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E-cadherin and NEDD9 expression in primary colorectal cancer, metastatic lymph nodes and liver metastases

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Abstract. In Croatia, colorectal cancer mortality rates in males are the third highest in Europe, after Hungary and Slovakia. The results for females rank Croatia in second place after Hungary. According to previous studies, the loss of E-cadherin expression and the higher expression of neural precursor cell-expressed developmentally downregulated 9 (NEDD9) are associated with a worse prognosis. The aim of the present study was to analyze the immunohistochemical expression of NEDD9 and E-cadherin as markers of metastatic potential using a tissue microarray. This retrospective study included 40 previously untreated patients, including 23 males and 17 females with a median age of 64.5 years (range 38-84), with colorectal cancer and synchronous liver metastases that underwent simultaneous colorectal and hepatic resection between January 1st 2006 and December 31st 2013, in the Clinical Hospital Center Sestre Milosrdnice (Zagreb, Croatia). The most frequent tumor stage was T3, while the most frequent nodal stage was N1. Microvascular invasion was present in 37.5% of patients, while perineural invasion was observed in 30% of patients. The immunohistochemical staining index of E-cadherin was highly positive in 87.5% samples of colorectal cancer, 67.7% of lymph nodes and 77.5% of liver metastases. In the primary tumor, highly positive NEDD9 expression was identified in 22.5% of patients. In lymph nodes, it was identified in 35.5% of patients, while in the liver, it was identified in 30% of patients. Significant positive correlations were observed between the percentage of positive lymph nodes and the immunohistochemical staining index of E-cadherin (ρ=0.372; P=0.039) and NEDD9 (ρ=0.451; P=0.011) in lymph nodes. After the conclusion of the study, 55% of the patients succumbed. No significant differences in survival rates were identified regarding the expression of E-cadherin and NEDD9 in the primary tumor, metastatic lymph nodes and liver metastases. Due to the small sample size and the negative results obtained, further research is required to implement these parameters as prognostic factors.

Introduction

Colorectal cancer (CRC) is the second most frequently diagnosed cancer and the third leading cause of cancer-related death in Croatia. According to the data obtained from the Croatian National Cancer Registry for 2015, there were 1,890 new cases in the male population, and 1,339 in the female population. During the same year, 2,056 people succumbed to CRC (1). Despite the existence of the Croatian national colorectal screening program, the trends in the rates of incidence and mortality still display an increase in CRC. At the time of primary diagnosis, 41% of patients have positive regional lymph nodes (LN) and 14% of patients have evidence of distant metastases (1).

Approximately 75-80% of all CRC cases are sporadic, while approximately 20% may be familial, due to low-penetrance genes without a clear pattern. Only 5% of CRCs are clearly inherited (familial adenomatous polyposis, Lynch syndrome, MUTYH-associated polyposis, Peutz-Jeghers syndrome, juvenile polyposis, Cowden syndrome and serrated polyposis). Most cases of hereditary CRC have an autosomal dominant inheritance pattern (except MUTYH-associated polyposis) (2). Relevant risk factors are as follows: Age (>50 years), lifestyle (high-fat, low-fiber-diet, obesity, physical inactivity, smoking and alcohol consumption), colorectal adenoma, inflammatory bowel disease (Crohn's disease and ulcerative colitis), and a family history of CRC. Primary tumor location is an important prognostic factor and has an effect on clinical presentation. The initial symptoms of left-sided CRC tumors are a change in bowel habits and bleeding; as these symptoms are more palpable, therefore they are identified, and CRC is diagnosed, at an earlier stage. Right-sided CRC tumors grow unnoticed

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until they are large, and symptoms are unspecific: Abdominal pain, vomiting and anemia.

Diagnosis should be confirmed with an endoscopically-guided biopsy. Diagnostic workup should include a complete blood count, liver and renal function tests, carcinoembryonic antigen and cancer antigen 19-9, multi-slice computed tomography of the chest, abdomen and pelvis, and magnetic resonance imaging of the pelvis (for rectal cancer). A multidisciplinary team approach insures that patients receive the best possible care. The pathology report should include the histology type, degree of differentiation, depth of bowel wall infiltration (T status), affected and examined LNs (N status) and presence of lymphovascular or perineural invasion (PNI). Clinical and pathological staging should be performed according to the latest edition of the International Union Against Cancer (UICC)/American Joint Committee of Cancer (AJCC) tumor-node-metastasis (TNM) classification for CRC. Clinical outcomes have improved dramatically over the past 15 years due to the availability of more active chemotherapeutic agents, and anti-VEGF and anti-EGFR targeted agents, but also due to the development of different surgical approaches (including colon-first, liver-first, two-step or simultaneous resection) and the possibilities for the local ablative treatment of liver, lung and peritoneal metastases (including chemoembolization, radioembolization, stereotactic radiotherapy and radiofrequency ablation). The median survival rate for patients with metastatic CRC is currently about 30 months. This improvement is achieved due to continuum of care, which includes different possible combinations of patient treatment i.e., combinations of drugs and different time-frame of therapy. The latest findings are focused on microsatellite instability high CRC, which may be sensitive to programmed cell death protein 1 (PD-1) inhibitors.

It is critical to identify the molecular markers of CRC, which can be used to monitor or predict the progression and prognosis of patients with CRC, and to investigate these potential biomarkers as therapeutic targets.

A crucial role in the progression and aggressiveness of CRC is attributed to epithelial-mesenchymal transition (EMT), a reversible developmental process that includes the dissolution of adherens junctions and loss of apicobasolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties. During the EMT process, cancer cells lose the expression of cellular adhesion proteins such as epithelial (E-) cadherin and γ-catenin (3). E-cadherin is a member of the large cadherins family of calcium-dependent cell adhesion proteins. This single-pass transmembrane glycoprotein, encoded by the CDH1 gene on chromosome 16q22.1, has a molecular weight of 120 kDa. The mature protein comprises a long extracellular domain with five E-cadherin repeats (EC1-5), a short transmembrane domain, and a cytoplasmic domain that includes juxtamembranous p120, and γ- and β-catenin binding sites (4).

Predominantly expressed at the basolateral membrane of epithelial cells, where its function is primarily cell-cell adhesion, E-cadherin has been shown to be essential during morula compaction and the subsequent epithelial tissue organization, which is achieved through hemophilic interactions between cadherin molecules, first among adjacent cells (trans-interaction) and then within the same cell by lateral association (cis-interaction) (5). Malignant epithelial cells undermine the function of E-cadherin in several ways, including gene mutations, epigenetic silencing by promoter hypermethylation, loss of heterozygosity, transcriptional silencing and microRNAs that regulate expression, transport and protein turnover at the cell surface (6-8).

Neural precursor cell-expressed developmentally downregulated 9 (NEDD9) protein is a member of the non-catalytic scaffolding proteins family (9), which also includes CASS1/BCAR1/p130Cas, CASS3/EFS/Sin and CASS4/HEPL. These proteins show the conservation of similar domain structures; an N-terminal Src homology 3 (SH3) domain that binds protein substrates (e.g., FAK, PYK2) and contains polyproline motifs, and a large substrate domain incorporating multiple YxxP motifs, which are phosphorylated by the Src family kinases to create binding sites for proteins with SH2 domains. The serine-rich region likely folds into a 4-helix bundle and highly conserved carboxyl-terminal domain that mediates homo- and heterodimerization with CASS1/BCAR1/p130Cas. Although the protein is mainly cytoplasmic, small quantities are localized with centrosomes and the ciliary basal body. The signaling function of NEDD9 is integrin-dependent, regulated by the phosphorylation of serines, threonines and tyrosines in the structural domains. PP2A phosphatases are potential regulators of the NEDD9 phosphorylation status. NEDD9 has a molecular weight of 93 kDa and oscillates between a faster migrating form of 105 kDa in G1/S cells and a slower migrating form of 115 kDa in G2/M cells. Previous studies have identified the crucial role of NEDD9 in the coordination of signaling cascades, contributing to changes in cell adhesion, migration, invasion and EMT (9,10). In normal human tissue, the highest level of NEDD9 is expressed in the lungs and kidneys, which are rich in immature lymphoid cells, and in the fetal brain prior to downregulation in the adult brain (10). Many cell lines, such as epithelial tumor-, melanoma-, lymphoma- and glioblastoma-derived cell lines, express an abundance of NEDD9.

Due to its pleotropic functions (cell adhesion, migration, invasion and EMT), the elevated expression of NEDD9 has emerged as a predictor of poor outcome, metastatic potential and chemoresistence in multiple cancer types (breast cancer, gastric cancer, glioblastoma, head and neck squamous cell carcinoma, hepatocellular carcinoma, non-small cell lung cancer, ovarian cancer, renal cancer, pancreatic ductal adenocarcinoma, prostate cancer and T-cell leukemia) (10-19). NEDD9 is a *bona fide* melanoma metastasis gene that enhances invasion *in vitro* and metastasis *in vivo* of both normal and transformed melanocytes (14). A growing body of preclinical data supports the theory that altered NEDD9 function is associated with other human diseases, such as stroke, Alzheimer's disease and autosomal dominant polycystic kidney disease.

The data regarding NEDD9 and E-cadherin expression in CRC are insufficient. However, a study has shown that overexpression of NEDD9 positively mediates the canonical Wnt/ß-catenin signaling pathway in CRC and it also negatively regulates membrane expression of E-cadherin (3). There is also a paper in which NEDD9 is identified as differentially expressed gene, associated with cyclin D1, which can be a molecular target for the treatment of CRC, because it interacts with their corresponding anti-neoplastic drugs (20). Therefore, our study aimed to analyze the immunohistochemical

NEDD9 and E-cadherin expression in a tissue microarray of nonmetastatic and metastatic CRC, and to determine whether their expression is associated with the clinical behavior and prognosis of CRCs.

Patients and methods

Patient information. Following approval by the Ethical Committee of Clinical Hospital Center Sestre Milosrdnice (Zagreb, Croatia), a total of 40 pairs of formalin-fixed, paraffin-embedded (FFPE) primary CRC and corresponding matched liver metastasis tissue specimens were retrieved from the tissue bank of the Ljudevit Jurak Department of Pathology and Cytology. The patients gave their written informed consent for the use of their biological materials and data in research. The patients had no history of the familial aggregation of CRC, had not been previously treated with chemo- or/and radiotherapy, and had undergone simultaneous colorectal and hepatic resection between January 1st, 2006 and December 31st, 2013. Tumor staging was performed according to the 7th edition of the TNM classification for CRC. The follow up deadline was December 31st, 2015. The survival time was calculated from the date of surgery to the follow up deadline, or the date of death. The clinicopathological features of patients are summarized in Table I.

Immunohistochemistry. Immunohistochemical analyses were performed by two board-certified pathologists who were blinded to the clinical data of the patients. Paraffin-embedded tissue sections (thickness 3-5 μm) were deparaffinized for 2 h at 60°C and then washed with distilled water after two and three changes of xylene and ethanol, respectively. Immunohistochemical staining was performed using the microwave streptavidin immunoperoxidase (MSIP) protocol, and by use of the labelled streptavidin-biotin (LSAB) method on a TechMate™ Horizon automated immunostainer (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) (11). Sections were incubated with rabbit anti-human NEDD9 poly-clonal (dilution 1:100, cat. no. ab37161; Abcam, Cambridge, UK) and mouse anti-human E-cadherin monoclonal (dilution 1:50, clone NCH-38; Dako; Agilent Technologies, Inc.) antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Abcam) for 1 h at room temperature. Sections were then washed with PBS and the antigen-antibody complex was visualized.

The reactions were determined in epithelial tumor components, as well as from the epithelial components of metastatic tumors in the LN and liver (Fig. 1). Positive reactions were determined at the site of strongest activity ('hot spot') under a magnification, x400 for a total of 1,000 tumor cells. The 'hot spot' was established following inspection of the whole section at a magnification of x40. The results for E-cadherin were presented semi-quantitatively using an immunohistochemical staining index (ISI), obtained by multiplying the intensity of reaction with the percentage of cells with a positive reaction. The range of ISI was from 0 to 9: 0, represents no reaction, 1-4 represents a low E-cadherin reaction, 5-9 represents a high E-cadherin reaction. The intensity of the reaction was scored as follows: 0, no reaction; 1, weak reaction; 2, moderate

Table I. Clinical description of the investigated sample (Dukes D, total n=40).

Parameter	n	%
Sex		
Male	23	57.5
Female	17	42.5
Localization of primary tumor		
Colon	28	70.0
Rectosigmoid junction	2	5.0
Rectum	10	25.0
T status		
T1	0	0
T2	3	7.5
T3	34	85.0
T4	3	7.5
N status		
N0 or Nx	9	22.5
N1	18	45.0
N2	13	32.5
Surgical margins		
Negative	39	97.5
Positive	1	2.5
Microvascular invasion		
Absent	25	62.5
Present	15	37.5
Perineural invasion		
Absent	28	70.0
Present	12	30.0
Outcome		
Survived	18	45.0
Succumbed	22	55.0

T, tumor; N, lymph node.

reaction; and 3, strong reaction. The percentage of immuno-reactive tumor cells was scored as follows: 0, for no reaction; 1, 0-10% of positive tumor cells; 2, >10-50% of positive tumor cells; and 3, >50% of positive tumor cells (21). The results for NEDD9 were presented semi-quantitatively and scored in the following way: 0, no reaction; 1, weak reaction in 0-10% of tumor cells; 2, moderate reaction in >10-25% of tumor cells; and 3, strong reaction in >25% of tumor cells (11).

Statistical analysis. The normality of data distribution was assessed with the Kolmogorov-Smirnov test, and appropriate non-parametric tests were used in the following statistical analyses. Spearman's ρ and Kendal's τ-b correlation coefficients for nominal-ordinal correlation were used to analyze associations between E-cadherin and NEDD9 expression in the primary tumor, LN and liver with other clinical variables. A log-rank (Mantel-Cox) test of the equality of survival distributions was performed for the expression of E-cadherin and NEDD9 in the primary tumor, LN and liver in relation
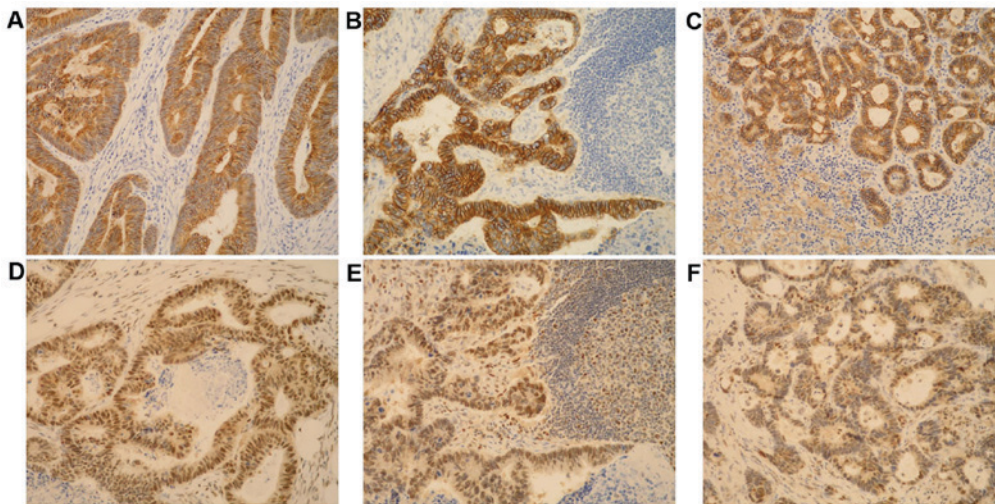
Figure 1. High intensity immunohistochemical expression of E-cadherin in (A) the primary tumor, (B) LN metastasis and (C) liver metastasis. High intensity immunohistochemical expression of neuronal precursor cell-expressed developmentally downregulated 9 in (D) the primary tumor, (E) LN metastasis and (F) liver metastasis. Magnification, x200. LN, lymph node; E-cadherin, epithelial cadherin.

to the survival outcome. The outcomes were illustrated with Kaplan-Meier survival curves. P<0.05 was considered to indicate a statistically significant difference. The data analysis software SPSS Statistics, version 23.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses and the production of graphical images.

Results

A clinical description of the investigated sample is shown in Table I. Of the patients, 57.5% were male. Median (interquartile range, IQR) age was 64.0 (57.3-73.5) years. Of the tumors, 70.0% were localized in the right colon. Median (IQR) tumor size was 50.0 (36.3-60.0) mm. Among the patients, 85.0% had T3 stage, and 22.5% had N0 or Nx stage. Microvascular invasion was positive in 37.5% of patients, and PNI in 30.0% of patients. Death occurred in 55.0% of patients, with a median (IQR) survival time of 620.5 (164.3-964.8) days. The median (IQR) percentage of positive LN was 13.9% (0.0-44.1%).

The E-cadherin and NEDD9 expression scores in the investigated samples are shown in Table II. 87.5% of the patients had a strong expression of E-cadherin in the primary tumor, 67.7% in the LN, and 77.5% in the liver. Highly positive NEDD9 expression in the primary tumor was identified in 22.5% of patients, while 35.5% had highly positive NEDD9 expression in the LN, and 30.0% in the liver.

The correlation coefficients for E-cadherin and NEDD9 expression in the primary tumor, LN and liver with clinical characteristics are shown in Table III. Significant positive correlation was noted between the percentage of positive LN with the ISI for E-cadherin in the LN (ρ=0.372; P=0.039) and with NEDD9 expression in LN (ρ=0.451; P=0.011), indicating that higher expression is significantly associated with a higher percentage of positive LN. Kaplan-Meier survival curves with log-rank tests for the expression of E-cadherin and NEDD9 in the primary tumor, LN and liver in relation to the survival outcome are shown in Fig. 2. There was no significant prediction of mortality associated with the expression of E-cadherin and NEDD9 at any location, indicating that for this tumor

stage (Dukes D), other prognostic markers are likely to be more clinically relevant.

Discussion

Left- and right-sided CRCs differ with respect to biology, epidemiology, pathology and clinical presentation. It is expected that most patients with synchronous liver metastases have right-sided CRC.

Various studies have shown controversial results regarding the expression levels of E-cadherin in CRC. Yun *et al* (22) studied stage III CRC, and found positive expression in 98.3% of samples. Dorudi *et al* (23) found that 81.2% of well and moderately differentiated tumors expressed strong positivity for E-cadherin, while 85.7% of poorly differentiated tumors were E-cadherin-negative. Three studies (Miladi-Abdennadher *et al* (24), Palaghia *et al* (25) and Elzagheid *et al* (26) identified a marginally lower expression (74.3, 67.69 and 59%, respectively). We attribute the results of Elzagheid *et al* (26) to the inclusion of all CRC stages in their study. In contrast to the aforementioned studies, Gu *et al* (27) identified that only 20% of patients with metastatic CRC had positive E-cadherin expression. The limitation of our study is reflected in the fact that we assessed only the membranous expression of E-cadherin. Elzagheid *et al* (28) assessed the cytoplasmic expression of E-cadherin. Tóth *et al* (29) used a scale based only on the percentage of immunopositive cells. Palaghia *et al* (25) used two scoring systems that were initially established for gastric carcinoma.

Our results show concordance with the results of Elzagheid *et al* (26), Khoursheed *et al* (30) and Roca *et al* (31), who reported that E-cadherin expression was not associated with tumor stage. Nevertheless, Ghadimi *et al* (32) reported a significant association between reduced E-cadherin and lower tumor grade, but did not identify a clear correlation between the loss of E-cadherin expression and the depth of tumor infiltration into the intestinal wall. Kwak *et al* (33) showed that the reduced expression of E-cadherin was associated with advanced stage tumors (P=0.029), while Lugli *et al* (34)

Table II. E-cadherin and NEDD9 expression in the investigated sample (Dukes D, total n=40).

ISI	n	%
E-cadherin in primary tumor		
0	2	5.0
1	3	7.5
2	35	87.5
E-cadherin in lymph nodes		
0	4	12.9
1	6	19.4
2	21	67.7
E-cadherin in liver		
0	1	2.5
1	8	20.0
2	31	77.5
NEDD9 in primary tumor		
0	8	20.0
1	9	22.5
2	14	35.0
3	9	22.5
NEDD9 in lymph nodes		
0	7	22.6
1	5	16.1
2	8	25.8
3	11	35.5
NEDD9 in liver		
0	3	7.5
1	11	27.5
2	14	35.0
3	12	30.0

ISI, immunohistochemical staining index; NEDD9, neuronal precursor cell expressed developmentally downregulated 9.

demonstrated that the loss of membranous E-cadherin was associated with a higher T-stage (P=0.03). Similar results were reported by Miladi-Abdennadher et al (24), who reported that E-cadherin expression was correlated with tumor size (P=0.02). Although the stage of the tumor was determined for every patient, the relatively small size of the group represented a limitation of the afore-mentioned study.

However, Roca et al (31) found no association between E-cadherin expression and LN metastasis. Similar to the present study, Kwak et al (33) demonstrated an association between E-cadherin expression and LN metastasis (P=0.004). Lugli et al (34) reported that in mismatch repair-proficient CRC, the loss of membranous E-cadherin was independently associated with a higher N stage (P<0.0001). In MLH1⁻ CRC, the loss of membranous E-cadherin was associated with a higher N stage (P=0.05) (34). Node-positive cancers exhibited significant loss of E-cadherin (P<0.001) according to Karamitopoulou et al (35). Ozgüven et al (36) found that reduced E-cadherin expression was significantly associated with LN metastasis (P=0.01). A borderline association of

E-cadherin expression and LN metastasis (P=0.09) was reported by Elzagheid et al (26). Kim et al (37) reported that E-cadherin expression may serve as a predictive marker for tumor invasion and LN metastasis.

E-cadherin expression was increased in up to 40% of liver metastases, compared with only 17% of metastatic LNs that were studied by Ikeguchi et al (38), whose results are consistent with those of the present study. The results of Kim et al (39), who analyzed patients that had undergone curative surgery for primary CRC and liver metastases, showed that E-cadherin expression in the tumor center was greater than that of the tumor margin, in the primary tumor and liver metastases (P<0.001, P=0.006, respectively). A likely explanation is the possibility that tumor cells regain epithelial features in distant metastases. Dorudi et al (23) and Mohri (40) postulated that negative E-cadherin expression was associated with liver metastasis. Nanashima et al (41) reported that negative E-cadherin expression tended to be associated with a poor prognosis. Kaihara et al (42) reported that LN metastasis and the decreased expression of E-cadherin were associated with liver metastasis. Elzagheid et al (28) reported that the E-cadherin membranous (MI) and cytoplasmic index (CI) were significantly higher in liver metastases compared to other anatomic sites (MI, P=0.034; CI, P=0.022). Truant et al (43) demonstrated that the expression of E-cadherin significantly increased in metastases compared with normal liver tissue. Gagliardi et al (44) compared liver metastases with their corresponding primary tumors, and found a complete loss of E-cadherin expression in 50% of liver metastases, while 86% of the primary tumors associated with the liver metastases exhibited strong expression.

A connection between survival rate and the reduced expression of E-cadherin was found by Kwak et al (33) and Kang et al (45), but was without statistical significance in a multivariate analysis; Lee et al (46) identified that the aberrant expression of E-cadherin in the invasive margin was a significant and independent risk factor for disease-free and overall survival in multivariate analysis, while Yun et al (22) reported that decreasing E-cadherin expression was associated with a poor outcome in terms of overall survival in univariate (P=0.016), but not multivariate (P=0.303, risk ratio=1.984, 95% confidence interval=0.539-7.296), analysis (46). The present study did not identify any statistically significant association between the survival rate and E-cadherin expression. Regarding the controversial results of E-cadherin expression, it should be noted that in various papers that we have mentioned, the research procedures were performed using various monoclonal antibodies, devices (instruments), classifications, and scoring systems (cut-off values). Therefore, there are many points that could have affected the difference in the results.

Studies of the immunohistochemical expression of NEDD9 in human tissue samples are few in the literature. Xia et al (47) found that NEDD9 expression is increased in ~50% of CRC samples, compared with normal colorectal tissue. Li et al (48) noted the high expression of NEDD9 in 68 of 92 CRC samples, compared with 12 of 92 in normal tissues (P<0.01). It was found that NEDD9 was significantly associated with an advanced TNM stage (P=0.014), pT grade (P=0.009), pN (P=0.013) and pM status (P=0.047). Patients with a higher NEDD9 expression had a significantly shorter overall survival rate (P<0.01) (48).

Table III. Coefficients for the correlation between E-cadherin and NEDD9 expression in the primary tumor, lymph nodes and liver with clinical characteristics.

	E-cadherin			NEDD9		
Expression of site	Primary tumor	Lymph nodes	Liver	Primary tumor	Lymph nodes	Liver
Age (years)						
ρ	0.245	-0.048	0.305	-0.084	-0.139	0.052
P	0.127	0.799	0.055	0.605	0.457	0.748
n	40	31	40	40	31	40
Tumor size (cm)						
ρ	-0.166	-0.281	-0.265	-0.025	0.08	0.175
P	0.305	0.126	0.098	0.88	0.669	0.281
n	40	31	40	40	31	40
T status						
ρ		-0.191			0.047	0.299
P		0.304			0.803	0.061
n	40	31	40	40	31	40
N status						
ρ	0.263	0.345	0.084	0.106	0.261	0.195
P	0.102	0.057	0.604	0.515	0.156	0.227
n	40	31	40	40	31	40
M status						
ρ						
P						
n	40	31	40	40	31	40
Surgical margins						
τB	0.06	-0.334	0.086	-0.108	-0.255	0.015
P	0.711	0.066	0.598	0.507	0.166	0.929
n	40	31	40	40	31	40
Microvascular invasion						
ρ	0.292	-0.022	0.176	-0.053	-0.041	0.108
P	0.067	0.907	0.278	0.743	0.825	0.508
n	40	31	40	40	31	40
Perineural invasion						
ρ	0.07	0.041	0.225	0.039	0.192	-0.094
P	0.668	0.826	0.163	0.81	0.3	0.563
n	40	31	40	40	31	40
Positive lymph nodes (%)						
ρ	0.2	0.372	-0.016	0.026	0.451	0.175
P	0.217	0.039[a]	0.922	0.872	0.011[a]	0.281
n	40	31	40	40	31	40
Sex						
τB	0.177	0.249	0.107	0.129	0.207	0.217
P	0.263	0.157	0.497	0.378	0.217	0.143
n	40	31	40	40	31	40
Survival time (days)						
ρ	-0.137	-0.075	-0.292	-0.035	-0.229	0.068
P	0.398	0.688	0.067	0.829	0.215	0.676
n	40	31	40	40	31	40

[a]$P<0.05$. ISI, immunohistochemical staining index; T, tumor; N, lymph node; NEDD9, neuronal precursor cell expressed developmentally downregulated 9; M, metastasis; ρ, Spearman's rank correlation coefficient; τB, Kendall's τ-b correlation coefficient.
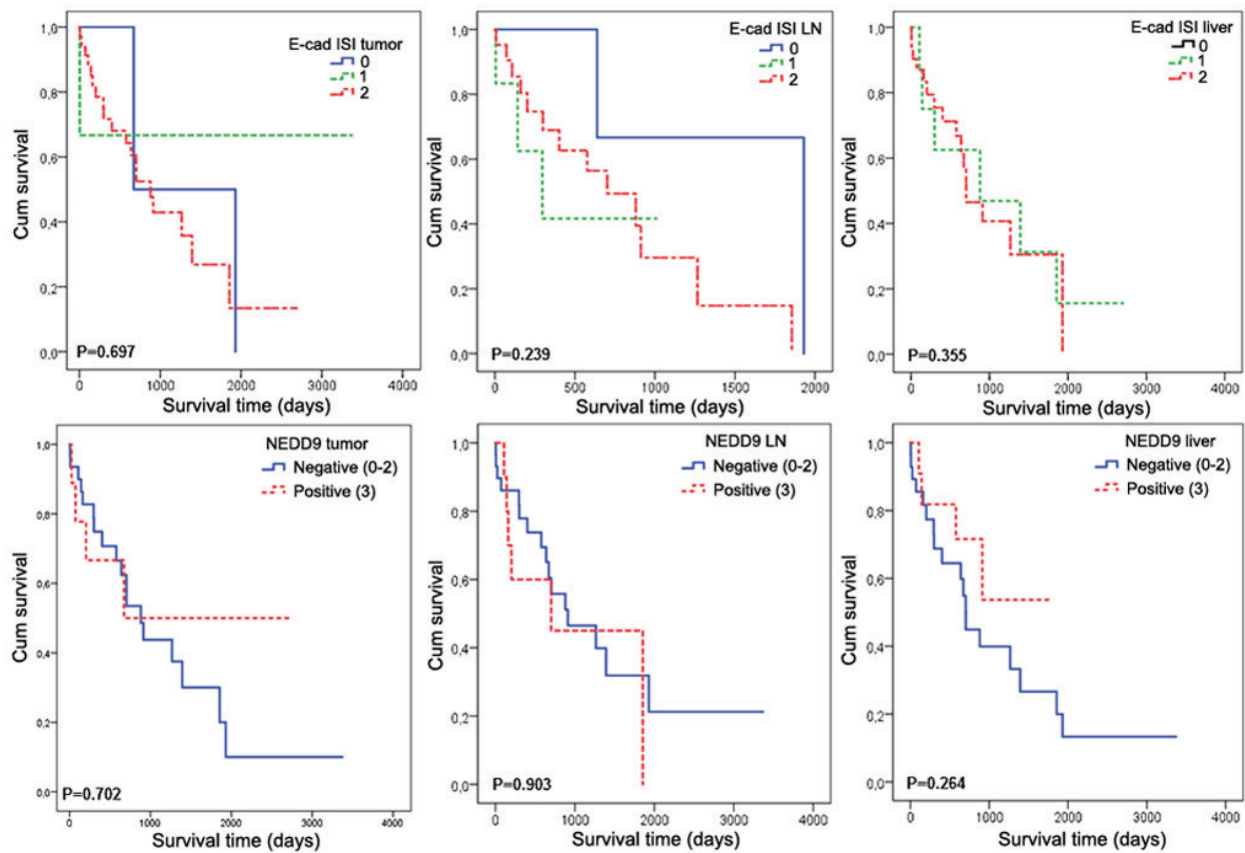
Figure 2. Expression of E-cadherin and NEDD9 in the primary tumor, metastatic LNs and liver metastasis compared with the survival outcome, as demonstrated by Kaplan-Meier survival curves with P-values from the log-rank test. E-cad, E-cadherin; ISI, immunohistochemical staining index; LN, lymph node, NEDD9, neuronal precursor cell-expressed developmentally downregulated 9.

The present study identified a strong expression of NEDD9 in 22.5% of primary CRC tumors, 35.5% of LNs and 30% of liver metastases. As did Li *et al* (48), we found a significant positive correlation between positive LN and NEDD9 expression. The difference in the expression of NEDD9 was also noted in cell lines (primary cell line SW480, and LN metastatic cell line SW620, derived from the same patient) (49). To the best of our knowledge, there are no published data on the expression of NEDD9 in CRC liver metastasis. The expression is similar to that of LN. Further studies of the expression of NEDD9 in liver metastases are needed. Potentially due to the small study cohort, no connection between the expression of NEDD9 in the primary tumor, LNs or liver metastases with survival rate was identified in the present study. Limitations of our study mostly arise from the small sample size. However, the registry of Croatian patients that had a resection of their CRC does not exist. Furthermore, there are no universally accepted guidelines for the treatment of CRC, i.e., a similar case will be treated rather differently in various institutions. This situation poses unsurmountable challenges for the accruement of a larger Dukes D patients' group. Since 2014, the EGFR testing has become a standard part of pathological reports. If we were performing the research now, we would include the results of EGFR as a factor determining the sampling of our groups. Clinical significance/relevance lies in the possibility of an improved distinction among patients who will experience more benefits of anti-EGFR therapy. Similar studies about the expression of E-cadherin and benefit of anti-EGFR therapy were published in settings of patients with lung adenocarcinoma (50,51). However, the patients included from 2014 still would not have a sufficient follow-up data at this point. Due to our objectively limited resources and the impossibility to use CEA-controlled oncolytic adenovirus (it is not available in Croatia), CEA was not used, although we recognize it as 'one of the most important factors'. Additional limitations to our study arise from the absence of data on E-cadherin and NEDD9 expression in CRC cell lines and animal models of CRC. In the future diagnostic procedures, if the equipment, samples, and experienced, professional staff would be provided to our institution, we intend to use this method (52).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PJ designed and performed the research, wrote the manuscript and analyzed the clinical data. PR and MPB collected and analyzed the samples. MM performed the statistical analysis. BK contributed to the design of the research, supervised the experiments and wrote the manuscript.

Ethics approval and consent to participate

All of the samples were collected at the time of diagnosis and after obtaining written informed consent. All experiments were performed in accordance with the Declaration of Helsinki and were approved by the Ethics Committee of Clinical Hospital Center Sestre Milosrdnice.

Patient consent for publication

The patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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