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Loss of E-cadherin expression predicts disease recurrence and shorter survival in colorectal carcinoma

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The traditional staging system is currently inadequate for identifying those patients with colorectal carcinoma (CRC) who carry a high risk for poor outcome. In this study, the expression of E-cadherin was evaluated in CRC to determine its correlation with clinico-pathological variables, and association with disease outcome in patients with long-term follow-up. The present series consisted of tissue samples obtained from 230 patients with stage I, II, III, or IV CRC treated during 1981–1990 at Turku University Hospital. Archival paraffin-embedded samples were used to build up tissue microarray blocks, and E-cadherin expression was assessed by immunohistochemistry using an automated staining system. Different grading systems were tested for expression of E-cadherin. Fifty-nine percent of all tumors were positive for E-Cadherin. There was no significant correlation between E-cadherin expression and gender ($p < 0.83$), localization ($p < 0.45$), tumor invasion ($p < 0.32$), or histologic grade ($p < 0.41$). However, loss of E-cadherin expression was significantly associated with older age ($p < 0.03$) and lymph node involvement ($p < 0.02$), and with borderline significance with advanced stage ($p < 0.09$) and tumor metastasis ($p < 0.09$). In univariate (Kaplan–Meier) survival analysis, positive E-cadherin significantly ($p = 0.009$) predicted longer disease-free survival (DFS), and the same was true with disease-specific survival (DSS) as well ($p = 0.007$). In multivariate (Cox) survival analysis, E-cadherin retained its significance as independent predictor of DFS (HR = 1.56; 95% CI 1.01–2.42, $p = 0.043$), but not DSS. A sub-group analysis revealed that E-cadherin expression also predicts DFS ($p < 0.01$) and DSS ($p < 0.04$) in stage II CRC. Our results implicate the usefulness of E-cadherin expression in predicting disease recurrence and long-term survival in CRC.

Key words: E-cadherin expression; colorectal cancer; prognosis; recurrence; adjuvant therapy.

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The traditional staging system is currently inadequate for identifying those patients with colorectal carcinoma (CRC) who carry a high risk for poor outcomes, and this might lead to potential under-treatment or over-treatment in many situations (1). Thus, there is a need to identify more effective predictors at genomic and proteomic levels than the traditional staging system to aid therapeutic decision-making (2, 3). Unfortunately, there has been no major improvement in patient survival despite the advances made in our understanding of the risk factors and pathogenesis as well as in development of new chemotherapy practices (4). In fact, approximately, 40–60% of CRC patients who

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undergo resection for potential cure will have advanced loco-regional disease and are classified as either stage II or stage III (5). However, tumors of the same stage can follow significantly different clinical courses, indicating a necessity for identification of novel prognostic factors, including molecular markers (6, 7) e.g., for better targeting of the treatment options. It has been shown that if diagnosed at an early stage, CRC is a potentially curable disease (8). Therefore, it is also important to identify clinically useful biomarkers that can detect CRC at an early stage.

Studies conducted during the past two decades have revealed that abnormal regulation of cadherins contributes to cancer progression, angiogenesis, cancer cell invasion, and metastasis. Therefore, cadherins and their regulators are potential candidates for diagnostic and prognostic predictors as well as possible therapeutic targets (9). The most compelling data for involvement of the cadherin family in cancer progression are available for E-cadherin. The causal relationship between E-cadherin dysfunction and cancer progression has been convincingly shown both *in vitro* and *in vivo*. Furthermore, the clinical relevance of E-cadherin deficiency has been confirmed by immunohistochemical means in most human cancers (10).

Under normal conditions, E-cadherin-catenin complex provides cell-cell adhesion. E-cadherin protrudes outside the cell membrane and adheres to E-cadherin from neighboring cells through calcium-dependent homophilic interaction. 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 (11). These complexes are typically found in the adherens junctions (12, 13), but also elsewhere (14, 15). 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 (16–18) and catenins (19–22) 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 (23), and E-cadherin acts as an invasion and metastasis suppressor molecule in cancer (24, 25).

As it was not too extensively studied, we evaluated E-cadherin expression in a series of CRC, and its relationships with several clinicopathological features, disease recurrence and long-term outcome.

PATIENTS AND METHODS

The present series consisted of tissue samples obtained from 230 patients with stage I, II, III, or IV CRC who had undergone bowel resection during 1981–1990 at Turku University Hospital (TUH), available for study at the archives of the Department of Pathology. Immunohistochemical staining was done at the Department of Pathology, Garyounis University, Benghazi, Libya. All pertinent clinical and histopathologic data of the patients were collected from the patients' case records and summarized in Table 1. All patients have been prospectively followed-up until death or when last seen alive at their clinical visit (March 2007), with the median FU-time of 77.0 months (range 2.0–263 months). The study was approved by the TUH Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Samples were collected with the endorsement of the National Authority for Medico-legal Affairs.

Tissue microarray (TMA)

Archival paraffin-embedded CRC samples were used to build up TMA blocks for immunohistochemical staining. Areas of invasive tumor with the lowest degree of differentiation, abundant in cells with the highest number of mitoses were chosen from the original blocks. Necrotic and autolytic areas and areas containing predominantly the stromal tissue were avoided. For tumors producing abundant intra- or extra-cellular mucin, invasive areas with the highest number of epithelial cells were chosen. These representative areas were marked by an experienced pathologist on hematoxylin and eosin (H&E)-stained slides from selected paraffin blocks, and a cylinder of tissue 1 mm in diameter was cut with a TMA instrument (Beecher Instruments, Sun Prairie, WI, USA) into a new paraffin block. This size of tissue section (1-mm wide) was equal to the often used three cores, 0.6-mm wide (26–29). As the core was larger than usual, sampling differences were less than in 0.6 mm cores. Serial 4-μm sections were then cut from the TMA paraffin blocks. The sections were mounted on ChemMate^{TM} Capillary Gap plus Slides (Grey) by Dako, Glostrup, Denmark. Normal colorectal mucosa was selected adjacent to, but at least 2-mm apart from the malignant tissues of the section. If available, another normal sample was obtained from normal colorectal mucosa at either of the resection

Table 1. Clinicopathologic characteristics of the patients

Characteristic	No. of patients (%)
Gender	
Male	108 (47)
Female	122 (53)
Age (years)	
< 65 years	102 (44)
> 65 years	128 (56)
Primary tumor status	
T1	9 (4)
T2	22 (10)
T3	131 (57)
T4	68 (29)
LNN involvement	
No	159 (69)
Yes	71 (31)
Metastasis	
No	194 (84)
Yes	36 (16)
Stage	
I	30 (13)
II	129 (56)
III	35 (15)
IV	36 (16)
Histologic grade	
Gr I	35 (15)
Gr II	171 (74)
Gr III	24 (11)
Localization	
Right colon	68 (30)
Left colon	75 (33)
Rectum	87 (37)
Recurrence during the follow-up	
Yes	85 (37)
No	118 (51)
Unknown	27 (12)
Status at the end of follow-up	
Alive	73 (32)
Dead as result of disease	118 (51)
Dead from other cause(s)	39 (17)

margins in the surgical specimens. So, usually two normal controls were available. Lymphatic follicles and hyperplastic and inflamed areas were avoided. To obtain enough mucosa for tissue array, tangentially cut areas were avoided.

E-cadherin immunostaining

Formalin-fixed, paraffin-embedded primary colorectal tumor tissue was obtained from 230 patients. Sections were cut serially at 5 μm for routine HE staining and for immunohistochemical (IHC) analysis. IHC analysis was done using the automatic system (BenchMark XT; Ventana Medical Systems, Inc. Tucson, AZ, USA). This fully automated processing of bar code-labeled slides included baking of the slides, solvent-free deparaffinization, antigen retrieval in a cell conditioning buffer CC1 (Mild: 36 min conditioning, and standard: 60 min conditioning), incubation with (the monoclonal anti-E-cadherin antibody (clone ECH-6; Ventana Medical Systems), for 32 min, at 37 °C. Application of ultraView^{TM} Universal DAB (a biotin-free, Multimer-based detection system for the specific and sensitive detection of mouse IgG, mouse IgM, and rabbit IgG primary antibodies). UltraView DAB includes: ultraView Universal HRP, ultraView Universal DAB Inhibitor, ultraView Universal DAB Chromogen, ultraView Universal DAB H_2O_2, and ultraView Universal DAB Copper. Counterstaining with hematoxylin (2021) took 4 min, and post-counterstaining with bluing reagent (2037) took 4 min as well. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

Evaluation of E-cadherin staining

The evaluation of staining of all TMAs was performed with a light microscope at the magnification of ×40, blinded by the information on tumor grade, stage or clinical outcome. The typical expression patterns of E-cadherin are illustrated in Fig. 1. Three different grading (A, B, and C) systems were applied to assess the patterns of E-cadherin expression in tumor cells. In system A, the membranous staining was graded into four categories: (0) no expression, no detectable staining in < 10% of the membrane (1) weak, but detectable discontinuous staining present in 10–39% of the membranes (2) moderate, clearly positive discontinuous staining present in 40–90% of the membranes and (3) intense continuous staining of the membrane creating a honeycomb pattern (30). In system B, cytoplasmic staining was graded in two categories: (i) no/weak expression and (ii) moderate/strong expression. Finally, in grading C, E-cadherin expression was categorized simply as negative or positive. All three systems were statistically tested, and the negative/positive grading (C) seemed to provide the most meaningful correlates of E-cadherin with the clinically relevant data.

In calculating the staining indexes: membrane index, the intensity of staining and the fraction of positively stained cells were taken into account, using the following formula:

$$I = 0 \times f0 + 1 \times f1 + 2 \times f2 + 3 \times f3$$

where I is the staining index, f0–f3 are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and 3 (31).

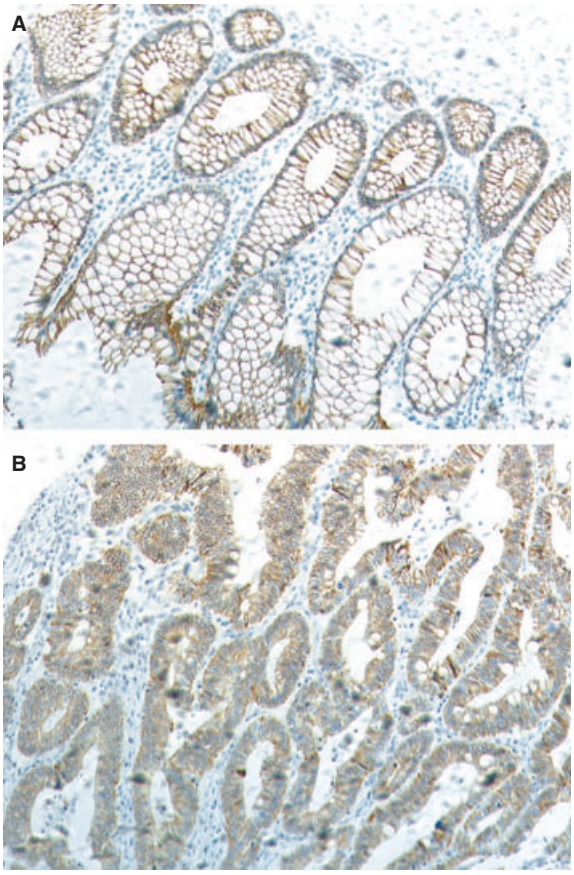

Fig. 1. E-cadherin expression patterns in CRC. (A) Expression of Ecadherin in normal colonic epithelium. (B) Expression of E-cadherin in primary colorectal carcinoma.

Three different grading systems to evaluate the expression of E-cadherin were tested; in addition to the above described 4-grade system, two other two grade systems were applied: (i) negative/weak *vs*. moderate/strong, and (ii) negative *vs*. positive. The latter grading system proved to be most useful and was adopted for all statistical calculations. To ensure reproducibility, random measurements of some samples were tested twice by one of the observers (AE) analyzing the sections, after a few days (intra-observer variation), and the estimations showed good correlation and reproducibility (Pearson's $r = 0.80$).

Statistical analysis

Statistical analyses were performed using the IBM SPSS® Statistics (IBM Company, New York, NY, USA) and STATA (StataCorp., TX, USA) software packages (IBM PASW Statistics for Windows, version 18.0.3 and STATA/SE 11.1). Frequency tables were analyzed using the chi-squared test, with likelihood ratio or Fischer's exact test being used to assess the significance of the correlation between the categorical variables. Odds ratios and their 95% confidence intervals (95% CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann–Whitney or Kruskal–Wallis) for 2- and multiple independent samples, respectively. Analysis of variance (ANOVA) was only used for deriving the mean values (and their 95% CI) of each individual stratum. Univariate survival analysis for the outcome measure [disease-specific survival (DSS), disease-free survival (DFS)] was based on Kaplan–Meier method, with log-rank (Mantel-Cox) comparison test. To assess the value of E-cadherin as an independent predictor, multivariate survival analysis was performed, using the Cox proportional hazards regression model controlling for the confounding by the following variables: age, sex, tumor localization, T, grade, (for DFS), and recurrence as additional variable (for DSS). In all tests, the values $p < 0.05$ were regarded statistically significant.

RESULTS

The expression pattern of E-cadherin was predominantly membranous in normal colonic epithelium and in the tumor area as well. The staining patterns of E-cadherin in CRC lesions are illustrated in Fig. 1.

E-cadherin expression related to clinicopathological features

Gender, localization, tumor invasion, or grade had no significant relationship with the expression of E-cadherin (Table 2). However, age was significantly associated with E-cadherin expression in that tumors of younger patients expressed E-cadherin more than tumors of the older patients ($p < 0.03$). Interestingly, lymph node (LNN) involvement was also significantly associated with loss of E-cadherin expression ($p < 0.01$) in that 54% of the tumors with LNN involvement tested negative for E-cadherin, whereas 64% of the cases expressing E-cadherin had no LNN involvement.

Similarly, loss of E-cadherin expression also showed a (borderline) correlation with the stage and tumor metastasis. Early stage tumors were E-cadherin positive, whereas tumors with advanced stage showed loss of expression ($p < 0.09$). A similar trend was noticed for metastasis, which were less frequent among

Table 2. The association of E-cadherin expression (negative/positive) with the clinicopathologic variables

Clinicopathologic feature	Significance
Gender	0.83
Age	**0.03**
Localization	0.45
T	0.32
N	**0.01**
M	0.09
Stage	0.09
Grade	0.41
Recurrence	**0.02**
DSS of 5 years	**0.005**
DSS of 10 years	**0.02**
DFS	**0.009**
Alive or not	**0.03**

DFS, disease-free survival; DSS, disease-specific survival.
Significant associations are bolded.

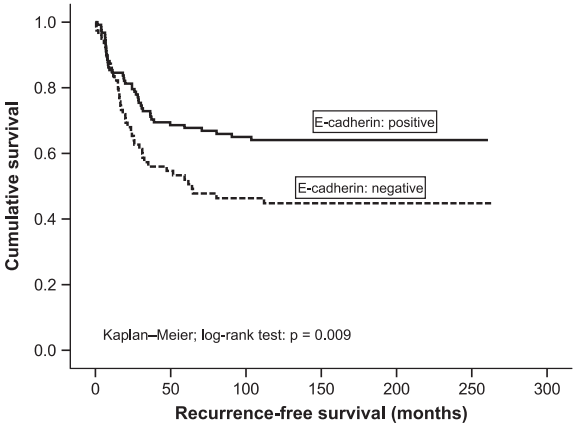

Fig. 2. E-cadherin expression (negative/positive) as determinant of disease-free survival (DFS) in univariate (Kaplan–Meier) analysis of stage I–IV CRC patients.

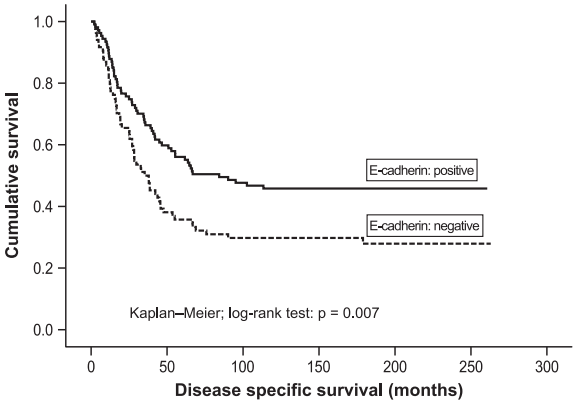

Fig. 3. E-cadherin expression (negative/positive) as determinant of disease-specific survival (DSS) in univariate (Kaplan–Meier) analysis of stage I–IV CRC patients.

tumors with positive E-cadherin expression ($p < 0.09$). Interestingly, 77% of the patients with tumors expressing E-cadherin showed objective response to treatment, in contrast to the fact that 60% of the non-responders showed loss of E-cadherin expression ($p < 0.04$, data are not shown). E-cadherin expression was also clearly associated with disease recurrence after treatment, in that the patients with no E-cadherin expression in their original tumor developed recurrence earlier (mean: 88 months) than those with positive expression of E-cadherin (mean: 114 months) ($p = 0.03$). The same trend was observed in the overall survival times; patients with E-cadherin-positive tumors survived significantly longer ($p = 0.01$).

Survival analysis

In Kaplan–Meier survival analysis, there was a highly significant ($p = 0.009$) difference in DFS between patients with E-cadherin positive tumors (longer DFS) and those with negative tumors (Fig. 2). The same was true with DSS (Fig. 3), patients with E-cadherin-positive tumors living significantly longer ($p = 0.007$).

To assess the value of E-cadherin as an independent predictor, a multivariate survival analysis was done, using the Cox proportional hazards regression model (stepwise backwards approach) controlling for confounding by the following covariates: gender, age, tumor localization, T, grade, (for DFS), and recurrence as an additional variable for DSS. In the Cox model, the independent predictors of DFS were: sex ($p = 0.012$) (in favor of women) and tumor invasion (T) ($p = 0.016$), as well as E-cadherin, with HR = 1.56, 95% CI 1.01–2.42 ($p = 0.043$). In a similar model for DSS, only age ($p = 0.006$) and disease recurrence ($p = 0.0001$) proved to be independent predictors of DSS, whereas E-cadherin was removed from the stepwise model because of lost significance.

We also performed these survival analyses in the sub-group of stage II tumors, because they

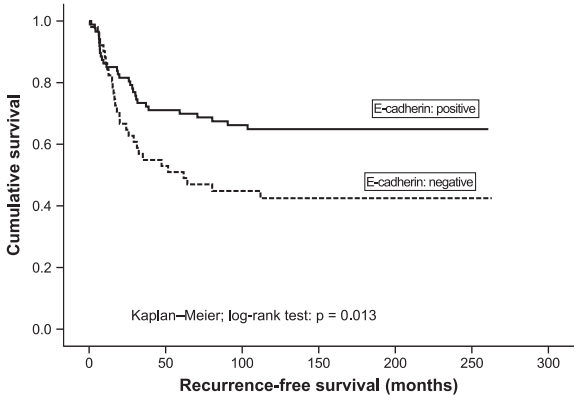

Fig. 4. E-cadherin expression (negative/positive) as determinant of disease-free survival (DFS) in univariate (Kaplan–Meier) analysis of stage II CRC patients.

comprise 56% of all CRCs in this cohort. It turned out that E-cadherin expression predicts disease recurrence also in stage II CRC. Kaplan–Meier survival analysis showed a significant ($p = 0.01$) difference in DFS between patients with positive tumors (longer DFS) and those with negative tumors (Fig. 4). At 5-year follow-up, 30% of the patients with E-cadherin positive tumors showed recurrence as compared with 55% of patients with no E-cadherin expression. The same was true with DSS (Fig. 5), patients with E-cadherin-positive tumors had significantly longer survival ($p = 0.04$); 63% of those with E-cadherin-positive tumors were alive at 5 years as compared with only 42% of the patients whose tumors had no E-cadherin expression.

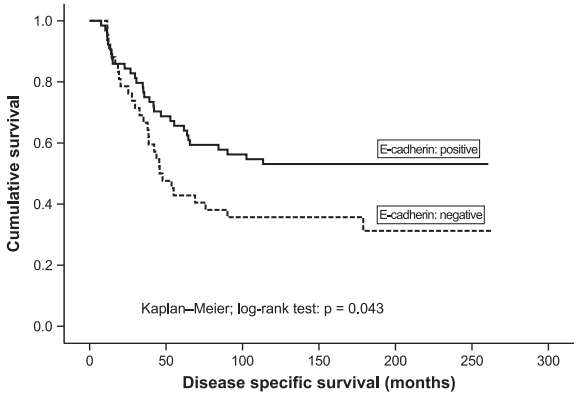

Fig. 5. E-cadherin expression (negative/positive) as determinant of disease-specific survival (DSS) in univariate (Kaplan–Meier) analysis of stage II CRC patients.

DISCUSSION

The present cohort of CRC patients enrolled at Turku University Hospital (Finland) is unique in that the follow-up of the patients covers an unusually long period (up to almost 25 years). Accordingly, we could calculate 5- and 10-year survival figures with good statistical power, because enough cases with substantially longer follow-up are included in this cohort. This makes the series different from many of the CRC studies, where only short survivals are reported. In addition, we used different approaches to analyze the expression of E-cadherin. It is well established that early CRCs can be cured with radical surgical resection alone (32). Unfortunately, however, some of the patients who undergo curative resection subsequently present with relapse and eventually die of their disease (33). Prediction of disease outcome in individual patients after curative resection is still far from reliable (34). The present data suggest that E-cadherin expression studied by IHC could be helpful in this prediction, and more rational decisions can be done as soon as we learn more accurate prediction of the disease outcome in individual patients (2, 35).

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 (36–39). The aim of the present study was to cast further light on the issues related to prognostication of CRC while assessing the value of quantitative E-cadherin expression profiles as independent prognostic factors. In this study, we focused on stage I–IV disease where molecular and other markers may help pinpointing a sub-group of patients, who would eventually benefit from the use of adjuvant therapy for their disease. This important decision involves a careful weighing of the risks of toxicity and complications against the potential curability of the disease (40). On the basis of the present results, we do believe that the grading system classifying CRCs as E-cadherin positive or negative is the clinically most relevant approach.

In the present study, several interesting and important observations were made, all implicating that the quantitatively measurable E-cadherin expression of cancer cells are of significant prognostic value in stage I–IV CRC. First, a negative expression of E-cadherin was more common among advanced stage tumors. A similar trend was observed between E-cadherin and LNN involvement; 54% of the tumors with LNN involvement showed no expression of E-cadherin, whereas 64% of the cases expressing E-cadherin had no LNN involvement. This is in alignment with study by Fang et al. (41), who observed that loss of E-cadherin expression was closely associated with advanced stage and LNN involvement in CRC. The same also applies to tumor metastasis, distant metastasis being more frequent among tumors with no E-cadherin expression. A similar observation has been previously reported by Ikeguchi et al. (42), who found that 80% of patients who developed haematogenic metastasis had tumors with both enlarged nuclei and reduced E-cadherin expression. In fact, Ochiai et al. (43) introduced a formula for predicting liver metastasis in patients with CRC; a combination of dysadherin, E-cadherin, and matrilysin was shown to be the best predictor of liver metastasis. These data closely parallels the experimental models, where loss of E-cadherin expression in transgenic mice was associated with the development of invasive CRC from well-differentiated adenomas (44).

These observations implicate E-cadherin as a biologic factor that might affect the behavior of the tumor cell population, but unfortunately, less well known are the molecular events responsible for progression and metastasis of CRC. There are several studies on genetic abnormalities of proto-oncogenes (K-Ras) (45) and tumor suppressor genes (p53 and APC) (46, 47) or, alternatively, molecular epigenetic changes (E-cadherin, p16 and RASSF1A) in CRC (48–50). Indeed, reduced expression of E-cadherin owing to aberrant CpG island hypermethylation has been regarded as one of the main molecular events involved in the dysfunction of the cell–cell adhesion system (51), as well as in invasion and metastasis (50, 52). However, a study by Liu et al. (53) failed to find evidence for promoter methylation of the E-cadherin gene as the cause of significant down-regulation of E-cadherin expression in CRCs. They concluded that methylated E-cadherin gene as a biomarker in CRC needs further validation. In fact, multiple mechanisms other than genetic and epigenetic silencing of E-cadherin could serve as alternative ways for interfering with the normal E-cadherin function under pathological conditions. As reviewed by van Roy and Berx (54), E-cadherin is removed from the plasma membrane by endocytosis and recycled to the sites of new cell–cell contacts. Abnormal activation of proto-oncogenes, such as c-Met, Src, and EGFR, results in increased phosphorylation of tyrosine residues in the cytoplasmic domain of E-cadherin, which leads to recruitment of the E3-ubiquitin ligase Hakai and subsequently mediates internalization and ubiquitin-dependent degradation of E-cadherin (55).

Obviously, one of the most important observations of the present study is the one linking E-cadherin expression with the disease outcome, i.e., appearance of recurrence and long-term DSS. This is clinically relevant for several reasons. Because of the fact that a substantial proportion of CRC patients with different stage of disease are at high risk for recurrence, it would be of paramount importance to find out reliable markers that would accurately predict those patients to become considered for adjuvant therapy.

In the present cohort, 38–40% of the patients eventually developed disease recurrence during the median follow-up time of 18.8 months (= median DFS for all patients with recurrent disease) (Figs 2 and 4). This is a substantially high rate particularly for a group of LNN-negative (stage II) CRC patients. Importantly, the mean DFS was significantly ($p = 0.032$) longer among patients with E-cadherin-positive tumors than in those with no E-cadherin expression. Importantly, E-cadherin maintained its significance as an independent predictor of DFS also in multivariate (Cox) model, adjusted for classical prognostic predictors. This fully substantiates the observations reported recently by Ngan et al. (56), who showed both in univariate- and multivariate survival analyses that loss of E-cadherin (and CD44) expression was significantly associated with shorter survival than did the high expression profiles, especially in stage II CRC, and loss of both markers had the worst impact on patient prognosis.

In univariate (Kaplan–Meier) survival analysis, E-cadherin was also a significant predictor of DSS. Not unexpectedly, E-cadherin expression was more often negative in patients, who eventually died of their disease as compared with those who were alive at the completion of the follow-up, and this difference was significant ($p = 0.007$). These data clearly implicate that CRCs with loss of E-cadherin expression are at high risk for local or distant recurrence and, because of the high adverse prognostic impact of disease recurrence; these patients are also more likely to die of their disease. To avoid this, these patients should be appropriate candidates for intensive follow-up and targeted therapeutic strategy.

Interestingly, the present study also confirmed that an objective response to treatment was markedly better among patients with E-cadherin-positive tumors than in the negative counterparts. This also feasibly explains the observations why E-cadherin was a significant predictor of disease recurrence. Accordingly, patients with E-cadherin-positive tumors developing recurrence are also likely to respond better to treatment and thus probably would gain a survival advantage. Indeed, this was shown to be the case in the present cohort, where long-term DSS was significantly better among the patients with positive E-cadherin expression in their primary tumors.

Taken together, the present results revealed declining E-cadherin expression in advanced disease stages, reflecting a tendency toward metastatic phenotype. Furthermore, positive E-cadherin expression was associated with a clinical benefit of treatment, in contrast to progressive disease among E-cadherin-negative cases, suggesting that E-cadherin expression might make the tumor cells more susceptible to therapy. Finally, loss of E-cadherin expression in CRC tumors seems to be associated with less favorable DFS and long-term DSS as compared with E-cadherin-positive tumors, possibly implicating some differences in the inherent malignancy of CRC that become manifested after prolonged follow-up.

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