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Original Article

Prognostic implications of adhesion molecule expression in colorectal cancer

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Abstract: Research on the expression of adhesion molecules, E-cadherin (ECAD), CD24, CD44 and osteopontin (OPN) in colorectal cancer (CRC) has been limited, even though CRC is one of the leading causes of cancer-related deaths. This study was conducted to evaluate the expression of adhesion molecules in CRC and to determine their relationships with clinicopathologic variables, and the prognostic significance. The expression of ECAD, CD24, CD44 and OPN was examined in 174 stage II and III CRC specimens by immunohistochemistry of TMA. Negative ECAD expression was significantly correlated with advanced nodal stage and poor tumor differentiation. Multivariate analysis showed that both negative expression of ECAD and positive expression of CD24 were independent prognostic factors for disease-free survival (DFS) in CRC patients ($P<0.001$, relative risk [RR] = 5.596, 95% CI = 2.712-11.549; $P = 0.038$, RR = 3.768, 95% CI = 1.077-13.185, respectively). However, for overall survival (OS), only ECAD negativity showed statistically significant results in multivariate analysis ($P<0.001$, RR = 4.819, 95% CI = 2.515-9.234). Positive expression of CD24 was associated with poor OS in univariate analysis but was of no prognostic value in multivariate analysis. In conclusion, our study suggests that among these four adhesion molecules, ECAD and CD24 expression can be considered independent prognostic factors. The role of CD44 and OPN may need further evaluation.

Keywords: Colorectal cancer, E-cadherin, CD24, CD44, osteopontin

Introduction

Colorectal cancer (CRC) is the second most common type of cancer and a major cause of cancer-related morbidity and mortality in the Western world [1]. In South Korea, CRC is the fourth most common type of cancer, and the incidence is increasing because of Westernization of the diet [2]. Although this disease is surgically curable in the early stages, tumors are frequently asymptomatic until the metastatic stage, which is associated with high mortality. Therefore, increasing efforts are being made to improve screening and prevention strategies for CRC and to enhance our capabilities of predicting clinical outcome. Currently, several molecular markers are being evaluated and established for a wide variety of tumors, including CRC. These markers have potential diagnostic, prognostic, or even therapeutic implications. It is widely known that tumor cell adhesion molecules involved in cell-cell and cell-extracellular matrix (ECM) adhesion are involved in the development of invasive and metastatic phenotypes.

E-cadherin (ECAD) is a member of the calcium-dependent adhesion molecule (CAM) family. ECAD mediates homophilic cell-cell adhesion in epithelial tissues and is localized to adjacent cell membranes. Decreased expression of ECAD has been correlated with a high tumor grade, an advanced stage of malignancies, and poor prognosis [3]. Low ECAD expression, or a lack of ECAD expression, is associated with dedifferentiation and metastasis [4, 5]. Loss of ECAD expression may result in reduced cell adhesion and increased invasion [6].

CD24 is a cell adhesion molecule that has been implicated in metastatic tumor progression of CRC. Strong cytoplasmic CD24 expression correlates significantly to shortened survival in CRC patients without distant metastases [7].

Osteopontin (OPN) is an integrin–binding protein that provides an anchor for osteoclasts to attach to the mineral surface of the bone matrix [8]. OPN-overexpressed colon cancer cells have been shown to be more proliferative *in vitro* and the tumors tend to grow faster *in vivo* [9]. OPN can bind to various cell surface receptors, including vitronectin (a_nb_3 integrin) and the hyaluronic acid receptor, CD44.

CD44 is a member of the immunoglobulin family that increases the metastatic potential of tumor cells [10]. The incubation of cells with CD44 antibody inhibits the binding of OPN and reduces migration [9].

Studies involving ECAD, CD24, CD44 and OPN or a combination of studies, have demonstrated that these molecules are responsible for tumor progression and metastasis, but the significant prognostic importance of these markers in CRC remains controversial.

This study was conducted to evaluate the expression of ECAD, CD24, CD44 and OPN in CRC and to determine the relationships with various clinicopathologic variables and potential prognostic significance.

Materials and methods

Patients and specimens

One hundred seventy-four patients with stage II-III colorectal carcinoma (CRC) who were treated between 2001 and 2004 were included in this retrospective study. The ethical use of human tissue for research was approved by our Institutional Review Board. For the purpose of this study, patients who died from other causes were excluded. None of the patients had undergone pre-operative chemotherapy or radiotherapy. After surgery, patients with stage III tumors were treated with 5-fluorouracil-based chemotherapy, whereas patients with stage II tumors were treated only upon patient request. The median follow-up period was 43.5 months (range, 2-112 months) for all patients.

All tissue samples were formalin-fixed and paraffin-embedded. Hematoxylin and eosin (H&E) stained slides, pathologic reports, and other medical records were reviewed to confirm the diagnosis and clinicopathologic parameters, including age, gender, tumor location, tumor size, depth of invasion (T), Nodal stage (N), degree of tumor differentiation, lymphovascular invasion (LI), and patient survival.

Tissue microarray (TMA) construction

For the purpose of performing effective detection, tissue microarray slides were used for this study. For preparation of these slides, the most representative area was carefully selected and marked on an H&E-stained slide. The TMA was assembled using a tissue microarray set (TMA01; TMA-Tech of Korea, Seoul, Korea). Tissue cores (3.0 mm in diameter) were punched from the original blocks and then inserted into new TMA cassettes (each of which contained 30 holes). Serially-sectioned slides were then produced. Each tissue microarray slide held 30 specimens, which could be analyzed simultaneously with minimum variation during the staining process. Each specimen was round in shape and 3.0 mm in diameter, with a sufficient amount of tissue for pathologic analysis.

Antibodies

This study used commercially-available monoclonal antibodies, as follows: anti-human E-cadherin antibody (clone EP700Y; Neomarkers, Fremont, CA, USA); CD44 antibody (clone 156-3C11; Neomarkers, Fremont, CA, USA); anti-human OPN antibody (clone RB-9097-PO; Neomarkers, Fremont, CA, USA); and anti-human CD24 antibody (clone SN3b; Neomarkers, Fremont, CA, USA).

Immunohistochemical staining procedure

For each antibody, deparaffinized tissue sections were placed in 10 mM citrate buffer (pH 6.0) and heated for antigen retrieval. Following incubation with each antibody at room temperature for 1 hour, the slides were subsequently incubated with a biotinylated secondary horse anti-mouse IgG antibody for 30 minutes and detected with avidin-conjugated horseradish peroxidase (DAKO, Carpenteria, CA, USA). The color was then developed using 3,3'-diaminobenzidine (DAB; ScyTek, Logan, UT, USA). Mayer's hematoxylin was then added as a counterstain. Lymph nodes and palatine tonsils were used as positive controls and a stain without primary antibodies was used as a negative control. Stains were performed in two sets of microarray blocks and discrepancies in the

Table 1. Correlations between the adhesion molecules and clinicopathologic data

Characteristics	ECAD			CD24		
	Negative	Positive	*P*	Negative	Positive	*P*
Age (yr)						
<60	22 (31.4%)	48 (68.6%)	0.169	12 (17.1%)	58 (82.9%)	0.615
≥60	23 (22.1%)	81 (77.9%)		21 (20.2%)	83 (79.8%)	
Gender						
Male	28 (27.2%)	75 (72.8%)	0.631	17 (16.5%)	86 (83.5%)	0.319
Female	17 (23.9%)	54 (76.1%)		16 (22.5%)	55 (77.5%)	
Tumor differentiation						
Well-to-moderate	40 (24.2%)	125 (75.8%)	**0.037***	33 (20.0%)	132 (80.0%)	0.136
Poor	5 (55.6%)	4 (44.4%)		0 (0.0%)	9 (100.0%)	
Tumor size						
<5 cm	29 (27.1%)	78 (72.9%)	0.637	15 (14.0%)	92 (86.0%)	**0.035***
≥5 cm	16 (23.9%)	51 (76.1%)		18 (26.9%)	49 (73.1%)	
Tumor location						
Left	11 (25.6%)	32 (74.4%)	0.961	8 (18.6%)	35 (81.4%)	0.945
Right	34 (26.0%)	97 (74.0%)		25 (19.1%)	106 (80.9%)	
T stage						
1, 2	2 (25.0%)	6 (75.0%)	0.826	0 (0.0%)	8 (100.0%)	
3, 4	43 (25.9%)	123 (74.1%)		33 (19.9%)	133 (80.1%)	
Nodal stage						
0	10 (14.3%)	60 (85.7%)	**0.004***	16 (22.9%)	54 (77.1%)	0.283
1, 2	35 (33.7%)	69 (66.3%)		17 (16.3%)	87 (83.7%)	
LI						
Absent	5 (11.4%)	39 (88.6%)	**0.011***	7 (15.9%)	37 (84.1%)	0.550
Present	40 (30.8%)	90 (69.2%)		26 (20.0%)	104 (80.0%)	
Characteristics	CD44			OPN		
	Negative	Positive	*P*	Negative	Positive	*P*
Age (yr)						
<60	22 (31.4%)	47 (67.1%)	0.057	11 (15.7%)	59 (84.3%)	0.455
≥60	19 (18.3%)	85 (81.7%)		21 (20.2%)	83 (79.8%)	
Gender						
Male	27 (26.2%)	76 (73.8%)	0.309	22 (21.4%)	81 (78.6%)	0.223
Female	14 (19.7%)	56 (78.9%)		10 (14.1%)	61 (85.9%)	
Tumor differentiation						
Well-to-moderate	35 (21.2%)	129 (78.2%)	**0.007***	28 (17.0%)	137 (83.0%)	**0.038***
Poor	6 (66.7%)	3 (33.3%)		4 (44.4%)	5 (55.6%)	
Tumor size						
<5 cm	25 (23.4%)	81 (75.7%)	0.729	20 (18.7%)	87 (81.3%)	0.897
≥5 cm	16 (23.9%)	51 (76.1%)		12 (17.9%)	55 (82.1%)	
Tumor location						
Left	6 (14.0%)	37 (86.0%)	0.188	7 (16.3%)	36 (83.7%)	0.680
Right	35 (26.7%)	95 (72.5%)		25 (19.1%)	106 (80.9%)	
T stage						
1, 2	3 (37.5%)	5 (62.5%)	0.697	1 (12.5%)	7 (87.5%)	0.814
3, 4	38 (22.9%)	128 (77.1%)		31 (18.7%)	135 (81.3%)	
Nodal stage						

0	14 (20.0%)	56 (80.0%)	0.456	8 (11.4%)	62 (88.6%)	0.052
1, 2	27 (26.0%)	76 (73.1%)		24 (23.1%)	80 (76.9%)	
LI						
Absent	9 (20.5%)	35 (79.5%)	0.710	8 (18.2%)	36 (81.8%)	0.967
Present	32 (24.6%)	97 (74.6%)		24 (18.5%)	106 (81.5%)	

*P<0.05.

immunostaining results were evaluated by staining with conventional paraffin tumor sections.

Evaluation of results of immunohistochemical staining

Two pathologists, who were unaware of the clinical parameters or outcomes for each patient, independently reviewed the immunohistochemically-stained sections. For the scoring of all molecules, 10 fields in the tumor frontier region were selected randomly and examined with high-power magnification. All discrepancies were resolved by joint review of the slides in question.

For ECAD expression, staining was scored as strong when immunoreactivity in the tumor region showed a similar membranous staining to its normal counterpart in more than 75% of the cells. Discontinuous membranous staining in 25-75% of the cells was scored as moderate staining. Absence of membranous staining or positive immunoreactivity in less than 25% of the cells was graded as weak or none [11]. Presence of more than 10% of distinct cell membrane staining was considered positive for CD24 and CD44 [12]. For OPN, granular cytoplasmic staining more intense than or equal to the adjacent normal colon epithelia in >10% of tumor cells was considered positive [12, 13].

Statistical analysis

All statistical analyses were performed using SPSS (version 15.0; SPSS, Chicago, IL, USA). Statistical significance was defined as a p value <0.05. Statistical associations between the levels of protein expression of ECAD, CD24, CD44 and OPN and other clinicopathologic factors were analyzed by the chi-square or Fisher's exact test when the chi-square test was not applicable. The correlations between protein expression of ECAD, CD24, CD44 and OPN were assessed by non-parametric analysis applying the Spearman rank correlation. In addition, this study examined the association between the expression status of the four adhesion molecules (ECAD, CD24, CD44 and OPN). The non-parametric Spearman's rho correlation coefficient was used to analyze the association between these four markers. Univariate and Multivariate analysis was performed using the Cox proportional hazards model to evaluate disease-free survival (DFS) and overall survival (OS).

Results

Mean age of the 174 patients was 60.78 years (30-86). Of the patients, 103 (59.2%) were men and 71 (40.8%) were women. The patients' characteristics are shown in **Table 1**. The tumors were located in the left side of the colon, including the descending, sigmoid, rectosigmoid colon, and rectum in 43 patients (24.7%) and in the right or transverse colon in 131 patients (75.3%). The sizes of the tumors ranged from 0.5-12 cm (mean, 4.95 cm). Histologically, 165 cases were classified as well- or moderately-differentiated (94.8%). Seventy cases (42.7%) were classified as stage II, and 104 cases (59.8%) as stage III.

Clinicopathologic correlations

Loss of membranous ECAD expression was observed in 25.9% of stage II and III CRC cases (45/174). CD24, CD44 and OPN expression was noted in 81.0% (141/174), 75.9% (132/174) and 81.6% (142/174), respectively, of stage II and III CRC cases (**Figure 1**; **Table 1**).

Loss of membranous ECAD expression was associated with poor tumor grade (P = 0.037), higher N stage (P<0.004), and presence of lymphatic invasion (P = 0.011). A significant increase in CD24 expression was detected in larger tumors (≥5 cm vs. <5 cm; P = 0.035). However, there was no association between CD24 expression and the other clinicopathologic features, including T stage, N stage, or lymphatic invasion. CD44 and OPN expression were associated with poor tumor differentiation (P = 0.007), but not associated with T stage, N stage, stage, and lymphatic invasion.

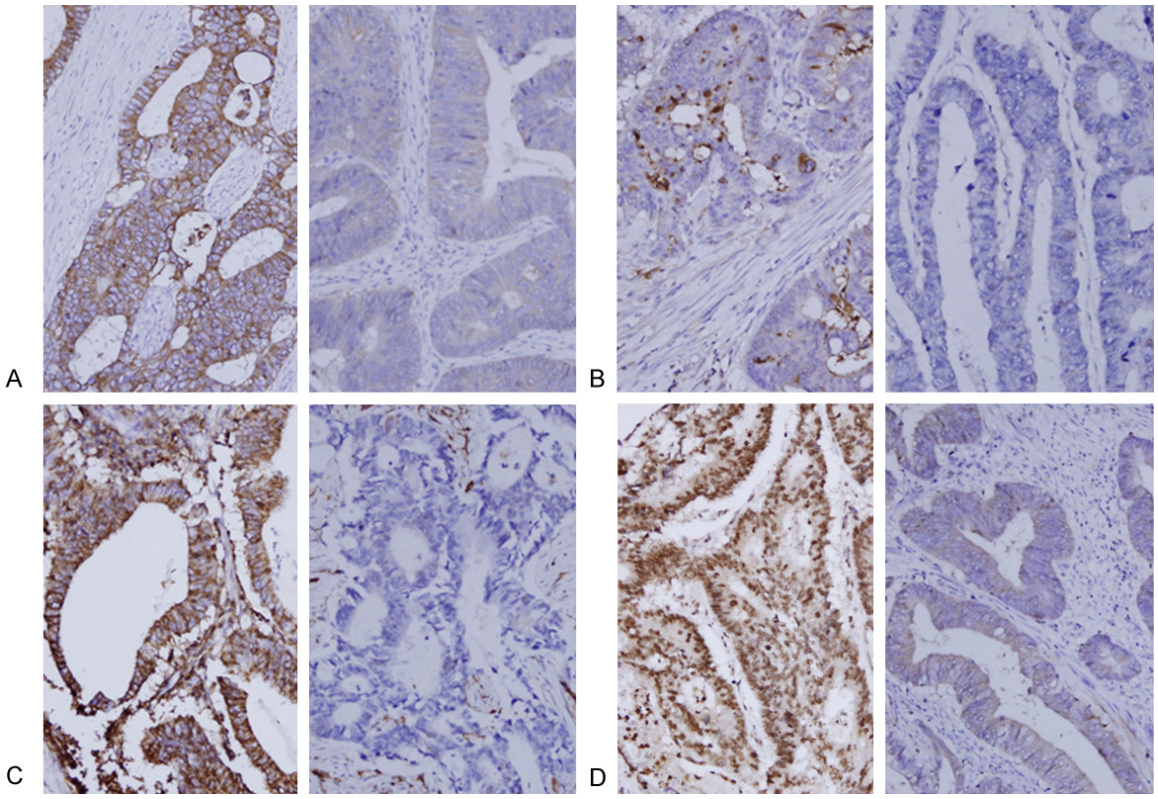

Figure 1. Representative sections of immunohistochemical stains of ECAD, CD24, CD44 and OPN. Positive (left column) and negative (right column) immunohistochemical stains of ECAD (A), CD24 (B), CD44 (C) and OPN (D), respectively (A-D, ×200).

Table 2. Spearman's rho correlation coefficients (*P* value) for the association between the expression status of four pairs of adhesion molecules

	ECAD	CD24	CD44	OPN
ECAD		-0.018 (0.815)	0.260 (0.001)	0.025 (0.748)
CD24	-0.018 (0.815)		0.061 (0.421)	-0.078 (0.304)
CD44	0.260 (0.001)	0.061 (0.421)		0.051 (0.504)
OPN	0.025 (0.748)	-0.078 (0.304)	0.051 (0.504)	

Association between the loss of ECAD expression and expression of CD24, CD44 and OPN

Loss of membranous ECAD expression was associated with loss of CD44 expression (Spearman's rho = 0.26, *P* = 0.001), but was not associated with CD24 or OPN expression. Expression of CD24 and OPN showed no association with other adhesion molecules (**Table 2**).

Role of adhesion molecules as prognostic factors in CRC

Negative expression of ECAD (*P*<0.001), positive expression of CD24 (*P* = 0.017), advanced N stage (*P* = 0.005) and present LI (*P* = 0.017) showed statistically poor disease-free survival in univariate analyses (**Table 3**). In multivariate analyses, negative expression of ECAD (P< 0.001), positive expression of CD24 (P = 0.038), and advanced N stage were revealed as statistically poor prognostic factors.

For overall survival, negative expression of ECAD (P<0.001), poor tumor differentiation (P< 0.001), advanced N stage (P = 0.013), and present LI (P = 0.042) were related to poor prognosis in univariate analyses (**Table 4**). Negative expression of ECAD (P<0.001) and poor tumor differentiation (P = 0.012) were significant poor prognostic factors in multivariate analyses. Positive expression of CD24 showed high relative risk (RR = 30,792, 95% CI = 1.502-631.258, P = 0.026) in univariate analysis, but had no significant association in multivariate analysis.

Table 3. Univariate and multivariate analyses for prognostic value of disease-free survival (DFS)

Characteristics	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P*	RR (95% CI)	*P*
Age (yr)		0.13		
<60	1.000			
≥60	0.588 (0.296-1.170)			
Gender		0.616		
Male	1.000			
Female	0.834 (0.410-1.697)			
Tumor differentiation		0.052		
Well-to-moderate	1.000			
Poor	3.279 (0.992-10.839)			
Tumor size		**0.034***		
<5 cm	1.000			
≥5 cm	2.484 (1.073-5.754)			
Tumor location		0.643		
Left	1.000			
Right	1.213 (0.536-2.742)			
T stage		0.730		
1, 2	1.000			
3, 4	1.420 (0.194-10.424)			
Nodal stage		**0.005***		**0.037***
0	1.000		1.000	
1, 2	3.558 (1.468-8.624)		2.569 (1.303-6.408)	
Lymphovascular invasion		**0.017***		
Absent	1.000			
Present	1.550 (1.081-2.223)			
ECAD		**<0.001***		**<0.001***
Positive	1.000		1.000	
Negative	5.444 (2.697-10.987)		5.596 (2.712-11.549)	
CD24		**0.017***		**0.038***
Negative	1.000		1.000	
Positive	1.550 (1.081-2.223)		3.768 (1.077-13.185)	
CD44		0.156		
Negative	1.000			
Positive	0.579 (0.272-1.232)			
OPN		0.663		
Negative	1.000			
Positive	1.240 (0.472-3.261)			

*$P<0.05$. Cox proportional hazard regression. RR, relative risk; CI, confidence interval; ECAD, e-cadherin; OPN, osteopontin.

Discussion

ECAD

In renal cell carcinoma, along with bladder, prostate, and colorectal cancer, reduced expression of ECAD has frequently been observed in cancer progression [5, 11, 14-19]. For CRC, several previous studies have reported relationships between lost or reduced expression of ECAD and clinicopathologic factors, such as tumor grade, tumor stage, metastasis, and patient survival [3-5, 11, 15, 20, 21].

In the current study, reduced ECAD expression was observed in 25.9% of the tumors (45 of

Table 4. Univariate and multivariate analyses for prognostic value of overall survival (OS)

Characteristics	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P*	RR (95% CI)	*P*
Age (yr)		0.345		
<60	1.000			
≥60	0.751 (0.415-1.359)			
Gender		0.758		
Male	1.000			
Female	0.909 (0.495-1.668)			
Tumor differentiation		**<0.001***		**0.012***
Well-to-moderate	1.000		1.000	
Poor	7.277 (3.175-16.678)		3.266 (1.295-8.237)	
Tumor size		0.126		
<5 cm	1. 1.000			
≥5 cm	1.490 (0.779-2.849)			
Tumor location		0.573		
Left	1.000			
Right	1.225 (0.605-2.483)			
T stage		0.484		
1, 2	1.000			
3, 4	0.658 (0.204-2.127)			
Nodal stage		**0.013***		
0	1.000			
1, 2	2.448 (1.209-4.958)			
Lymphovascular invasion		**0.042***		
Absent	1.000			
Present	2.632 (1.036-6.687)			
ECAD		**<0.0011***		**<0.001***
Positive	1.000		1.000	
Negative	5.492 (2.989-10.091)		4.819 (2.515-9.234)	
CD24		**0.026***		
Negative	1.000			
Positive	30.792 (1.502-631.258)			
CD44		**0.005***		
Negative	1.000			
Positive	0.414 (0.225-0.761)			
OPN		0.817		
Negative	1.000			
Positive	1.108 (0.464-2.646)			

*$P<0.05$. Cox proportional hazard regression. RR, relative risk; CI, confidence interval; ECAD, e-cadherin; OPN, osteopontin.

174), which was less than results of previous studies (37.8%-45.8%) [4, 5, 22]. This discrepancy may be due to differences in the evaluation system for immunopositivity or variations between the cases examined. In our study, we selected cases pathologically diagnosed as stages II and III.

Dorudi et al. reported that ECAD expression is closely related to the stage and tumor grade, with more aggressive cancers displaying markedly reduced expression of ECAD [4]. Ghadimi et al. reported a significant relationship between reduced ECAD and lower tumor grade, but did not find a clear correlation between loss of ECAD expression and depth of tumor infiltration into the intestinal wall (T-category) [5]. In a multivariate study of 84 CRC cases, Roca et al. revealed that ECAD expression was not associated with pathologic parameters, such as

tumor stage, tumor grade, or lymph node metastasis, but the loss of ECAD was an independent, adverse prognostic marker was an independent, adverse prognostic marker [21]. A study involving 1420 CRC cases using the TMA technique showed that loss of ECAD expression is associated with higher T stage, higher N stage, presence of vascular invasion, and worse survival in mismatch repair (MMR)-proficient CRC and with higher N stage and worse survival in multiL homolog 1 (MLH1)-negative CRC [20]. In this study, Lugli et al. also concluded that loss of membranous ECAD was an independent adverse prognostic factor in CRC.

Recently, in a study of 140 stage II and III CRC cases, Ngan et al. reported an inverse relationship between ECAD expression and tumor differentiation and showed that loss of expression of ECAD was an independent adverse prognostic factor in CRC [11].

In the present study, ECAD was identified as an independent prognostic factor for disease free survival (DFS) and overall survival (OS) in CRC.

CD24

Recently, CD24 has been shown to be a prognostic marker in gastric adenocarcinoma [7]. CD24 is a small, heavily glycosylated, mucin-like cell surface protein. It functions as an alternative ligand of P-selectin, an adhesion receptor expressed on activated endothelial cells and platelets, and can thus enhance the metastatic potential of CD24-expressing tumor cells [7].

Conflicting reports exist regarding the potential relationship between variant CD24 expression and the prognosis of patients with CRC. Weichert et al. reported strong cytoplasmic CD24 expression correlated significantly to shortened patient survival in the group of CRC cases without distant metastases [7]. This report emphasized the importance of the finding of cytoplasmic CD24 as an independent marker of high-risk patients. The study suggested that this information could be used to individualize patient care (e.g., closer patient monitoring for disease recurrence or progression after surgery). However, another study by Ahmed et al. reported that CD24 shows early upregulation and nuclear expression but is not a prognostic marker in CRC [23].

In the present study, positive expression of CD24 was associated with independent poor prognosis for disease-free survival. Also it showed poor prognosis for overall survival in univariate analyses.

CD44

CD44 is a transmembrane glycoprotein involved in cell-to-cell and cell-to-matrix interactions [10]. CD44 is located on chromosome 11p13. The human *CD44* gene consists of at least 20 exons. Conflicting conclusions exist regarding the potential relationship between variant CD44 expression and the prognosis of patients with CRC [24]. Using CD44s, Asao et al. reported that the loss of CD44s expression is a sensitive marker for lymph node metastasis in CRC [24]. A multivariate analysis involving 74 CRC cases by Huh et al. indicated that CD44s expression is an independent predictor of overall survival [25]. Another study involving 72 CRC cases using an immunoenzymatic assay did not show any significant relationship between tumor CD44s levels and patient outcome [26]. In the present study, CD44 showed better overall survival in univariate analyses, in contrast to previous studies. However, in multivariate analyses, CD44 lost its significance as a prognostic marker.

OPN

OPN is a secreted glycophosphoprotein expressed by a number of cell types, including epithelial cells. Recent evidence has linked OPN with tumorigenesis, notably with the regulation of cell motility, invasion, and metastasis formation [9, 27, 28]. OPN-overexpressed colon cancer cells were shown to be more proliferative *in vitro* and the tumors tend to grow faster *in vivo* [9]. In a multivariate regression study, Rohde et al. found that OPN expression alone is an independent poor prognostic predictor in CRC [29].

In the current study, OPN expression was significantly higher in well-to-moderately differentiated tumors than poorly differentiated tumors ($P = 0.038$). However, OPN expression was not related to clinicopathologic variables, such as tumor size, stage, and lymph node metastasis. OPN also showed no statistical association with DFS and OS. This result is in contrast to previous reports [29, 30]. Further studies with larger scale will reduce this controversy.

In conclusion, negative ECAD expression and positive CD24 expression are associated with poor prognosis in CRC. OPN expression does not appear to be associated with long-term outcome and CD44 expression needs further investigation. ECAD and CD24 should be utilized clinically as they demonstrate useful prognostic significance in CRC.

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Disclosure of conflict of interest

None.

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