

The mean age of patients on the date when surgery was performed was 64.58 (SD±7.20) years (range from 40 to 81 years). Sixty-nine case were included in the study, 42 of them being male (60.87%) and 27 women (39.13%). In 5 (7.25%) cases tumors were located in the right colon, while 60 (86.95%) tumors were found in the left colon, and 4 (5.80%) tumors at the transverse colon. Tumor size changed between 2.5 and 10.0 cm (mean 5.25 cm SD±1.58). Twenty-four cases (34.78%) were Duke's stage B2, 33 (47.83%) were C2 and 12 cases (17.39%) were Duke's stage D. Three (4.35%) patients had well differentiated tumors, 59 (85.51%) had moderately differentiated, 7 (10.14%) patients had poorly differentiated, and 8 (11.59%) had mucinous type adenocarcinoma. Lymphatic and venous invasion were observed in 64 (92.75%) and 50 (77.96%) cases respectively. Lymph node involvement was observed in 45 (65.22%) and liver metastasis in 10 (10.49%) of cases. The clinicopathological features are summarized in Table 1.

Statistically we found a correlation between grade of differentiation and lymphatic invasion ($p<0.0001$), venous invasion ($p=0.082$), peritoneal infiltration ($p<0.0001$), Duke's stage ($p=0.042$) and liver metastasis ($p=0.068$). A statistical correlation was noted between Duke's stage and lymphatic invasion ($p=0.006$), venous invasion ($p<0.0001$), peritoneal infiltration ($p<0.0001$), lymph node involvement ($p<0.0001$) and liver metastasis ($p<0.0001$). Statistically significant correlations were observed between venous invasion and recurrence of the disease (Fisher's exact test $p=0.032$), between liver metastasis and recurrence (Fisher's $p=0.006$) and between Duke's

stage and recurrence of the disease (Pearson's test p=0.002) (Figure 2).

Table 1: Clinicopathological features of the cases

	n	%
Gender		
Male	42	60.87
Female	27	39.13
Age	40-81(Mean: 64.58 SD±7.20)	
Tumor size	2.5-10 (Mean: 5.25 SD±1.58) cm	
Histology		
Well-differentiated	3	4.35
Moderate	59	85.51
Poor	7	10.14
Type		
Non-mucinous	61	88.41
Mucinous	8	11.59
Lymphatic invasion		
Yes	64	92.75
No	5	7.25
Venous invasion		
Yes	50	72.46
No	19	27.54
Serosal invasion		
Yes	66	95.65
No	3	4.35
Lymph node metastasis		
Yes	45	65.22
No	24	34.78
Peritoneal invasion		
Yes	9	13.04
No	60	86.96
Liver Metastasis		
Yes	10	14.49
No	59	85.51
Dukes' stage		
B2	24	34.78
C2	33	47.83
D	12	17.39
Recurrence		
Yes	21	33.33
No	42	66.67
Death of colorectal cancer		
Yes	12	19.05
No	51	80.95

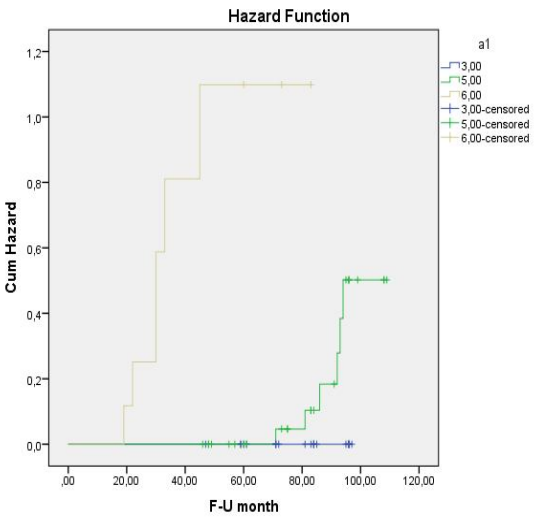

Figure 2: Duke's stage correlates with survival of patients with colorectal carcinoma (Kaplan-Meier curve for survival).

Immunohistochemical findings

In the normal colon mucosa, all the epithelial cells expressed claudin-1 along the cell membrane but not in the cytoplasm. The expression of claudin-1 in epithelial cancer cells was membranous (Figure 1a). In 23 (33.33%) cases the immunoreactivity was weak, moderate in 16 (23.19%) and strong in 7 (10.14%) cases, while 23 cases (33.33%) were negative. A statistically significant relationship was found between claudin-1 expression and histological type of the tumors (Pearson's test p=0.046). The expression pattern of E-cadherin was predominantly membranous in normal colonic epithelium and in tumor area as well. In the present study, CRCs exhibited strong E-cadherin immunoreactivity in 32 (53.6%) cases, moderate in 21 (30.45%) and weak in 16 (23.19%)(Figure 1b). Low expression of E-cadherin was associated with a less differentiated tumor type (p=0.046). Statistically an association was observed between claudin-1 and E-cadherin expression (Pearson's test p=0.002). β-catenin immunohistochemical expression was detected in the membranous and in some cases in the nucleus of carcinoma cells. Specifically, 24 out the 69 (34.78%) were strongly positive, 16 (23.19%) were moderately positive, 27 (39.13%) reacted weakly and 2 patients (2.90%) were negative. Low expression of β-catenin was related with Dukes' stage (p=0.019). Statistically an association was observed between claudin-1 and β-catenin expression (Pearson's test p=0.009). A statistically significant correlation was noted between E-cadherin and β-catenin expression (Fisher's test p<0.0001). Some tumor specimens, regardless membranous expression of β-catenin, had positive staining in the nucleus of tumor epithelia cells. In 27/69 (39.13%) of tumor cases we observed β-catenin expression, whereas no nucleus was stained in normal colonic epithelial cells (p<0.0001). In the whole study population all these cases with tumor cells showing membranous expression of β-catenin had a better overall survival than those without it, however, this difference was no statistically significant (p>0.05) (Figure 3). The nuclear expression did not correlate with any clinicopathological parameter. CD31 immunoreactivity was universally detected in small vessels and capillaries (Figure 1c). Microvessels are represented by brown capillaries or small clusters, which stand out sharply from other tissue. At the tumor site the MVD ranged from 6 to 55 (median 24 SD±8.5). Statistically an association was observed between CD31 expression and grade of differentiation and peritoneal infiltration (Mann-Whitney test, p=0.0018 and p=0.035 respectively). CD105 immunostaining was rarely expressed in normal mucosal vessels (Figure 1d); however, in the cancer tissue, CD105 was intensely expressed in vascular endothelial cells of tumor, ranged from 12 to 79 (median 33 SD±15.7).

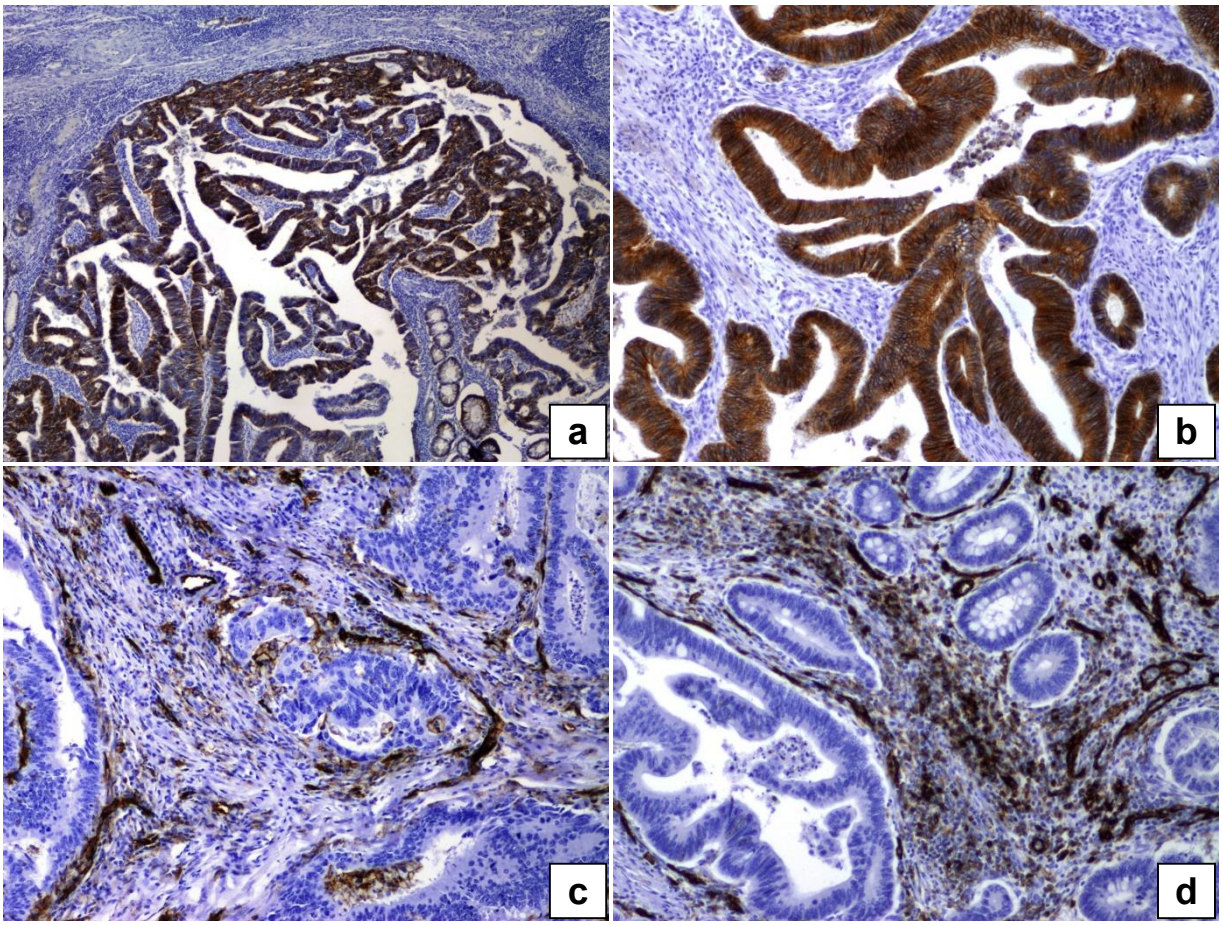

Figure 1: [a]: Strong immunoreactivity of Claudin-1 in colorectal adenocarcinoma (x100). **[b]:** E-cadherin strong immunostaining in well-moderate adenocarcinoma of the colon (x200). **[c]:** CD31 staining in vessels of the stroma in colorectal adenocarcinoma (x100). **[d]:** CD105 immunoexpression in the neo-vasculature of colorectal adenocarcinoma (x200)

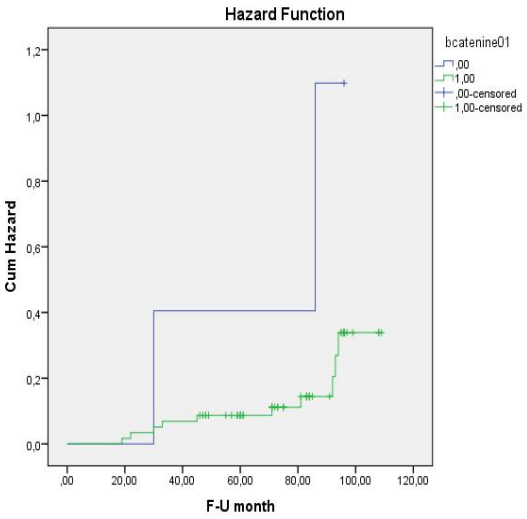

Figure 3: β-catenin expression correlates (borderline) with survival of patients with colorectal cancer (Kaplan-Meier curve).

CD105 was lower in mucinous adenocarcinomas compared with non-mucinous (p=0.01). The CD105 immunoreactivity was also expressed in the cytoplasm of neoplastic epithelial cells. Specifically, 11 out the 69 (15.94%) cases were moderately positive, 19 (27.54%) were weakly positive and 69 (56.52%) cases were negative. A statistical significant relationship was observed between CD105 vessel immunoexpression and venous invasion (Mann-Whitney's test, $p<0.0001$), peritoneal infiltration ($P<0.0001$), liver metastasis ($p<0.0001$), lymph node metastasis (p=0.039), and relapse of the disease after surgical resection and adjuvant chemotherapy (p=0.010). Univariate and multivariate analyses showed positive statistical relation between CD105 MV counts and overall survival ($p<0.0001$), indicative of CD105 as an independent predictor of survival (Figure 4). Statistically a positive correlation was found between CD105 tumor epithelium expression and E-cadherin

($p<0.0001$) and β-catenin ($p=0.068$), but no correlation was observed with claudin-1 expression ($p=0.299$).

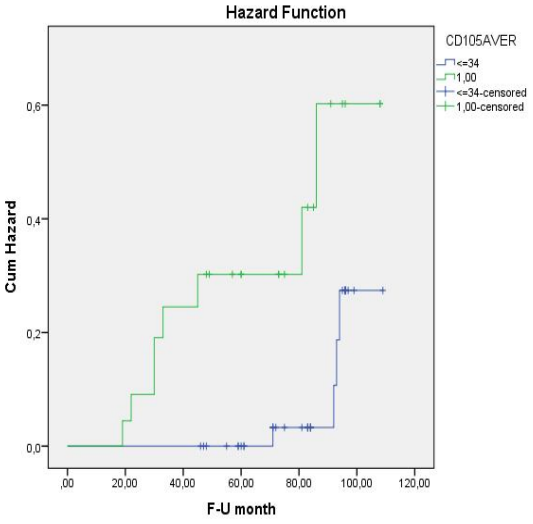

Figure 4: Endoglin (CD105) microvessel density (MVD) correlates significantly with survival of patients with colorectal cancer (Kaplan-Meier survival curve)

DISCUSSION

Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer death worldwide [6]. The main prognostic factors in colorectal cancer are tumor size, lymph node involvement, grade of differentiation and distant disease spread [34, 35]. Other important factors include invasion of blood and/or lymphatic vessels and penetration or perforation of the bowel wall [34, 35]. Molecular markers may improve clinicopathologic staging and provide basis to guide novel therapeutic strategies which target specific tumor-associated molecules according to individual tumor biology, however, so far, no ideal molecular marker has been found to predict disease progression [36]. This study is a continuation of our efforts to further elucidate the biology of CRC and to identify more effective prognostic factors than the traditional staging system to aid therapeutic decision-making [37-39]. To our knowledge there are no reports concerning the correlation between claudin-1, E-cadherin/β-catenin complex expression and neoangiogenesis related markers (e.g. CD105 and CD31).

Tight junction associated proteins including the large family of claudins are critical for maintaining cell-cell adhesion in epithelial cells [1]. For some of the family member, it has been shown that they also play critical roles in proliferation and tumor signaling pathways [4, 5]. However, their role in cancer progression remains largely unexplored. Several claudins have shown to be differentially expressed in various types of cancer and have been suggested as possible biomarkers and targets for cancer therapy [8, 9, 15]. Differing data have been published concerning claudin-1 expression in colorectal tumors, describing both downregulation as well as overexpression of the protein. More frequently increased protein levels were found in adenomas and adenocarcinomas [16-18]. In an immunohistochemical study of de Oliveria et al, claudin-1 expression has been shown to be increased in neoplastic tissue compared to normal colon mucosa [16]. Similarly, in the study of Dhawan et al, claudin-1 staining was been found to be significantly increased in neoplastic colonic cells and in metastatic lesions as compared with normal colon epithelium while localization has changed from cell membrane to cytoplasm and nucleus. Both the density and expression rate of claudin-1 was more prominent in tumor cells. Another interesting finding in that study was the nuclear staining of claudin-1 in 58% of liver and 35% of lymph node metastasis [40]. Huo et al, in their study demonstrated that the expression of claudin-1 at the mRNA and protein levels was found increased in the CRC tissue in comparison to that in the normal tissue specimens [18]. In our study, 66.7% of the cases expressed immunoreactivity to claudin-1, and only in 10.14% of the cases the staining was strong, so there are downregulation of claudin-1 expression in CRC tissues, in agreement with other reports [10-13]. It is logical to expect this down-regulation because tumorigenesis is accompanied by disruption of tight junctions, with resultant loss of cohesion, invasiveness and lack of normal process of differentiation. We also noted no immunoreactivity of claudin-1 in poorly differentiated malignancies and mucinous adenocarcinomas in contrast to well-differentiated tumors that exhibit a claudin profile that closely resembles normal tissue, in line with other reports [10-13]. The decreased of diminished expression of claudin-1 in the small group of mucinous cases may also be related to the loss of cohesion as stated before.

In CRC, there are reports that correlate claudin-1 with some clinicopathological features, while others such no find such a correlation. In particular, it has been shown that claudin-1 overexpression was correlated with grade of differentiation [10-13, 40], morphological type [40], tumor size [40], tumor invasion [13, 40], venous invasion [13, 40] and metastasis [13, 40]. However, other investigators did not find any correlation of caludin-1 expression with tumor size [12, 17, 18], depth of invasion [12], venous invasion [12], lymph node metastasis [18] or liver metastasis [12]. In the present work, although the number of cases was limited, we observed an association between claudin-1 expression and the histological type of the tumor, in the way that percentage of tumor cells was higher in non-mucinous that in mucinous ($p=0.046$) cancers, in accordance with other report [41]. We also failed to

demonstrate any correlation between expression of claudin-1 and tumor differentiation (p=0.820), Dukes' stage (p=0.72), poor outcome (p=0.701) and/or other clinicopathologic parameter. The discrepancies might be explained by differences in methodology, the case numbers, and antibodies used.

The adhesion of cells is a complex interaction between different adhesion molecules such as cadherins and catenins. Decreased levels of immunohistochemically detectable E-cadherin has been shown in colorectal carcinoma [20, 25]. Previous immunohistochemical studies have described abnormal expression of E-cadherin in the majority of the CRCs. Most studies show an overall decrease in the expression of E-cadherin compared to adjacent normal mucosa [20, 26], in agreement with our results. Several studies have demonstrated that loss of E-cadherin expression is associated with tumor size, histopathology, growth patterns, and worse prognosis [20, 25, 26, 42]. On the other hand, other authors failed to demonstrate any correlation between and/or expression of E-cadherin with conventional staging, tumor differentiation, invasive metastatic potential or prognosis [43], in line with our results. The discrepancies might be explained by differences in methodology or antibodies used, and the number of cases. Although E-cadherin acts as an invasion-suppressor gene, the lack of correlation with standard prognostic factors may be evidence that interaction between E-cadherin and colorectal tumor behavior is complicated [42, 43]. Decreased β-catenin expression has been shown to disrupt homotypic cell adhesion and contributes to cellular motility and invasiveness, and a variable expression of β-catenin in CRCs indicates tumor progression [25, 26, 43]. Horkko et al, found that the intensity of nuclear immunostaining of β-catenin was increased at the invasive margin in advanced Duke's stage tumors. However, there was no association between β-catenin intensity and tumor budding [44]. Lugli et al, have shown that nuclear β-catenin expression and loss of membranous E-cadherin expression can be independent adverse prognostic factors in CRC [45]. In the present study, we found a significant disturbed distribution of both E-cadherin and β-catenin immunoreactivity in CRC compared to normal epithelial cells. Statistically a significant relationship was found between E-cadherin and β-catenin expression (p<0.0001) in CRC, indicating a strong association between these two molecules. In addition we observed a positive correlation between E-cadherin and claudin-1 expression (p=0.001) and between β-catenin and claudin-1 (p=0.008) indicating that the expression of claudin-1, E-cadherin and β-catenin was similarly mediated in the CRC tissue specimens. These results suggest that some common signaling pathways might regulate tight and adherence junctions. In the present study, strong expression of membranous staining of β-catenin indicates better patients' outcome. This association in our study although statistically is not significant, clearly indicates an obvious susceptibility. Statistical significance may be reached if additional cases can be recruited. Previous immunohistochemical studies of β-catenin in colorectal cancers have shown contradictory results with respect to nuclear staining and clinical outcome. In some cases, nuclear β-catenin staining was predictive of worse survival [45, 46], but others did not produce any supportive evidence [47], in line with the present investigation. Differences in results are probably due to case numbers, variation in antigen retrieval, staining procedures and evaluation systems.

MVD has been found to be higher in primary tumor compared with the corresponding normal colorectal tissue. However, the significance of MVD in prediction of tumor aggressive behavior and patients' prognosis remains controversial, probably due to the difference in selection of endothelial markers or technique of MVD quantification [30-32]. In the present study, we assessed MVD with PCAM-1 (CD31) marker in CRC tissues. Consistent with previous studies [30-32], we found a significant increase of MVD in CRCs compared with their corresponding normal mucosa. We did not see a correlation between tumor stage (p=0.32) or patients' survival (p=0.83), which is in agreement with other reports [30, 32]. An interesting finding in the present work is the association of CD31-MVD with the grade of differentiation (p=0.018) and peritoneal infiltration (p=0.035). The Kaplan Meier survival analysis indicates that MVD obtained using CD31 showed no significant correlation with survival, so CD31 was not correlated with prognosis, in accordance with other studies [32, 33].

Endoglin (CD105) is mainly expressed on proliferating endothelial cells and consequently better reflects the occurrence of new vessels, and is over-expressed on tumor-associated vascular endothelium [29, 31]. It may thus represent a more specific and sensitive marker for tumor angiogenesis than the commonly used panedothelial markers CD31, CD34, or von Willebrand factor. In our study, endoglin microvessel immunostaining was consistently present in all the cases studied. CD105 stained small vessels with high sensitivity in the tumor, but the blood vessels in normal tissue did not stained with CD105, this is in agreement with previous studies where CD105 was expressed mainly in proliferating blood vessels [30, 32, 33] In the present report, using multivariate analysis, microvessel count by CD105 showed a statistically significant correlation with the presence of vessel invasion (p<0.0001), peritoneal invasion (p<0.0001), lymph node metastases (p<0.039) and liver metastases

($p<0.0001$), independent of tumor stage. This is consistent with other studies that showed that microvessel count is an independent prognostic factor [48, 49], reinforcing the premise that CD105 might be considered for further therapeutic trials as anti-angiogenic therapy. It has been demonstrated that accumulation of mRNA for CD105 is up-regulated in colon carcinoma tissues, in comparison with normal and dysplastic colon mucosa. This overexpression of CD105 is positively correlated with disease progression, confirming that the CD105-MVD is a prognostic marker in colon carcinoma [49, 50], in line with our observations. We noted that CD105-MVD was correlated with relapse of the disease after surgical resection and adjuvant chemotherapy ($p<0.010$).

Other interesting finding in the present report, is the presence of CD105 in cancer epithelial cells and its association with E-cadherin and b-catenin ($p<0.0001$ and $p<0.068$ respectively). Recently, CD105 has been reported to play a role in the regulation of adhesion, motility and invasion of normal and transformed cancer cells. It has been suggested that loss of endoglin (CD105) in cancer cells causes cell detachment and may be associated with cancer progression [51].

Endoglin (CD105) is not only expressed in the cell surface but its soluble form can also be detected in the blood. Takahashi et al, [52], observed that increased serum endoglin was associated with metastasis in patients with solid tumors including colorectal and breast carcinomas; and, in CRC patients, the difference in endoglin levels between the metastasis-negative patients and the metastasis-positive patients was statistically significant.

We showed in this study that: a) down-regulation of claudin-1 in CRC was correlated statistically with E-cadherin and β-catenin; b) claudin-1 expression was associated with the histological type of the tumor; c) β-catenin expression appeared to reduce the risk of poor outcome of the patients with CRC; d) Endoglin (CD105) expression was associated with venous invasion, lymph node and liver metastases; e) CD105 tumor cells expression was related with E-cadherin and β-catenin, but not with claudin-1; and f) increased CD105-MVD was a strong predictor of disease recurrence and poor patients' survival. Further investigations are required to understand better the roles of claudin-1, E cadherin, β-catenin, and endoglin in colorectal cancer progression and malignancy, and to determine how dysregulation of these proteins may alters responses to therapeutic intervention.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

We thank the Department of Oncology of the University Hospital of Ioannina for providing the medical files of patients, and Mrs. Ariana for her technical assistance.

AUTHOR'S CONTRIBUTIONS

US carried out the immunohistochemistry and drafted the manuscript. EI, CS, and DT participated in collecting samples and clinical data. LL and JK analyzed the data (statistics). AM designed the study and finalized the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. Prog Biophys Mol Biol 2003; 81:1-44.
2. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2001; 2: 285-293.
3. Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occluding in fibroblasts. J Cell Biol 1998; 143: 391-401.
4. Soler AP, miller RD, Laughlin KV, Carp NZ, Klurfeld DM, Mullin JM. Increased tight permeability is associated with the development of colon cancer. Carcinogenesis 1999; 20: 1425-1431.
5. Martin TA, Jiang WG. Tight junctions and their role in cancer metastasis. Histol Histopathol 2001; 16: 1183-1195.
6. Peto J. Cancer epidemiology in the last century and the next decade. Nature 2001; 411: 390-395.
7. Riddell RH, Petras RE, Williams GT, Sobin LH. Tumors of the Intestines (Atlas of Tumor Pathology (AFIP) 3rd Series) American Registry of Pathology, Washington DC, pp 133-189, 2002.
8. Tokes AM, Kulka J, Paku S, Szik A, Packa C, Novak PK, Szilak K, Kiss A, Schaff A. Claudin-1, -3, and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. Breast Cancer Res 2005; 7: 296-305.
9. Tsukuhara M, Nagai H, Karmiakito T, Kawata H, Takayashiki N, Saito K, Tanaka A. Distinct expression pattern of claudin-1 and claudin -4 in intraductal papillary mucinous tumors of the pancreas. Pathol Int 2005; 55: 63-69.
10. Resnick MB, Konkin T, Routhier J, Sabo E, Pricolo VE. Claudin-1 is a strong prognostic indicator in stage II colonic cancer: a tissue microarray study. Mod Pathol 2005; 18: 511-518.

11. Matsuoka T, Mitomi H, Fukui N, Kanazawa H, Saito T, Hayashi T, Yao T. Cluster analysis of claudin-1 and -4, E-cadherin, and b-catenin expression in colorectal cancer. J Surg Oncol 2011; 103: 674-686.
12. Yoshida T, Kinugasa T, Akagi Y, Kawahara A, Romeo K, Shiratsuchi I, Riju Y, Gaotanda Y, Shirouzu K. Decreased expression of claudin-1 in rectal cancer: a factor for recurrence and poor prognosis. Anticancer Res 2011; 31: 2517-2525.
13. Shibutani M, Noda E, Maeda K, Nagahara H, Ohtani H, Hirakawa K. Low expression of claudi-1 and presence of poorly differentiated tumor clusters correlate wit poor prognosis in colorectal cancer. Anticancer Res 2013; 33: 3301-3306.
14. Iacobuzio-Donahue CA, Maitra A, Shen-Ong GI, van Heek T, Ashfaq R, Meyer R, Walter K, Berg K, Hollingsworth MA, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Discovery of novel tumor markers of pancreatic cancer using global gene expression technology. Am J Pathol 2002; 160: 1239-1249.
15. Fluge O, Bruland O, Akslen LA, Lillehaug JR, Varlang JE. Gene expression in poorly differentiated thyroid carcinomas. Thyroid 2006; 16: 161-175.
16. de Oliveria SS, de Oliveria IM, DeSouza V, Morgado-Diaz JA. Claudins: up-regulation in human colorectal cancer. FEBS Lett 2005; 279: 6179-61-85.
17. Gröne J, Weber B, Staub E, Heinze M, Klaman I, Pilarsky C, Hermann K, Castanos-Velez E, Röpcke S, Mann B, Rosenthal A, Buhr HJ. Differential expression of genes encoding tight junction proteins in colorectal cancer: frequent dysregulation of claudin-1, -8, and -12. Int J Colorectal Dis 2007; 22: 651-659.
18. Huo Q, Kinugasa T, Wang L, Huang J, Zhao J, Shibaguchi H, Kuroki M, Tanaka T, Yamashita Y, Nabeshima K, Iwasaki H, Kuroki M. Claudin-1 protein involved in the tumorigenesis of colorectal cancer. Anticancer Res 2009; 29: 851-858.
19. Mess ST Mennigen R, Spicker T, Rijcken E, Senninger N, Hafer J, Bruewer M. Expression of tight and adherens junction proteins in ulcerative colitis associated colorectal carcinoma: upregulation of claudin -1, claudin-3, claudin-4 and β-catenin. Int J Colorectal Dis 2009; 24: 361-368.
20. Bezdekova M, Brychtova S, Sedlakova E, Langova K, Brychta T, Belej K. Analysis of Snail-1, E-cadherin and claudin-1 expression in colorectal adenomas and carcinomas. Int J Mol Sci 2012; 13: 1632-1643.
21. Niessen CM, Gottardi CJ. Molecular components of the adherens junctions. Biochem Biophys Acta 2008; 1778: 562-571.
22. Wheelock MJ, Johnson KR. Cadherins as modulators of cellular phenotype. Annu Rev Cell Dev Biol 2003; 19: 207-235.
23. van Aken E, De Wever O, Correia de Rocha AS, Marcel M. Defective E-cadherin/catenin complexes in human cancer. Virchows Aech 2001; 439: 725-731.
24. Takayama T, Shiozaki H, Shibamoto S, Oka H, Kimura Y, Tamura S, Inoue M, Monden T, Ito F, Monden M. Beta-catenin expression in human cancers. Am J Pathol 1996; 148: 39-46.
25. Hiscox S, Jiang WG. Expression of E-cadherin, alpha, beta and gamma catenin in human colorectal cancer. Anticancer Res 1997; 17: 1349-1354.
26. Ghadimi BM, Behren J, Hoffmann I, Haeusch W, Birchmeier W, Schlag PM. Immunohistological analysis of E-cadherin, alpha-, beta- and gamma-catenin expression in colorectal cancer: implication for cell adhesion and signaling. Eur J Cancer 1999; 35: 60-65.
27. Takeichi M. Cadherins in cancer: implications for invasion and metastasis. Curr Opin Cell Biol 1993; 5: 806-811.
28. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Ann Rev Cell Dev Biol 2004; 20: 781-810.
29. Duff SE, Ki C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. FASEB J 2003; 17: 984-992.
30. Akagi K, Ikeda Y, Sumiyoshi Y, Kimura Y, Kinoshita J, Miyazaki M, Abe T. Estimation of angiogenesis with anti-CD105 immunostaining in the process of colorectal cancer development. Surgery 2002; 131: S109-S113.
31. Fonsatti E, Sigalotti L, Arslan P, Altomonte M, Maio M. Emerging role of endoglin (CD105) as a marker of angiogenesis with clinical potential in human malignancies. Curr Cancer Drug Targets 2003; 3: 427-432.
32. Saad RS, Liu YL, Nathan G, Celebrezze J, Medich D, Silverman JF. Endoglin (CD105) a vascular endothelial growth factor as a prognostic marker in colorectal cancer. Mod Pathol 2004; 17: 197-203.
33. Romani AN, Borghetti AF, Del Rio P, Sianesi M, Soliani P. The risk of developing metastatic disease in colorectal cancer. J Surg Oncol 2006; 93: 446-455.
34. Hamilton SR, Honen A. Pathology and genetics tumors of the digestive system. World Health Organization Classification of Tumors. IARC Press, Lyon, 2000.
35. Dukes CE, Bussey HJR. The spread of rectal cancer and its effect in prognosis. Br J Cancer 1958; 12: 309-320.
36. Cascinu S, Georgoulias V, Kerr D, Manghan T, Labianca R, Ychou M. Colorectal cancer in the adjuvant setting: perspectives on treatment and the role of prognostic factors. Annu Oncol 2003; 14 Suppl 2 :ii25-ii29.
37. Mitselou A, Ioachim E, Skoufi U, Tsironis D, Nesseris I, Lampri E, Vougiouklakis T, Briassoulis E, Arvanitis LA. Association between thrombospondin-1, angiogenesis related markers, and extracellular matrix components with colorectal cancer outcome. The Open Journal of Colorectal Cancer 2012, 5:38-46
38. Mitselou A, Skoufi U, Briasoulis E, Arvanitis D, Vougiouklakis T, Tsimofiannis KE, Ioachim E. Association of syndecan-1 with angiogenesis related markers, extracellular matrix components, and clinicopathological features in colorectal carcinoma. Anticancer Res 32: 3977-3985, 2012.
39. Mitselou A, Ioachim E, Skoufi U, Tsironis C, Tsimogiannis K E., Skoufi C, Vougiouklakis T, Briasoulis E. Predictive Role of Thymidine Phosphorylase Expression in Patients with Colorectal Cancer and its Association with Angiogenesis-related Proteins and Extracellular Matrix Components. In vivo 26: 1057-1068, 2012.
40. Dhawan P, Singh AB, Deane NG, No YR, Shiou SR, Schmidt C, Neff J, Washington MK and Beauchamp RD. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest 2005; 115: 1765-1776.

41. Ersoz S, Mungan S, Cobanoglu U, Turgutalp H, Ozoran Y. Prognostic importance of Claudin-1 and Claudin-4 expression in colon carcinomas. Pathol Res Prac 2011; 201: 285-289.
42. Elzagheid A, Buhmeida A, Laato M, El-Faitori O, Syrjanen K, Collan Y, Pyrhonen S. Loss of E-cadherin expression predicts disease recurrence and shorter survival in colorectal carcinoma. APMIS 2012; 120: 539-548.
43. Buhmeida A, Elzagheid A, Algais A. Expression of the cell-cell adhesion molecule β-catenin in colorectal carcinoma and their metastasis. APMIS 2008; 116: 1-9.
44. Hörkkö TT, Klintrup K, Mäkinen JM, Näpänkangas JB, Tuominen HJ, Mäkelä J, Karttunen TJ, Mäkinen MJ. Budding invasive margin and prognosis in colorectal cancer-no direct association with beta catenin expression. Eur J Cancer 2006; 42: 964-971.
45. Lugli A, Zlobec I, Minoo P, Baker K, Tornillo L, Terraciano L, Jass JR. Prognostic significance of the wnt signaling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: a tissue microarray-based analysis. Histopathology 2007; 50: 453-464.
46. Wong SCC, Lo ESF, Chan AKC, Lee KC, Hsiao WL. Nuclear beta catenin as a potential prognostic marker in patients with colorectal cancer from Hong Kong. J Clin Pathol Mol Pathol 2003; 56: 347-352.
47. Chung GG, Provost E, Kielhorn EP, Charette LA, Smith BL, Rimm DL. Tissue microarray analysis of β-catenin in colorectal cancer shows nuclear phosphor-β-catenin is associated with a better prognosis. Clin Cancer Res 2001; 7: 4013-4020.
48. Minhajat R, Mori D, Yamasaki F, Sugita Y, Satoh T, Tokunaga O. Endoglin (CD105) expression in angiogenesis of colon cancer: analysis using tissue microarrays and comparison with other endothelial markers. Virchows Arch 2006; 448: 127-134.
49. Choi HJ, Hyun MS, Jung GJ, Kim SS, Hong SH. Tumor angiogenesis as a predictor in colorectal carcinoma with special reference to mode of metastasis and recurrence. Oncology 1998; 55: 575-581.
50. Bellone G, Granigui C, Vizio C, Mauri FA, Prati A, Solero D, Dughera L, Ruffini E, Gasparri G, Camandona M. Abnormal expression of endoglin and its receptor complex (TGF-β1 and TGF-β receptor II) as early angiogenic switch indicator in premalignant lesions of the colon mucosa. Int J Oncol 2010; 37: 1153-1165.
51. Liu Y, Jovanovic B, Pins M, Lee C, Bergan RC. Overexpression of endoglin in human prostate cancer suppresses cell detachment, migration and invasion. Oncogene 2002; 21: 8272-8281.
52. Takahashi N, Kawanishi-Tabata R, Haba A Tabata H, Haruta Y, Tsai H, Sean BK. Association of serum endoglin with metastasis in patients with colorectal, breast, and other tumors, and suppressive effect of chemotherapy on the serum endoglin. Clin Cancer Res 2001; 7: 524-532.