

Correlation of multiple proteins with clinic-pathological features and its prognostic significance in colorectal cancer with signet-ring cell component

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Abstract. – **OBJECTIVE:** Primary colorectal cancer (CRC) with signet-ring cell (SRC) component is a distinct tumor of colon and rectum, and its prognosis is very poor. Reliable markers to predict the poor clinical outcome of this subgroup cancer remains undetermined. Therefore, we perform this study to investigate the prognosis value of seven proteins in CRC with SRC component .

PATIENTS AND METHODS: This study involved 117 patients diagnosed with CRC with SRC component between January 2008 and August 2015 at Fudan University Shanghai Cancer Center. The samples from these patients were analyzed by immunohistochemistry to reveal the expression levels of p53, p21, E-cadherin, COX-2 (Cyclo-oxygenase-2), Bcl-2, CD44 and Ki-67. Kaplan-Meier analysis and log-rank testing were performed to estimate survival. Subsequently, a Cox proportional hazard model was used to calculate hazard ratios for the risk of death.

RESULTS: The p21, p53, COX-2, E-cadherin, Bcl-2, CD44 and Ki-67 expression were detected in 60.5%, 61.3%, 69.7%, 68%, 28.6%, 62.2% and 75.6% of the samples, respectively. The positive expression of p53 ($p=0.017$) and the negative expression of COX-2 ($p=0.001$) or E-cadherin ($p=0.047$) in CRC with SRC component were significantly associated with decreased overall survival, but the other expression levels were not. In a multivariate analysis, the negative expression of COX-2 was found to be an independent prognostic factor for poorer overall survival (hazard ratio, 0.37; 95% confidence interval, 0.19 to 0.75, $p=0.003$)

CONCLUSIONS: The COX-2 positive expression in CRC patients with SRC component had a poorer outcome than patients who were COX-2-negative. Therefore, COX-2 could be considered as an indicator for appropriate treatment and intensive follow-up in these subgroup patients.

Key Words

Colorectal signet-ring cell carcinoma, COX-2, Prognostic factors.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females¹, and its incidence rates are rapidly increasing in Eastern Asia and Eastern Europe². Colorectal signet-ring cell carcinoma (SRCC) is a rare but distinctive histological subtype of CRC. Population-based studies^{3,4} have indicated that SRCC approximately accounts for 0.1-2.4% of CRC patients. According to the World Health Organization definition⁵, SRCC is an adenocarcinoma in which a substantial amount (>50% of the tumor) of the signet-ring cell is retained within the tumor. However, we have also seen colorectal adenocarcinomas with the presence of signet-ring cells (or mucin) in less than 50% of the tumor. Currently, the features of CRC with the signet-ring cell (SRC) component and the overall outcomes of these patients have not been well determined.

During the past decades, much effort has been placed on investigating the multiple mechanisms involving carcinogenesis and development of CRC, since the identification of novel molecular prognostic factors with a distinct prognosis outcome would be a great help to improve the prediction of clinical outcome and determine the appropriate therapeutic approach⁶⁻¹⁰. In this study, we analyze the expression of p21, p53, Cyclooxygenase-2 (COX-2),

E-cadherin, CD44 and the Ki-67 protein which have been cited as prognostic factors in patients with CRC, and determine the predictive role of these molecular markers in CRC with SRC component.

Patients and Methods

Patients and tissue

A total of 117 CRC patients with SRC component who underwent surgical resection at Fudan University Shanghai Cancer Center between January 2008 and August 2015 were enrolled in the study. All specimens were fixed in 10% formalin, embedded in paraffin, and sectioned into 4- μ m slices. Tissues 5 cm or above from the resection margin were used as controls. None of the patients received any radiotherapy, chemotherapy, or immunotherapy before surgery, and were confirmed pathologically after surgery. All patients were staged according to TNM Stage (7th AJCC 2009). Permission to use the tissue sections for research purposes was obtained and approved by the Ethics Committee of Fudan University Shanghai Cancer Center.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded primary tumors were obtained from all patients. Tissue slides were routinely stained with hematoxylin and eosin. For immunohistochemistry analysis, the slides were subjected to antigen retrieval in Target Retrieval Solution, pH 9 (DAKO, Denmark A/S, Glostrup, Denmark) with PT Link (DAKO, Denmark A/S, Glostrup, Denmark). Tissues were incubated with mouse monoclonal antibody anti-p21 (dilution 1:100, clone p21, DAKO, Denmark A/S, Glostrup, Denmark), mouse monoclonal antibody anti-p53 (dilution 1:100, clone p53, DAKO, Denmark A/S, Glostrup, Denmark), mouse monoclonal antibody anti-E-cadherin (dilution 1:100, clone NCH-38, DAKO, Denmark A/S, Glostrup, Denmark), mouse monoclonal antibody anti-COX-2 (dilution 1:100, Carpinteria, DAKO, Denmark A/S, Glostrup, Denmark), rabbit polyclonal antibody anti-bcl-2 (dilution 1:50, Thermo Scientific, Rockford, IL, USA), mouse monoclonal antibody anti-CD44 (dilution 1:100, clone CD44, DAKO, Denmark A/S, Glostrup, Denmark), mouse monoclonal antibody Ki-67, clone MIB-1 (DAKO, Denmark A/S, Glostrup, Denmark). Negative controls were incubated

with mouse or rabbit IgGs (DAKO, Denmark A/S, Glostrup, Denmark). Detection was done with EnVision™ system (DAKO, Denmark A/S, Glostrup, Denmark).

Immunohistochemical Analysis

The expression was scored by two independent observers who had no knowledge of the clinical data. All membranous, cytoplasmic and nuclear staining were evaluated. Using the Ki-67 proliferation index, the percentage of positive cells out of 1,000 cells was calculated at the location where most positive cells expressed in tumor cell nuclei are distributed. Greater than 50% was determined as positive. For the other markers, the staining was graded into four groups: (+++) strong, (++) moderate, (+) weak, (-) negative. Tumors were regarded as immunopositive if 10% of tumor cells showed immunoreactivity.

Statistical Analysis

Statistical analyses of the immunohistochemical staining were performed using SPSS16.0 software (SPSS Inc., Chicago, IL, USA). Frequency data were analyzed with χ^2 (chi-square) test. Survival analysis was conducted using the Kaplan-Meier method. A comparison of survival curves was carried out using the log-rank test. Univariate and multivariate analyses were performed based on the logistic regression or Cox regression model with forward conditional variable entry ($p < 0.05$) and removal ($p > 0.10$). $p < 0.05$ was considered statistically significant.

Results

Patient Characteristics

Of the 117 study participants, 81 patients (69.2%) were male, and 36 patients (30.8%) were female, resulting in a male: female ratio of 2.25:1. The median age was 52.0 years (range, 24 to 87 years). Sixty-three patients (53.8%) had colon cancer, and fifty-four patients (46.2%) had rectum cancer. Of the 117 patients, 82 patients (70.0%) had more than 50% signet-ring cell component, which was diagnosed as colorectal signet-ring cell carcinoma, and 35 patients (30%) had less than 50% signet-ring cell component. In total, 1 patients were classified as stage I, 10 patients as stage II, 74 patients as stage III, and 32 patients as stage IV (Table I).

Table I. Clinicopathologic characteristics of 117 CRC patients with SRC component.

Variable	Value
Median age (range), y	52 (24-87)
Gender	
Male	81
Female	36
Primary tumor location	
Colon	63
Rectum	54
Signet-ring cell component	
≤50%	35
>50%	82
T stage	
T2	2
T3	58
T4	57
N stage	
N0	16
N1	21
N2	80
Metastasis	
No	85
Yes	32
Stage	
I	1
II	10
III	74
IV	32
Preoperative CEA levels	
Mean (range), U/ml	13.1 (0.08-377)
Preoperative CA 19-9 levels	
Mean (range), U/ml	68.1 (0.8-1993)
Lymphatic invasion	
Positive	70
Negative	47
Perineural invasion	
Positive	72
Negative	45

Correlation between the Clinicopathologic Factors and Expression of Molecular Markers

In all the 117 cases, 71 (60.7%) demonstrated positive results for p21, 70 (60.0%) for p53, and 78 (66.7%) for COX-2. And 80 (68.4%) showed positive results for E-cadherin, 34 (29.1%) for bcl-2, 72 (61.5%) for CD44, and 88 (75.2%) for Ki-67.

Of the seven proteins, p53 expression was detected more frequently in the large tumor ($p=0.031$, Table II) and was correlated with the presence of lymph node metastases ($p=0.043$, Table II). Negative E-cadherin and COX-2 expression were also correlated with the presence of lymph node metastases ($p=0.03$ and $p=0.049$, Ta-

bles III and IV, respectively). Additionally, negative E-cadherin was detected more frequently in younger patients ($p=0.041$, Table II). However, tumor location, CEA and CA 19-9 levels before surgery, T stage, vascular invasion and perineural invasion were not correlated with the expression of any of the examined proteins (Table II).

Correlations in Protein Expression and their Association with Survival

Univariate analyses revealed the positive expression of p53 ($p=0.017$, Figure 1) and the negative expression of COX-2 ($p=0.001$, Figure 2) and E-cadherin ($p=0.047$, Figure 3) in CRC with SRC component was significantly associated with decreased overall survival, but the other expression levels were not. In a multivariate analysis, the negative expression of COX-2 was found to be an independent prognostic factor for poorer overall survival (hazard ratio, 0.35; 95% confidence interval, 0.17 to 0.70, $p=0.003$, Table III).

Discussion

Primary colorectal SRCC is a rare entity accounting for less than 1% of all CRC¹¹⁻¹³. Multiple previous researches^{4,14-18} have shown that colorectal SRCC is associated with poor survival outcomes compared with mucinous adenocarcinoma (MA) and the more common adenocarcinoma (AC). Tan et al⁹ also demonstrated that CRC with SRC component is associated with poor prognosis in comparison with MA and AC. The identification of predictive and prognosis biomarkers will facilitate the selection of suitable patients and the personalization of treatment. However, to our best knowledge, no relevant studies have been published to identify the biomarkers of prognosis in colorectal SRCC. Due to the low incidence of primary colorectal SRCC, patients with a component of SRC, regardless of the extent, has been included for analysis in our study.

The oncosuppressor protein p53 is the most important protein in the signaling pathways of the cell cycle and apoptosis²⁰. Furthermore, the functional loss of p53 is proposed as a late event in the transition from adenoma to carcinoma. Liu et al²¹ evaluated 153 CRC patients and found that p53 protein expression is an independent predictor of shorter overall survival in patients with completely resected CRC. Lumachi et al²² also reported that p53 positive expression in colorectal

Table II. Correlation of multiple protein expression in primary tumor with demographic and clinic-pathological features.

Variable	P53					P21				
	N° pat.	Neg.	Pos.	X ²	p-value	N° pat.	Neg.	Pos.	X ²	p-value
Sex										
Male	81	31	50	0.4	0.53	81	31	50	0.12	0.73
Female	36	14	22			36	12	24		
Age (years)										
≤60	75	32	43	0.54	0.46	75	25	50	3.14	0.077
>60	42	15	27			42	21	21		
Tumor location										
Colon	65	22	43	2.43	0.12	65	19	46	6.24	0.013
Rectum	52	25	27			52	27	25		
Tumor size (cm)										
≤5	72	37	35	4.63	0.031	73	30	43	0.26	0.61
>5	45	14	31			44	16	28		
Lymphovascular invasion										
No	47	22	25	0.33	0.57	47	19	28	0.041	0.84
Yes	70	29	41			70	27	43		
Perineural invasion										
No	45	16	29	0.65	0.42	45	17	28	0.073	0.79
Yes	72	31	41			72	29	43		
Lymph node metastasis										
No	16	11	5	4.11	0.043	16	6	10	0.026	0.87
Yes	101	42	59			101	40	61		
CEA before surgery										
≤5	79	31	48	0.088	0.77	79	32	47	0.14	0.7
>5	38	16	22			38	14	24		
CA 19-9 before surgery										
≤27	81	32	49	0.048	0.83	80	29	51	1	0.32
>27	36	15	21			37	17	20		
Type of surgery										
Radical	85	34	51	0.004	0.91	85	36	49	1.2	0.27
Palliative	32	13	19			32	10	22		
T stage										
T2	2	0	2	1.61	0.45	2	0	2	1.32	0.52
T3	58	25	33			58	23	35		
T4	57	22	35			57	23	34		

To be continued

cancer was associated with a worse outcome. In agreement with these studies, we found that loss of p53 expression was significantly associated with tumor size and lymph node metastasis. And we also observed a significant association between p53 expression and poorer survival of CRC with SRC component in this study, but it becomes a non-significantly independent prognostic factor in multivariate analysis.

E-cadherin is a member of the cell adhesion molecules of the cadherin family that is also believed to act as a tumor suppressor gene, as its expression and function are downregulated or altered in many human cancers, and its re-expres-

sion decreases both the proliferative and invasive capacity of tumor cells. Yun et al²³ reported that negative E-cadherin expression was associated with a less favorable long-term prognosis. Elzagheid et al²⁴ reported that loss of E-cadherin expression was significantly associated with older age ($p < 0.03$) and lymph node involvement ($p < 0.02$). In univariate (Kaplan-Meier) survival analysis, negative E-cadherin significantly predicted shorter disease-free survival. Consistent with previous reports, loss of E-cadherin expression was significantly associated with younger age and lymph node metastasis. And univariate analysis had shown that negative expression of E-cadherin

Table II. Correlation of multiple protein expression in primary tumor with demographic and clinic-pathological features.

Variable	COX-2					E-cadherin				
	N° pat.	Neg.	Pos.	X ²	p-value	N° pat.	Neg.	Pos.	X ²	p-value
Sex										
Male	81	25	56	0.72	0.4	81	25	56	0.07	0.79
Female	36	16	20			36	15	21		
Age (years)										
≤60	75	24	51	0.17	0.68	75	30	45	4.17	0.041
>60	42	15	27			42	9	33		
Tumor location										
Colon	65	19	46	1.11	0.29	65	19	46	0.39	0.53
Rectum	52	20	32			52	18	34		
Tumor size (cm)										
≤5	72	22	50	0.037	0.85	72	21	51	0.52	0.47
>5	45	13	32			45	16	29		
Lymphovascular invasion										
No	47	17	30	0.28	0.59	47	14	33	0.12	0.73
Yes	70	22	48			70	23	47		
Perineural invasion										
No	45	15	30	0	1	45	13	32	0.25	0.62
Yes	72	24	48			72	24	48		
Lymph node metastasis										
No	16	2	14	3.87	0.049	16	2	14	4.69	0.03
Yes	101	38	63			101	41	60		
CEA before surgery										
≤5	78	26	52	0	1	78	29	49	3.34	0.068
>5	39	13	26			39	8	31		
CA 19-9 before surgery										
≤27	80	26	54	0.079	0.78	23	58	1.27	0.26	
>27	37	13	24			14	22			
Type of surgery										
Radical	84	26	58	2.57	0.11	72	12	60	19.4	0.001
Palliative	32	15	17			45	25	20		
T stage										
T2	2	0	2	1.088	0.58	2	0	2	1.82	0.4
T3	58	19	39			58	21	37		
T4	57	20	37			27	16	41		

To be continued

was a significant prognostic factor for overall survival in CRC with SRC component, but it became a non-significantly independent prognostic factor in multivariate analysis.

COX-2 is an inducible enzyme that regulates prostaglandin synthesis from arachidonic acid²⁵. Data indicated that COX-2 is involved in the regulation of apoptosis, angiogenesis and tumor cell invasiveness. Studies are conflicting regarding the prognostic significance of COX-2 in CRC patients. Sheehan et al²⁶ reported that COX-2 expression was correlated with higher Duke stage, larger tumor size, and more presence of LN metastasis. Furthermore, Al-Maghrabi et al²⁷ re-

ported a correlation between COX-2 expression and cancer recurrence. Yamauchi et al (Dis Colon Rectum 2002; 45: 98-103) reported that the cancer-free survival rate was lower in patients with COX-2-positive tumors. Miladi-Abdennadher et al²⁸ reported that COX-2 overexpression confers a reduced overall survival rate and is an independent factor predictive for prognosis in Tunisian patients with CRC. However, Lobo Prabhu et al²⁹ reported that negative COX-2 expression was related to lower disease-specific survival and disease-free survival in the rectal cancer cohort. Wu et al³⁰ evaluated 139 CRC patients and found that no correlation could be

Table II. Correlation of multiple protein expression in primary tumor with demographic and clinic-pathological features.

Variable	Bcl-2					CD44				
	N° pat.	Neg.	Pos.	X ²	p-value	N° pat.	Neg.	Pos.	X ²	p-value
Sex										
Male	81	56	25	0.42	0.52	81	32	49	0.12	0.73
Female	36	27	9			36	13	23		
Age (years)										
≤60	75	50	25	1.85	0.17	75	32	43	1.56	0.21
>60	42	33	9			42	13	29		
Tumor location										
Colon	65	48	17	0.6	0.44	65	23	42	0.59	0.44
Rectum	52	35	17			52	22	30		
Tumor size (cm)										
≤5	72	52	20	0.15	0.7	72	24	48	2.08	0.15
>5	45	31	14			45	21	24		
Lymphovascular invasion										
No	47	35	12	0.47	0.49	47	17	30	0.17	0.68
Yes	70	48	22			70	28	42		
Perineural invasion										
No	45	33	12	0.2	0.65	45	17	28	0.014	0.9
Yes	72	50	22			72	28	44		
Lymph node metastasis										
No	16	12	4	0.15	0.7	16	5	11	2.7	0.1
Yes	101	71	30			71	10	61		
CEA before surgery										
≤5	78	56	22	0.083	0.773	108	26	52	0.11	0.74
>5	39	27	12			39	19	20		
CA 19-9 before surgery										
≤27	80	56	24	0.11	0.74	81	28	53	1.69	0.19
>27	37	27	10			36	17	19		
Type of surgery										
Radical	85	61	24	0.1	0.75	85	35	50	0.97	0.33
Palliative	32	22	10			32	10	22		
T stage										
T2	2	2	0	1.41	0.49	2	0	2	1.34	0.51
T3	58	39	19			58	22	36		
T4	57	42	15			57	23	34		

To be continued

found between COX-2 expression and American Joint Committee on Cancer stage, depth of cancer invasion, or the presence of lymph node metastases. In the 118 (85%) patients whose cancers expressed COX-2 had better survival after 10 years follow-up, compared with those patients whose cancers did not express COX-2, but this observation did not reach a statistical significance. In our study, no significant difference in tumor size, lymphovascular invasion, perineural invasion, tumor stage was observed when comparing the cancers that did or did not express COX-2. We found that the negative of COX-2 was associated with a higher lymph node

metastasis. Univariate and multivariate analysis showed that negative expression of COX-2 was a significant prognostic factor for overall survival in CRC with SRC component.

Generally, Ki-67 expression is used as a marker of cell proliferation, and while it has been recognized as an independent prognostic factor in breast cancer^{31,32}, its prognostic value in CRC remains controversial. Lumachi et al²² reported that Ki-67 overexpression in colorectal cancer was associated with a worse outcome. However, Fluge et al³³ reported that high Ki-67 expression indicated improved relapsed-free survival in colon cancer. Shin et al³³ assessed 266 CRC

Table II. Correlation of multiple protein expression in primary tumor with demographic and clinic-pathological features.

Variable	Ki-67			X ²	p-value
	N° pat.	Neg.	Pos.		
Sex					
Male	81	56	25	0.42	0.52
Female	36	27	9		
Age (years)					
≤60	75	50	25	1.85	0.17
>60	42	33	9		
Tumor location					
Colon	65	48	17	0.6	0.44
Rectum	52	35	17		
Tumor size (cm)					
≤5	72	52	20	0.15	0.7
>5	45	31	14		
Lymphovascular invasion					
No	47	35	12	0.47	0.4
Yes	70	48	22		
Perineural invasion					
No	45	33	12	0.2	0.65
Yes	72	50	22		
Lymph node metastasis					
No	16	12	4	0.15	0.7
Yes	101	71	30		
CEA before surgery					
≤5	78	56	22	0.083	0.773
>5	39	27	12		
CA 19-9 before surgery					
≤27	80	56	24	0.11	0.74
>27	37	27	10		
Type of surgery					
Radical	85	61	24	0.1	0.75
Palliative	32	22	10		
T stage					
T2	2	2	0	1.41	0.49
T3	58	39	19		
T4	57	42	15		

patients undoing surgery, and also found that Ki-67 expression was not associated with colorectal cancer survival. In the current study used a 50% cut-off for defining Ki-67 protein positive phenotypes, as proposed by several authors. Ki-67 expression was only correlated with the type of surgery. Univariate and multivariate analysis had shown that positive expression of Ki-67 was not a significant prognostic factor for overall survival in CRC with SRC component.

Table III. Multivariate analyses of prognostic factors for colorectal cancer patients with signet-ring cell component.

Covariate	Multivariate analysis Hazard ratio (95%CI)	p-value
p53	2.01 (0.96-4.24)	0.066
COX-2	0.35 (0.17-0.70)	0.003
E-cadherin	0.79 (0.40-1.55)	0.487
p21	1.04 (0.54-2.02)	0.905
Bcl-2	0.70 (0.34-1.45)	0.342
CD44	0.99 (0.51-1.91)	0.977
Ki-67	0.80 (0.39-1.68)	0.561

p21 is known as one of the cell cycle inhibitors which plays a role through the p53 dependent or independent pathway. Sim et al³⁴ reported that high p21 expression at the pretreatment biopsy was associated with poorer tumor regression and short disease-free survival in rectal cancer patients treated with chemoradiotherapy. Bcl-2 is a gene involved in cell proliferation regulation; bcl-2 super expression can inhibit the apoptosis and induce tumorigenesis, and also it seems capable of inhibiting the therapeutic apoptosis. Torsello et al³⁵ reported that negative bcl-2 expression is associated with poorer survival in younger colorectal cancer patients. In the present study, univariate and multivariate analysis showed that negative bcl-2 expression was not related with poorer survival in CRC patients with SRC component. CD44, a cell hyaluronate receptor, is a surface marker for cancer stem cells, which implicates in the metastatic behavior of cancer cells. Hong et al³⁶ reported that low CD44 expression was correlated with increased tumor recurrence and short disease-free survival. Huang et al³⁷ reported that CD133 and CD44 proteins co-expression were shown to be the independent prognostic factors of colorectal cancers. In the present study, no significant association was found between CD44 expression and the prognosis of CRC patients with SRC component.

Conclusions

In this study, the positive expression of p53 and the negative expression of COX-2 or E-cadherin is significantly correlated with a poor prognosis in CRC patients with SRC component. Notably, the low HR (HR, 0.37; 95%CI, 0.19 to 0.75; $p=0.006$) associated with negative COX-2 expression from the multivariate analysis demonstrates its potential as an independent

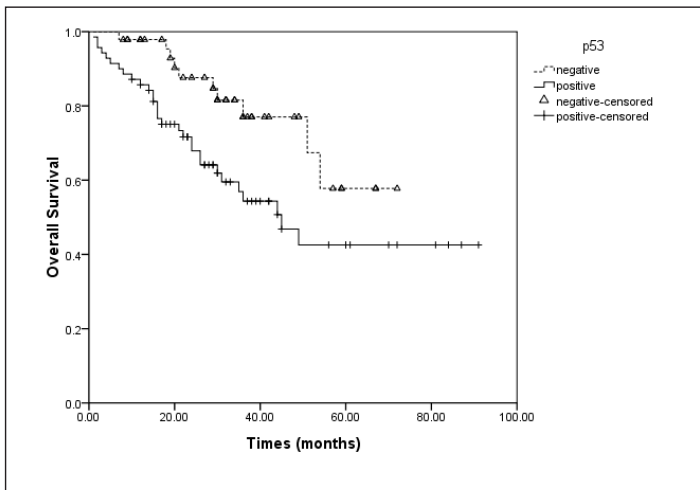


Figure 1. Univariate analyses for prognostic variable of overall survival based on p53 expression.

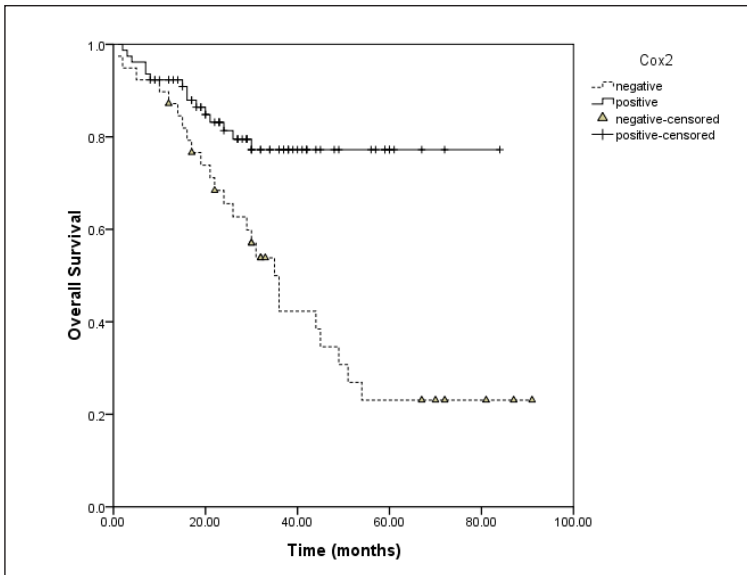


Figure 2. Univariate analyses for prognostic variable of overall survival based on COX-2 expression.

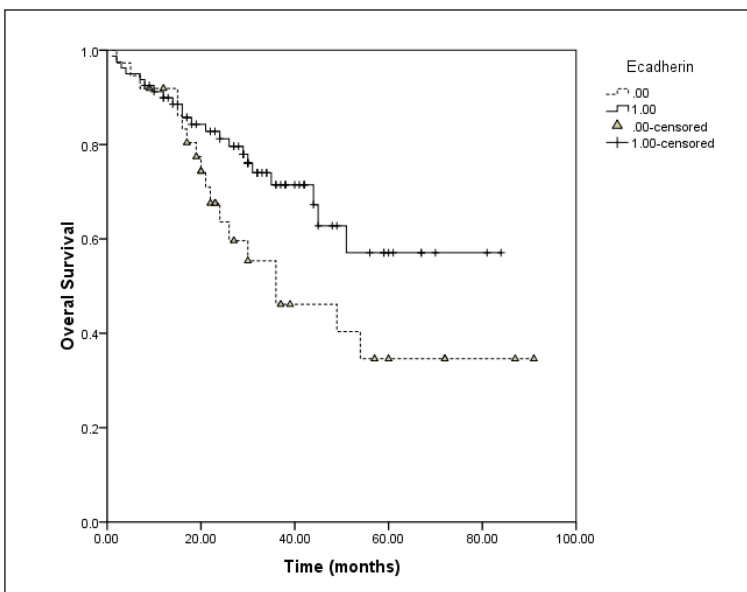


Figure 3. Univariate analyses for prognostic variable of overall survival based on E-cadherin expression.

prognostic factor for this subgroup patients. Based on these findings, we suggest that the number of expressed proteins may be helpful for identifying patients with a potentially poor prognosis, which will help to determine personalization of treatment for these patients.

Conflict of Interests

The Authors declare that they have no potential conflict of interests.

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