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Özgün Çalışma / Original Article

UZAK METASTAZLI REKTUM KANSERLERİ (EVRE IV) İNVAZİV TÜMÖR SINIRINDA E KADHERİN'İN DOKU EKSPRESYONUNDA AZALMA GÖSTERMEKTEDİR

RECTUM CANCERS WITH DISTANT METASTASES (STAGE IV) SHOW DECREASED TISSUE EXPRESSION OF E CADHERIN AT THE INVASIVE TUMOR FRONT

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ABSTRACT

PURPOSE: Rectal cancers (RC) are one of the most important causes of death worldwide. Survival of patients is mainly associated with the TNM stage. However, patients characterized by the same tumor stage often have prominent distinct survival. This is particularly a clinical challenge and new biomarkers are needed. In this research, we analyzed the prognostic role of E-cadherin (EC) in stage IV RC.

MATERIAL AND METHODS: Eighty-five stage IV RC patients operated at Kırıkkale University between 2001 and 2015 were included in this retrospective study. EC was scored using model A on immunohistochemical (IHC) stained sections. The relationship between the results and the clinicopathological characteristics was analyzed.

RESULTS: EC percentage was significantly downregulated in RCs classified as advanced pT (p=0.005), angiolymphatic invasion (p=0.034), stage IVb (p=0.006), high number of metastatic lymph nodes (p=0.039) and high grade (p=0.014). In univariate analysis, low EC patients had worse 5-year survival (RFS: 28.3%, p<0.001; OS: 41.2%, p<0.001). Multivariate analyzes confirmed that low EC is an independent worse survival parameter for RFS (Hazard ratio [HR]: 1.33 [1.15-3.46], p=0.001) and OS (HR: 1.57 [1.09–4.32], p=0.002).

CONCLUSION: Our study confirmed the prognostic significance of low EC in stage IV RCs. Therefore, we suggest that this parameter may be an indicator of worse prognosis in RCs. This biomarker can be easily defined on IHC stained slides and can use a molecular agent in RC therapy.

Keywords: *E cadherin, rectal cancer, prognostic markers, stage IV*

ÖZET

AMAÇ: Rektal kanserler (RK) dünya genelinde en önemli ölüm nedenlerinden biridir. Hastaların sağkalımı temel olarak TNM evresi ile ilişkilidir. Bununla birlikte, aynı tümör evresi ile karakterize edilen hastalar sıklıkla belirgin farklı sağ kalımlara sahiptir. Bu özellikle klinik bir çelişkidir ve yüksek riskli hastaları ayırt etmek için yeni biyobelirteçlere ihtiyaç vardır. Bu araştırmada, stage IV RK'da E cadherin (EC) 'nin sağ kalımdaki rolünü analiz ettik.

GEREÇ VE YÖNTEM: Bu retrospektif çalışmaya Kırıkkale Üniversitesi'nde 2001-2015 arasında cerrahi girişim uygulanan seksen beş RK hastası dahil edildi. EC, immünohistokimyasal (İHK) boyanmış kesitlerde model A kullanılarak skorlandı. Sonuçlar ve klinikopatolojik özellikler arasındaki ilişki analiz edildi.

BULGULAR: EC yüzdesi, ileri pT (p = 0,005), anjiyolenfatik invazyon (p=0,034), evre IVb (p=0,006), yüksek lenf nodu metastazı sayısı (p=0,039) ve ileri grade (p=0,014) olan RK'larda anlamlı derecede düşüktü. Tek değişkenli analizde, düşük EC'li hastalar 5 yıllık kötü sağkalıma sahipti (RFS: % 28,3, p <0,001; OS: %41,2, p<0,001). Çok değişkenli analizler, düşük EC'nin RFS (Hazard ratio [HR]: 1,33 [1,15 – 3,46], p=0,001) ve OS (HR: 1,57 [1,09 – 4,32], p=0,002) için bağımsız bir kötü hayatta kalma parametresi olduğunu doğruladı.

SONUÇ: Sonuçlarımız stage IV RK'larda düşük EC' nin prognostik önemini doğruladı. Bu nedenle, bu parametrenin RK'larda kötü prognozun bir göstergesi olabileceğini öneriyoruz. Bu biyobelirteç, İHK boyalı lamlar üzerinde kolaylıkla tanımlanabilir ve RK tedavisinde bir moleküler ajan olarak kullanılabilir.

Anahtar Sözcükler: *E cadherin, rektal kanser, prognostik belirteçler, stage IV*

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PURPOSE

In the western world, rectal cancers (RC) are the second most common cause of tumor death and the third most frequently carcinoma [1]. Despite advances in adjuvant therapy and surgery, 5-year survival rates remain below 20% in stage IV disease [2]. Tumor-Node-Metastasis (TNM) staging is the gold standard in the prognosis of RC patients. However, some patients with equal stages of the disease have a worse prognosis. Therefore, there is a great need to identify these cases in which different treatment options can be tried.

Epithelial-mesenchymal transition (EMT) is defined as one of the methods of metastasis such as local invasion, rupturing from main cancer and migration to distant sites. The differentiation of an epithelial cell into a mesenchymal cell is the main method of EMT, which is common in the normal inflammatory process [3]. Also, EMT can be considered as a physiological mechanism characterized by the proliferation of cancer cells, loss of tumor cell polarity and migration to the loco-regional area [4, 5]. Therefore, one of the first steps in the metastatic process can be considered as EMT. Several well-known molecular EMT agents of EMT have been described in the literature, but downregulation of E cadherin (EC) in tumor cells is considered to be a typical finding of EMT [6]. EC is a transmembranous protein with tumor suppressor activity that binds cells together. In the structure of the EC, this cell-cell adhesion is supported by forming a complex connection with the cell skeleton. However, the relationship between the cancer environment and EMT remains unclear [7, 8].

In the present study, we aimed to examine the prognostic value of EC to predict high-risk patients in stage IV RC.

MATERIAL AND METHODS

Study Design

The study was approved by the Health Research Ethics Committee of Kırıkkale University (2019.05.19). In this study, all procedures were consistent with 1964 Helsinki declaration and the ethical standard of the national/ institutional research committee and the. Informed consent was obtained from the patients individually. The authors did not have appropriate financial involvement and did not report a conflict of interest.

In this study, patients who were surgically resected for stage IV RC between 2001-2015 (n=85) in Kırıkkale University hospital were identified. This database included retrospectively collected data such as information on age, pT, size, number of metastatic lymph nodes, neural and vascular invasion, grade, stage and survival. RCs were categorized according to the following criteria: age (mean age 76, <76 and ≥76), pT (pT1/pT2 and pT3/pT4), size (mean size 5.5 cm; ≥ 5.5 cm and < 5.5 cm), metastatic lymph node number (<7 and ≥7), angiolymphatic invasion (no and yes), perineural invasion (no and yes), stage (stage IVA and stage IVB) and grade (High grade and Low/Moderate grade). All cases were re-evaluated according to American joint committee on cancer classification, 7th [9].

Processing of Tissues

Paraffin-embedded archival tumor samples were obtained from all patients. A tumor block showing the deepest invasively area was chosen from each patient. Cases were accepted only when there was sufficient tissue in the paraffin block for future studies. Two 4 micron thick sections (n = 85) were taken from each block and stained with hematoxylin & eosin (H&E) and EC. An experienced pathologist evaluated all sections.

Estimates of EC

EC was semi-quantitatively estimated on Immunohistochemical (IHC) stained sections according to Model A [22]. Model A recommends using the hot spot area, deepest invasive block, and invasive margin for pathological evaluation. Firstly, the area of the invasive margin was subjectively screened in an x10 magnification on IHC sections. The percentage of EC immunopositivity was scanned in all directions at invasively tumor margin in an x20 magnification to subjectively determine the low (<50%) EC and high (≥50%) EC area described by He [11]. The EC ratio was then noted in 10 high power fields (HPF) using an x20 magnification (0.785 mm^2) **(Figure 1).** EC staining was noted positive when the staining was completely around the cytoplasmic membrane of the cell as described by Dass [10]. If cancer cells did not show a blue-stained nucleus on IHC stained sections, these cells were excluded from the number to avoid the counting of brown cytoplasmic artefacts.

Immunohistochemical Study

A 4 μm (n = 85) section was taken from all blocks and a platinum-plated slide was placed (Dako). We used the Pt link (Dako) to apply the pretreatment methods. We obtained the heat-induced retrieval epitope by the target retrieval solution (Dako) at 97 ° C, pH 9 for 20 minutes. Staining was performed according to Autostainer link 48 (Dako). Peroxidase-blocking reagent (Dako) was used to blocked endogenous peroxidase activity. The mouse monoclonal EC (Clone Nch-38, 1: 50, Dako) was the primary antibody and we diluted with antibody diluent (EnVision Flex, Dako, S2022). We incubated the primary antibody for 30 minutes at room temperature and amplification was performed by Mause linker (Dako) for 20 minutes. Hrp reaction (Dako) was used to detect bound antibody, chromogen-diluted Dab reaction (Dako) in substrate buffer (Dako) was used for visualization. Meyer's hematoxylin (Germany, Darmstadt Merck) was used for counterstain and the slides were coverslipped by Pertex (Gothenburg, Sweden, Histolab).

Patients Follow-up

In this study, survival rates were considered in terms of outcome measures. Information about survival was obtained from archival records. The primary surgery

day was taken as the basis for calculating the endpoint time. The follow-up period was fourteen years in order to make a more reliable decision about the outcome of the patients. Recurrence-free survival (RFS) was defined as the time between primary surgery day and death for any reason and distant or local-regional recurrence. Overall survival (OS) was defined as the period between the primary surgery day and the day of death or the last follow-up for any reason. All patients whose last follow-up date was more than sixty months and all events after sixty months follow-up were censored at sixty months.

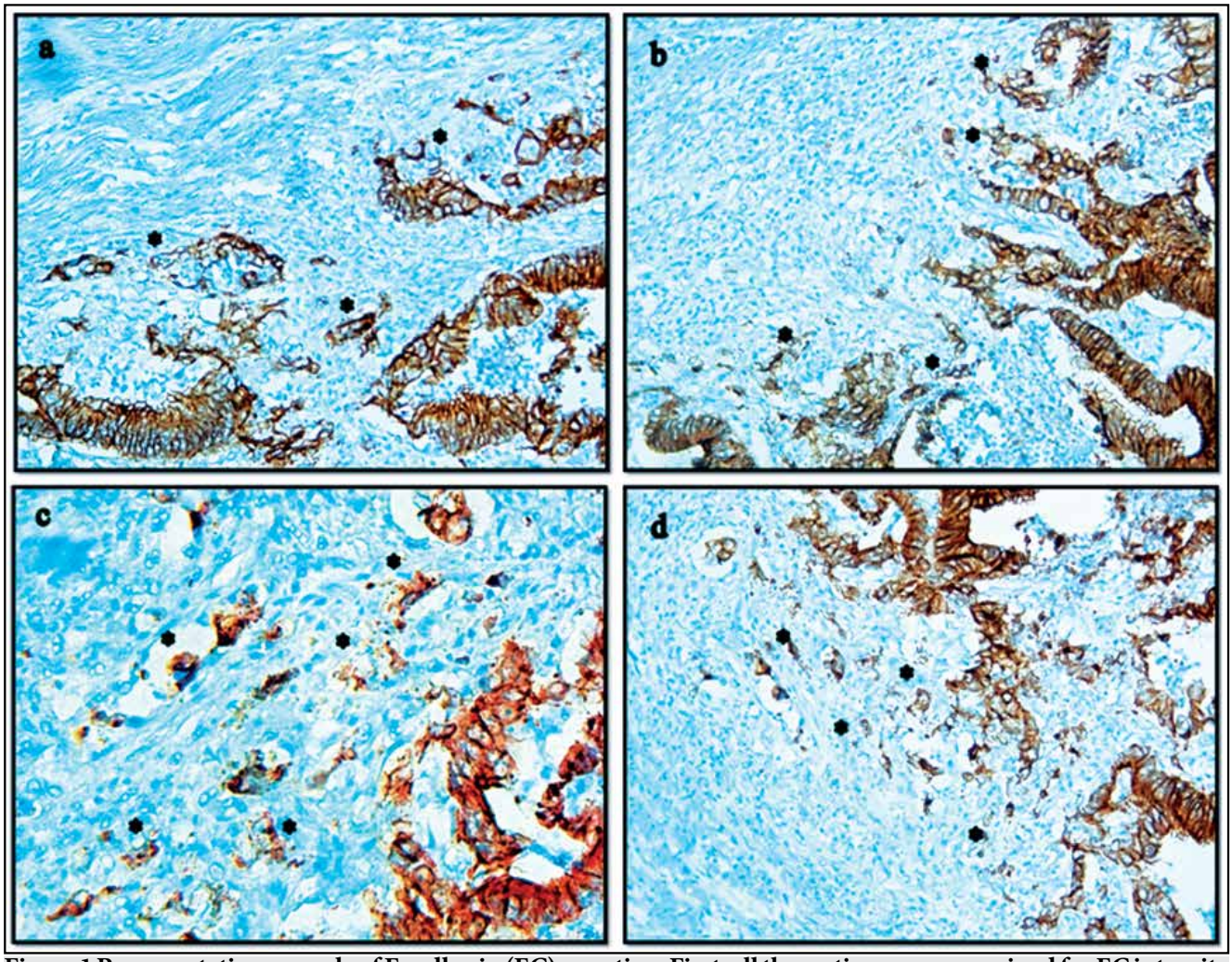

Figure 1 Representative example of E cadherin (EC) counting. First, all the sections were examined for EC intensity at an x10 magnification. Immunopositivity of the EC percentage (asterisk) was searched in both directions along the invasive tumor margin using an IHC stained section to subjectively determined the high (≥50%) (a-b) and low (<50%) (c-d) EC area. Then, EC was counted in 10 HPFs using an x20 objective field (0.785 mm²).

Statistical Evaluation

Descriptively data were listed using frequencies and percentages for categorical data andv standard deviation, means, and ranges for continuous data. Chi-Square test was used to analyze the relationships between clinicopathological and categorical variables of EC, and Fisher's Exact test was used when the Chi-Square test was not applied. Kaplan-Meier method was used to present survival curves and the Log-rank test was used to evaluate significant differences between univariate survival groups. Cox-regression model with a hazard ratio (HR) of 1.0 as a reference and a 95% confidence interval (CI) was used to analyze the multivariable survival groups. SPSS 21.0 (North Castle, IBM institute, ABD) was used for the analyses. All tests were two-sided and p-values less than 0.05 level were noted as significant.

RESULTS

Patients

Eighty-five cases that were surgically resected for RC were included in the study. 30 (35.3%) of the cases were male and 55 (64.7%)were female. The mean of age and size were 76.08±7.45 (range: 37-86) and 5.72±1.85 (range: 3-10), respectively. 36 (42.3%) of the cases were

detected as PT1/PT2, 49 (57.7%) as PT3/PT4, and 45 (53.0%) of the tumor was poorly differentiated and 40 (47.0%) was low/moderately differentiated.

Assessment of EC

EC was scored on IHC stained sections according to model A mentioned above. Sections were examined at low power magnification and the EC staining distribution was not homogeneous in the slides. A suitable block was selected from each tumor showing the deepest invasive front and good staining homogeneity. The tumors were divided into two groups with a cut-off of 50% described by He [11].

Table 1 The relationship between EC and prognostic factors (n=85)

		E cadherin (n=85) (%)		
		≥50%	**<50%**	**P-value**
PT-stage	**pT3/pT4**	15	21	
		41.6%	58.4%	**0.005***
	pT1/pT2	35	14	
		71.4%	28.6%	
Age	**≥76**	20	18	
		52.6%	47.4%	0.297
	<76	30	17	
		63.8%	36.2%	
AL invasion	**Positive**	17	20	
		45.9%	54.1%	**0.034***
	Negative	33	15	
		68.7%	31.3%	
Size	**≥5.7 cm**	21	19	
		52.5%	47.5%	0.264
	<5.7 cm	29	16	
		64.4%	35.6%	
PN invasion	**Positive**	22	20	
		52.3%	47.7%	0.233
	Negative	28	15	
		65.1%	34.9%	
LN Status	**≥7**	16	19	
		45.7%	54.9%	**0.039***
	<7	34	16	
		68.0%	32.0%	
Grade	**High grade**	18	22	
		45.0%	55.0%	**0.014***
	Low/Moderate grade	32	13	
		71.1%	28.9%	
Stage	**Stage IVB**	14	20	
		41.1%	58.9%	**0.006***
	Stage IVA	36	15	
		70.5%	29.5%	

*. The significant limit for Chi-square test was 0.05. Significant results are in italics. **Abbreviations:** EC: E-cadherin, PT-stage: Pathological tumor stage, PN: Perineural invasion, LN: Lymph Node, AL: Angiolymphatic invasion.

Statistical results were as follows: The mean and median percentage of EC was 51.75% ±9.50% (range 15%-75%) and 50%, respectively. Thirty-five (41.1%) of cases was low EC (<50%) and significant association was determined with advanced pT (p=0.005), angiolymphatic invasion (p=0.034), high metastatic lymph node number (p=0.039), stage IVb (p=0.006) and high grade (p=0.014). The relationship between EC and clinicopathological characteristics is listed in **Table 1.**

Table 2 Univariate and multivariate survival analysis of EC (n=85)

		Univariate survival analysis (n=85) (%)				Multivariate survival analysis (n=85) (%)			
		OS		RFS		OS		RFS	
		5-year (%)	P-value	5-year (%)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-Value
PT-stage			0.185		0.108		NC		NC
	pT3/pT4	37		46		-		-	
	pT1/pT2	62		53		-		-	
Age			0.824		0.683		NC		NC
	≥76 cm	45		58		-		-	
	<76 cm	54		41		-		-	
AL invasion			0.564		0.455		NC		NC
	Yes	50		53		-		-	
	No	49		46		-		-	
Size			0.368		0.277		NC		NC
	≥5.7 cm	43		50		-		-	
	<5.7 cm	56		49		-		-	
PN invasion			0.247		0.195		NC		NC
	Yes	40		47		-		-	
	No	59		52		-		-	
LN Status			0.124		**0.045***		0.324		0.092
	≥7	35		44		1		1	
	<7	64		55		2.64 (0.77-5.47)		2.72 (0.91-5.46)	
Grade			**0.033***		**0.029***		0.069		**0.048***
	High grade	32		43		1		1	
	Low/Mod-erategrade	67		56		3.28 (0.78-5.34)		1.49 (1.04-3.27	
Stage			**0.008***		**0.003***		**0.016***		**0.009***
	Stage IVa	27		40		1		1	
	Stage IVb	72		59		1.58 (1.12-2.68)		1.53 (1.11-2.58)	
EC			**<0.001***		**<0.001***		**0.002***		**0.001***
	Low	28		41		1		1	
	High	71		58		1.57 (1.09-4.32)		1.33 (1.15-3.46)	

*. The significant limit for Chi-square test was 0.05. Significant results are in italics. **Abbreviations:** EC: E-cadherin, PT-stage: Pathologic tumour stage, PN: Perineural invasion, LN: Lymph Node, AL: Angiolymphatic invasion, NC: Not calculable, CI: Confidence interval, HR: Hazard ratio, OS: Overall survival, RFS: Relapse-free survival

Follow-up
In the follow-up period of fourteen years, all of the patients died (100%; n=61 in low EC, n=24 in high EC), and seventy-six patients had relapsed (89.4%; n=50 in low EC, n=26 in high EC). The 5-year OS and RFS ratios were 28.3% and 41.2% in low EC cases, versus 71.7% and 58.8% in high EC patients, respectively **(Table 2).**

Survival Analyses
In univariate analysis, significant differences between survival groups were observed for RFS (p<0.005), OS (p<0.001). Lymph nodes status, grade and stage were associated with adverse outcomes for RFS and OS. In multivariate analysis, low EC was an independent poor prognostic parameter for RFS (HR=1.33 [1.15-3.46], p=0.001), OS (HR=1.57 [1.09-4.32], p=0.002). Grade and stage were the other parameters significantly associated with survival groups **(Table 2, Figure 2).**

DISCUSSION

The potential impact of EC in stage IV RC patients was investigated in our retrospective study. Our results suggest that there is a significant relationship between low EC and worse prognostic parameters and that EC plays an important role in the metastasis of cancer cells. If this result is confirmed in further clinical researches, EC may be preferred as a predictive marker in RC.

In recent years, RC has been described as one of the most common cancers not only in the Western world but in Northwest Asia. The sporadic disease has the majority of cases developing RC [12]. Molecular and biologic processes in RC play a significant role despite the researches of physiologic and etiologic elements [13]. All of the large intestine lumens are covered by colonic epithelial cells. These epithelial cells are separated from the epithelial layer by several phenotypic alterations called EMT [14]. EMT usually develops in embryogenesis, also in several pathological processes such as interstitial fibrosis of kidney, adhesion of endometrium and cancer. In recent studies, cancer progression, chemotherapy resistance, and metastasis-like properties have been associated with changes in EMT [15].

EC is a subtype of transmembrane proteins 1, plays a significant role in cell-cell adhesion, and is named EC when present in epithelial tissue [7, 8]. The invasion of surrounding tissues by tumor cells occurs as a result of reduced or lost EC which provides the connection between neighbouring cells, and this is a good example of EMT [15]. Cell migration and mobility in metastasis are supported by the loss of cellular integrity resulting from the appearance of mesenchymal properties during EMT. EMT-related features have been identified not only in RC [16] but in many human tumors, including gastric, oesophageal, breast and ovarian carcinomas [17-20].

Several markers such as N-cadherin, EC, Snail, and vimentin have been described as indicators of EMT [21]. However, according to literature, EC is the best-defined molecular biomarker of EMT, and the most specific feature of EMT was the loss of EC [21]. Therefore, EC may be a useful marker of poor differentiation, malignant phenotype, and invasiveness in RC. But, there is currently no consensus on the assessment of EC expression in RC patients. He et al. [11] examined 549 studies in a meta-analysis and unfortunately found significant differences in primary antibody dilution rates, clinical factors, number of patients, gender and age, and IHC methods. However, it is not clear in the literature whether the survival values of EC are different in RCs with these different parameters. In this study, we investigated a population of patients resected only for stage IV RC and performed pathological evaluation according to model A [22] to ensure standardization. In other words, unlike other studies, our patient population was quite homogeneous and standardization was high in our method.

In our analyses, downregulation of EC expression has

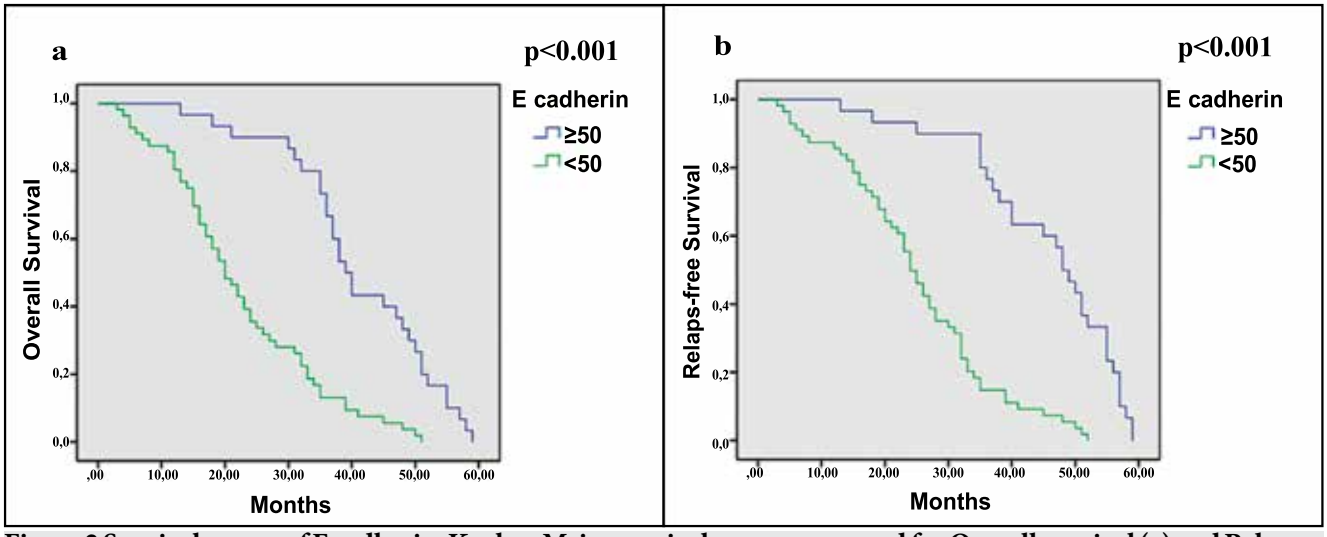

Figure 2 Survival curves of E cadherin. Kaplan-Meier survival curves were used for Overall survival (a) and Relapse-free survival (b). P-value is significant at the 0.05 level.

a poor survival in stage IV RC, which is in agreement with most previous studies. He et al. conducted a meta-analysis of 2730 colorectal cancer patients and found a significant association between decreased E-cadherin expression and prognosis [11]. In the subgroup analysis of this study, low E-cadherin expression was found to be significantly associated with poor prognosis in Asian countries rather than European countries. Also, E-cadherin was found to be associated with non-small cell lung cancer and gastric cancer in Asians in the literature, and this finding was not found in Europeans [23]. These findings suggest that there may be racial differences in E-cadherin expression in terms of prognostic factor. In our study, the fact that low EC expression is related to survival and that our country is from Asian countries supports the opinion that EC expression may contain racial differences.

The percentage of EC expression in RC ranges from 5% to 90% in the literature [11]. There are different opinion on the cut-off value of low E-cadherin expression. This may reflect no consensus on the evaluation of EC expression. He et al. reported that low E-cadherin was more associated with prognosis when the cut-off value was >50% [11]. In this study, we also used >50% as the cut-off value. We found that 41.1% of tumors had low EC expression, and low EC expression was significantly associated with survival. Therefore, we recommend that future studies use 50% as the cut-off value for EC.

As in our study, there was a significant correlation between E-cadherin expression and clinicopathologic features related to prognosis such as degree of differentiation, stage and lymph node status in the literature [11, 12]. These clinicopathological features also include perineural infestation and size [12]. However, our data showed decreased membranous EC expression in perineural invasive and large tumors, but this was not statistically significant Although reported studies have shown that EC expression in perineural invasion and large-size RC significantly decreased, there are contradictory studies that have not reported such a connection [11]. In this study, we estimated the percentage of EC in 10 HPFs, and the final percentage of ECE may have changed by this multiple-area counting method. Also, only an identifiable cancer cell nucleus was calculated in our study, so the ratio we calculated may have changed for this calculation method.

In this study, there are several limitations. There is a major limitation in the nature of the retrospective analysis. For example, it is impossible to overcome sampling bias because of the nature of retrospective research. Because the cases examined were previously sampled and used for diagnostic purposes. Also, the cases were treated before 2015 by previous methods that revealed the distinction between RC-treated studies today. We investigated EC using a block and a section that symbolize only a small portion of the entire tumor. Nevertheless, this study has been the largest EC study in Turkey.

CONCLUSION

EC expression decreased significantly in stage IV RCs and associated with poor prognostic features. Therefore, this marker can be a good prognostic parameter in patients who underwent curative surgery for RC. Adding this useful marker to the risk situation should be beneficial for good stratification for RC treatment.

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