E-cadherin expression in Libyan patients with colorectal carcinoma

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Abstract
Aim: The present study is undertaken to evaluate E-cadherin expression in a series of Libyan colorectal cancer cases to get an insight in its potential prognostic value in colorectal cancer in Libyan patients. Materials and Methods: A series of 81 Libyan patients with colorectal carcinoma were retrospectively studied. All carcinomas were selected from the archives of the Department of pathology, Benghazi University, derived from the period from January 2007 to December 2011. All tumors were classified using the histopathological criteria of the World Health Organization (WHO) classification, and staging was made according to the criteria of tumor-node-metastasis (TNM) classification of the International Union against Cancer. Results and Discussion: ImmunoHistocchemical (IHC) analysis was done using the automatic system (BenchMark XT, Ventana Medical System, Inc. Tucson, Arizona, USA). This fully automated processing of code-labeled slides included baking of the slides, solvent free deparaffinization, antigen retrieval in a cell conditioning buffer CCI (Mild: 36 minutes conditioning, and standard: Two staining indexes were calculated: the membrane index (MI) and cytoplasmic index (CI). These indices were calculated with both the intensity of staining and the fraction of positively-stained cells taken into account using the following formula: \[ I = 0 \times f_0 + 1 \times f_1 + 2 \times f_2 + 3 \times f_3 \] Our data showed that loss of E-cadherin expression is more frequently detected in older age group and in colorectal cancer patients with lymph node involvement; 75% of tumors with lymph node involvement showed negative expression of E-cadherin. One of the most important finding of the current study is the association of E-cadherin expression with the disease outcome. Conclusion: These data suggest that the loss of the E-cadherin function could be associated with invasiveness, lymph node metastasis and distant metastasis resulting in poor prognosis.

Keywords
Cadherin, Colorectal Carcinoma, Immunohistochemistry

1. Introduction
Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer deaths, with 1 million new cases diagnosed annually and more than half a million cases die from this disease, accounting for 8% of all cancer-related deaths worldwide.¹,²

Unfortunately, the conventional prognostic factors for patient survival and the traditional staging system are inappropriate for identifying those CRC patients who carry high risk of poor prognosis. Thus, there is a necessity for identification of more effective prognostic factors, including molecular markers to predict cancer outcome and improve
therapeutic decisions. Therefore, it has been reported that CRC is a potentially curable disease if diagnosed at an earlier stage. Therefore, it is important to recognize clinically useful biomarkers that can detect CRC at an early stage.

E-cadherin is a transmembrane Type I glycoprotein containing a cytoplasmic domain of 150 amino acids and an extracellular domain of 550aa. E-cadherin is involved in the generation and maintenance of adherens junctions (AJ) via hemophilic (E-cadherin-E-cadherin) interaction and most often homotypic (epithelial-epithelial cell interaction) adhesion. Therefore, the adhesion molecule E-cadherin, a cell surface glycoprotein plays an essential role in the maintenance of the normal structure and function of adult epithelial tissues. The extracellular domain interacts homotypically with the E-cadherin molecules of adjacent cells and maintains intercellular adhesion. Its cytoplasmic tail comprises a complex group of proteins including intracytoplasmic proteins, such as catenins. These complexes are classically distributed in the adherens junctions.

Studies conducted during the past decades have reported that the loss of these normal intercellular junctions precedes the tumor invasion and metastasis. Thus, loss of E-cadherin-mediated adhesion appears to be of important value in the neoplastic process, permitting cells to escape normal growth control signals, resulting in loss of differentiation and increased cell proliferation with invasive behavior.

As it was not too extensively studied, we evaluated E-cadherin expression in a series of Libyan colorectal cancer cases and its correlation with a variety of clinicopathological variables, disease recurrence and long term outcome to get insight in its potential prognostic value in colorectal cancer in Libyan patients.

2. Patients and Methods

A series of 81 Libyan patients with colorectal carcinoma were retrospectively studied. All carcinomas were selected from the archives of the Department of pathology, Benghazi University, derived from the period from January 2007 to December 2011, based on availability of representative paraffin blocks. Informed consent was obtained from all the patients and approval for the study was obtained from Institute Ethics Review Board. All the patients were followed up until death or when last seen alive at their clinical visit (June 2012) with the median FU-time of months (range: 3-142 month, mean: 45 month). The duration of follow-up and the outcomes at the end of follow-up were determined for each patient from hospital and clinic charts. One experienced pathologist confirmed all histological diagnoses. All tumors were classified using the histopathological criteria of the World Health Organization (WHO) classification, and staging was made according to the criteria of tumor-node-metastasis (TNM) classification of the International Union against Cancer (16). Clinical data of the patients are presented in Table 1.

2.1. E-cadherin Immunostaining

Formalin-fixed, paraffin-embedded primary colorectal tumor tissue was obtained from 81 patients. Sections were cut serially at 5µm for immunohistochemical (IHC) analysis. IHC analysis was done using the automatic system (BenchMark XT, Ventana Medical System, Inc. Tucson, Arizona, USA). This fully automated processing of code-labeled slides included baking of the slides, solvent free deparaffinization, antigen retrieval in a cell conditioning buffer CCI (Mild: 36 minutes conditioning, and standard: 60 minutes conditioning), incubation with Rabbit monoclonal anti-E-cadherin antibody, 7.0ml ready-to-use from Spring Bioscience (clone: SP64, Catalog no. M3641, 6920 Koll Center Parkway, CA 94566, USA), for 32 min, at 37ºC. Application of I-View™ DAB Detection Kit (Lot no. B05860AZ), which, includes: I-View DAB HRP, I-View DAB Inhibitor, I-View DAB Biotin, I-View DAB H2O2, and I-View DAB Copper. Counterstaining with haematoxylin II (C00758) was done for 4 minutes, and post-counterstaining with bluing reagent (B11129) was done for 4 minutes as well. After staining, the sections was dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

2.2. Evaluation of E-cadherin Staining

The evaluation of the E-cadherin staining was performed with a light microscope at X40 magnifications and with the evaluator blinded to the information on tumor grade, stage, or clinical outcome. Membranous and cytoplasmic staining was evaluated. For cell membrane staining, four categories were used, (3+++, 2++, 1+, -) (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 creates a honeycomb pattern. The cytoplasmic staining was also graded into four categories: (0) Negative, no detectable staining, (1) Weak, but detectable still staining, (2) Moderate, clearly positive but still weak, (3) Heavy staining, intense. Two staining indexes were calculated: the membrane index (MI) and cytoplasmic index (CI). These indices were calculated with both the intensity of staining and the fraction of positively-stained cells taken into account using the following formula:

\[ I = \text{I} = 0 \times f_0 + 1 \times f_1 + 2 \times f_2 + 3 \times f_3 \]

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 scores could vary between 0 and 3 (17, 18). The reproducibility of the evaluation of E-cadherin staining indices was tested by employing intra-observer reproducibility.

2.3. Statistical Analysis
Statistical analyses were performed using the IBM SPSS Statistics (IBM Company, NY, USA) and STATA (StataCorp., Texas, USA) software packages (IBM PASW Statistics for Windows, version 19). Frequency tables were analyzed using the Chi-square test, with likelihood ratio (LR) or Fischer’s exact test being used to assess the significance of the correlation between the categorical variables. Analysis of variance (ANOVA) was only used deriving the mean values (and their 95%CI) of each individual stratum. Univariate survival analysis for the outcome measure (DSS, DFS) was based on Kaplan-Meier method, with log-rank (Mantel-Cox) comparison test. In all tests, the values $p<0.05$ were regarded statistically significant.

### 3. Results

#### 3.1. Patterns of E-Cadherin Expression in CRC Samples

The expression pattern of E-cadherin was membranous and cytoplasmic in normal colonic epithelium and in the tumor area as well. Examples of the staining patterns of E-cadherin are illustrated in Figures 1a, b, c and d. Of the 81 tumors, 41 (51%) were considered negative (staining intensity 0; figure 1d), whereas 40 (49%) were considered positive (staining intensity $>1$; figure 1b, c). Strong expression of E-cadherin was noticed in normal colonic mucosa (figure 1a).

**Figure 1.** Immunohistochemical staining for E-cadherin expression in colorectal adenocarcinoma; (A) Normal colonic epithelium showed membranous expression of E-cadherin; (B) Adenocarcinoma of colon showed cytoplasmic expression of E-cadherin; (C) Adenocarcinoma of colon showed cytoplasmic and membranous expression of E-cadherin; (D) Adenocarcinoma of colon showed no expression of E-cadherin. Magnification of all samples is at (X40).

#### 3.2. Correlation of E-Cadherin Expression with Clinicopathological Features

The distribution of E-cadherin expression in tumor samples in relation to clinicopathological characteristics is presented in (Tables 1 and 2). Using different cut-off points (mean, median, and 2-tier score (0 Vs 1, 2, 3) and (0, 1, Vs 2,3). The present study revealed that a significant correlation between E-cadherin expression and tumor localization in that tumors arising in the right and left colon express E-Cadherin more than tumors arising in the Rectum ($p<0.043$), loss of E-Cadherin expression was more frequently detected in the Rectal adenocarcinomas. Moreover, E-Cadherin expression showed a significant correlation with the age ($p<0.002$), in that tumors of the younger patients ($<55$ years), expressed E-Cadherin more than tumors of the old patients, loss of E-Cadherin was more frequently detected in older patients ($>55$ years). Interestingly, Loss of E-Cadherin expression associated significantly ($p<0.039$) with lymph node metastasis.

**Table 1.** Correlation of E-Cadherin Expression (negative vs. positive) and Clinico-pathological Features of CRC samples.

<table>
<thead>
<tr>
<th>Features</th>
<th>Number of cases(%)</th>
<th>E-cadherin Expression</th>
<th>$p$-value</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Negative (0)</td>
<td>Positive (1,2,3)</td>
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<tr>
<td>Gender</td>
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<td>19 (49%)</td>
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<tr>
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<td>39 (49%)</td>
<td>20 (51%)</td>
<td>19 (49%)</td>
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<tr>
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<td>41 (51%)</td>
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<td>Age group (years)</td>
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<td>≤55</td>
<td>41 (51%)</td>
<td>21 (51%)</td>
<td>20 (49%)</td>
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<tr>
<td>&gt; 55</td>
<td>40 (49%)</td>
<td>20 (50%)</td>
<td>20 (50%)</td>
</tr>
<tr>
<td>Features</td>
<td>Number of cases(%)</td>
<td>E-cadherin Expression</td>
<td>p-value</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------</td>
<td>-----------------------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative (0)</td>
<td>Positive(1,2,3)</td>
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<td>Lymph node involvement</td>
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<td>20 (61%)</td>
<td>13 (39%)</td>
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<tr>
<td>No</td>
<td>30 (48%)</td>
<td>17 (57%)</td>
<td>13 (43%)</td>
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<td>Tumor grade</td>
<td>0.490</td>
<td>0.043</td>
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<td>Well</td>
<td>23 (29%)</td>
<td>10 (43%)</td>
<td>13 (57%)</td>
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<td>Moderate</td>
<td>48 (60%)</td>
<td>25 (52%)</td>
<td>23 (48%)</td>
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<td>Poor</td>
<td>9 (11%)</td>
<td>6 (67%)</td>
<td>3 (33%)</td>
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<td>Right colon</td>
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<td>10 (50%)</td>
<td>10 (50%)</td>
</tr>
<tr>
<td>Left Colon</td>
<td>42 (53%)</td>
<td>17 (40%)</td>
<td>25 (60%)</td>
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<td>Rectum</td>
<td>17 (22%)</td>
<td>13 (77%)</td>
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<td>Tumor Stage</td>
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<td>0.039</td>
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<tr>
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<td>24 (32%)</td>
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<td>13 (54%)</td>
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<td>11 (42%)</td>
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<td>Recurrence</td>
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<td>37 (51%)</td>
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<td>Distance Metastasis</td>
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<td></td>
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<tr>
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<td>50 (66%)</td>
<td>25 (50%)</td>
<td>25 (50%)</td>
</tr>
<tr>
<td>Yes</td>
<td>26 (34%)</td>
<td>15 (58%)</td>
<td>11 (42%)</td>
</tr>
</tbody>
</table>

Table 2. Correlation of E-Cadherin Expression (below mean vs. above mean) and Clinico-pathological Features of CRC samples.
On the other hand, tumor recurrence, tumor invasion, gender, distance metastasis and status at end point had no significant relationship with the expression of E-cadherin.

### 3.3. Survival Outcome of CRC Patients

In Kaplan-Meier survival analysis (at mean as cut-off point) there was a significant (p< 0.03) difference in DFS between patients who have E-cadherin expression above mean and those with E-cadherin expression below mean (Figure 2). Interestingly, 98% of the patients with tumors expressing E-cadherin above mean showed longer disease free survival in comparison with only 50% of patients with tumors expressing E-cadherin below mean. In Kaplan-Meier survival analysis (0 vs 1, 2, 3 as a cut-off point), there was a difference (p< 0.08) in DFS between patients with E-cadherin positive tumors (longer DFS) and those with negative tumors. At 4 year follow-up, 98% of the patients with E-cadherin positive tumors showed longer DFS as compared with 53% of patients with no E-cadherin expression (Figure 3).

Figure 2. E-cadherin expression (below mean vs. above mean) as determinant of disease-free survival (DFS) of CRC in univariate Kaplan Meier analysis (p<0.03, log rank).

Figure 3. E-cadherin expression (negative vs. positive) as determinant of disease-free survival (DFS) of CRC in Univariate Kaplan Meier analysis (p<0.08).

### 4. Discussion

In this study, we have examined the expression of E-cadherin in colorectal cancer of Libyan patients. In accordance with previous reports, we showed that the pattern of expression of E-cadherin was both membranous and cytoplasmic in primary colorectal tumors (19, 20). In addition, our data showed that loss of E-cadherin expression is more frequently detected in older age group and in colorectal cancer patients with lymph node involvement; 75% of tumors with lymph node involvement showed negative expression of E-cadherin.

The same observation was demonstrated by Fang et al. (21), who reported that loss of E-cadherin expression was associated with lymph node involvement. A similar finding has been reported by Miladi-Abdennader et al. (22), who observed that expression of E-cadherin was associated with age of patients at diagnosis and (tumor size) in colorectal cancer.

Results from the present study indicate that the expressions of E-cadherin were different in relation to the primary site of tumor. Rectal tumors tend to have negative E-cadherin expression whereas left site tumors showed increased E-cadherin expression. This finding is consistent with Aamodt et al. (23), who observed that there is difference between rectal and colon cancer in expression of E-cadherin. The reason for this difference regarding the prognostic value of E-cadherin between rectal and colon adenocarcinomas is difficult to explain, but could be attributed to different tumor biology within these two entities.

In the current study, we did not find any significant correlations between E-cadherin expression and both tumor differentiation and depth of primary tumor contrary to the studies reported regarding the expression of E-cadherin and tumor differentiation in colorectal carcinoma (24-26). All these observations implicate E-cadherin as biologic factor that might affect the behavior of the tumor cell population. Several studies have reported that down-regulation of E-cadherin in colorectal cancer is rarely attributed to E-cadherin gene mutation (27-28), a phenomenon commonly observed in diffuse-type gastric (29) and lobular breast carcinomas (30). Efstathiou et al. detected E-cadherin inactivating mutation found in only 7% of colorectal carcinoma cell lines. Also, structural mutations or loss of heterozygosity do not play a crucial role for E-cadherin inactivation in colon cancer. He concluded that other epigenetic events such as promoter methylation have been implicated (31-32).

One of the most important finding of the current study is the association of E-cadherin expression with the disease outcome. The mean DFS was significantly (p< 0.03) longer among patients with E-cadherin positive tumors than in those with negative E-cadherin expression. This is consistent with the study reported by Ngan et al.
who reported that loss of E-cadherin (and CD44) expression was significantly associated with shorter survival than did the high expression tumors and loss of both marker has been linked to poor prognosis in colorectal cancer (33).

Decreased levels of E-cadherin expression were reported in many immunohistochemical studies on epithelial malignancies (34-36). In some tumor types, including CRC, the loss of E-cadherin expression is associated with the loss of tumor differentiation and is shown to be correlated with an increased likelihood of distant metastasis (37). The down-regulation of E-cadherin is seen most prominently in carcinomas showing infiltrative growths associated with little intercellular cohesion, such as invasive lobular carcinoma of the breast and diffuse gastric adenocarcinoma including gastric signet-ring cell carcinoma (38-40). These data suggest that the loss of the E-cadherin function could be associated with invasiveness, lymph node metastasis and distant metastasis resulting in poor prognosis.

Therefore, the present study revealed that loss of E-cadherin expression in advanced stage of the disease stages leads toward metastatic phenotype and poor prognosis in colorectal cancer.

Limitation of the Study: This is only a preliminary study. To elucidate more about the significance of E-cadherin and its expression in CRC further studies need to be carried out regarding the expression patterns including the localization of beta-catenin. This will elucidate the important role of beta-catenin in the E-cadherin/catenins complex which is an important regulator of cell invasion and metastasis.

**Conflict of Interest**

The authors declare that they have no competing interests.

**Acknowledgment**

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