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Investigating the role of CD44 expression in the survival of colorectal cancer patients

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Abstract

Introduction: The CD44 is widely detected as a marker of cancer stem cells for colorectal cancer (CRC). However, its prognostic role in CRC and patient survival is still controversial.
Objectives: The present study aimed at investigating the role of CD44 expression in the survival of CRC patients.
Patients and Methods: The present retrospective study was conducted on 90 CRC paraffin blocks in the archive unit of the pathology department of Al-Zahra hospital. First, the patients' basic and clinical information and also their outcome and patients' survival were recorded. Then, the CD44 expression was evaluated and recorded by immunohistochemistry method.
Results: The CD44 expression was positive and negative in 42 (46.7%) and 48 (53.3%) cases, respectively. In addition, the positive CD44 expression was reported in 78.6% and 21.4% of survived and non-survived patients, respectively ($P=0.144$). The frequency of CD44 expression was higher in patients undergoing chemotherapy ($P=0.001$); however, none of the patients' other demographic and clinicopathological factors had a significant association with the CD44 expression ($P>0.05$). Moreover, it was found that the survival rate was higher in patients with a higher CD44 expression (HR=0.597; $P=0.452$); though, the CD44 expression and the other studied factors had no significant association with the patients' survival rate ($P>0.05$). Only metastasis significantly increased the chance of mortality (HR=7.639; $P=0.005$).
Conclusion: According to the results of the present study, more than 40% of CD44 expression was positive in CRC patients. In addition, the chance of the positive CD44 expression in patients undergoing chemotherapy (survived or non-survived) was significantly higher than the chance in patients without chemotherapy. Therefore, it may be considered as a poor prognostic marker in this cancer.

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Introduction

Colorectal cancer (CRC), as one of the most common cancers, is the second cause of cancer-related death in the world. Despite significant advances in the treatment process, the mortality rate is still reported to be high due to the patients' resistance to treatment or the recurrence of the disease (1). More than 30% of patients are presented with symptoms of metastatic disease and finally 50%-60% of them develop metastasis (2). In 2012, there were 1.4 million new cases of this disease and 694 thousand deaths were reported in this respect (3). Treatments for CRC can include a combination of surgery, radiotherapy and chemotherapy. The treatment of choice is surgery; however, it provides only the possibility of treating cancers confined to the colon wall while cancer spread throughout the body is usually not curable and the management focuses on improving the quality of life and symptoms of the disease such that the five-year survival is reported to be about 65% (3,4). Needless to say, the presented percentage depends on the progress of the cancer, the likelihood of removing all the cancer by surgery and the patients' overall health status. The recurrence and mortality reports reveal that the current treatments have encountered relative failure, as a result

Key point

This study was conducted on 90 colorectal cancer (CRC) patients. Outcome and survival of patients as well as the CD44 expression was recorded. The results of this study revealed that 46.7% of CD44 expression was positive in CRC patients. The chance of the positive CD44 expression in patients undergoing chemotherapy (survived or non-survived) was significantly higher than the chance in patients without chemotherapy. Therefore, CD44 expression may be considered as a poor prognostic marker in CRC patients.

of which researchers are in quest of new solutions to improve the patients' quality of life. In recent years, the hypothesis of cancer stem cells has been considered for the expression of cancer pathogenesis. Based on a large amount of evidence, human cancers are caused by stem cell disorders. In malignant tumors, the uncontrollable self-renewal of a small population of cancer stem cells leads to the development of cancer cells, that are abnormally differentiated and contribute to tumor growth (4). As these cells are resistant to anti-cancer drugs and radiotherapy, they play a role in the progress, recurrence and metastasis of cancers. According to this hypothesis as only a subset of cells causes tumor formation, new treatment methods have been presented with the aim of removing cancer stem cells or inducing their differentiation (5).

In this regard, the identification of these cells is one of the most significant fields of treatment at present. Studies have indicated that some cancer stem cell markers such as CD133, CD44, epithelial cell adhesion molecule and aldehyde dehydrogenase are markers with prognostic potential in different cancers (6). Among them, CD44 having a gene in chromosome 11p13 can be mentioned. This protein, recognized as a significant membrane receptor for hyaluronic acid, includes one end that binds to hyaluronic acid and the other end is in the cytoplasm and activates different biological activities of tumor such as differentiation, proliferation, invasion, cancer cell movement, chemotherapy resistance, and metastasis through the activation of mitogen-activated protein kinase, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), and Wnt pathways (7,8). Studies conducted regarding CD44, for example, have shown that this marker is involved in prostate cancer metastasis. Moreover, some other studies have indicated the association of the DC44 expression with poor survival rate, lymph node metastasis, distant metastasis and poor tumor differentiation in CRC while several studies stated the loss of CD44 expression as a poor prognostic factor for CRC patients (9). Therefore, this marker has received researchers' due attention in CRC. In addition, it seems that the association between its expression level and tumor malignancy can be illuminative in preventing metastasis by the early detection of cancer cells.

Objectives

The present study aimed at evaluating the role of this identifier as a specific marker in CRC and also evaluated its association with these patients' survival rate.

Patients and Methods

Study design

The current study was retrospective. The population of the study included CRC patients, whose tissue blocks were available from 2012 to 2017 in the archive unit of the pathology department of Al-Zahra hospital, Isfahan.

Patient enrollment

Ninety cases were considered as the sample according to the sample size formula at the 95% confidence level and considering the five-year survival rate of CRC from previous studies equal to 64% (10) and the error level of 0.1.

The inclusion criteria for the study were the CRC patients that either underwent or did not undergo chemotherapy. Moreover, their paraffin block was available in the archive unit of our hospital and had the appropriate block quality for sampling. In addition, the patient information was provided in the patient medical record.

Data collection and follow-up procedure

The researcher referred to the archive unit of the pathology department and randomly selected the samples that met the inclusion criteria of the study. The basic and clinical information including age, gender, family history of cancer, cancer type, tumor size, malignancy grade, metastasis, cancer recurrence and history of chemotherapy before the surgery and also CD44 expression status in the samples were evaluated and recorded.

In addition, the patient's family was contacted and asked about the patient's survival status and length to evaluate the patient outcome and survival rate.

Immunohistochemical determination of CD44

First, sections with a thickness of 4 µm from each paraffin block were placed on positively charged slides. Then, the sections were deparaffinized at 60°C using xylene and dehydrated by alcohol with decreasing degrees of 100, 90, 80 and 70, respectively.

Subsequently, antigens were recovered by heating the slides in Tris buffer with pH = 9 in a microwave at 95°C. After cooling the slides with 3% hydrogen peroxide solution, the internal peroxidases of the tissue were blocked, after which the primary CD44 antibody (Biocare Medical, USA, code: PM318AA) was incubated for 30 minutes at 37°C. Afterwards, secondary antibody was used in two steps and incubated for 10 minutes in each step. Finally, diaminobenzidine chromogen and hematoxylin were respectively conducted for the final staining of the cells and the staining of the background for one minute. At the end, after washing and dehydrating by alcohol with ascending degrees 70, 80, 90 and 100 respectively, the slides were mounted and ready to be observed under microscope. Tonsil tissue was used as a positive control. The CD44 staining was examined in the entire available area and cells whose membrane and cytoplasm were stained brown were considered as the positive CD44 expression.

The report of the CD44 expression in specific immunohistochemical staining was as follows: first, the intensity of staining was checked with the eye lens N.100 and was graded 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) staining intensity. Then, the slides were examined

with the eye lens N.40 to count and express the percentage of cells whose membrane and cytoplasm were stained and were then graded 1, 2, 3 and 4 for stainability of less than 25%, 25-50%, 50-75%, and more than 75%, respectively. At the end, the two numbers obtained from the first and second stages were added together and the ultimate number was a score of 7. If the final number was within the range of 0-3, the CD44 expression was considered negative and if it was within the range of 4-7, the CD44 expression was considered positive (Figure 1).

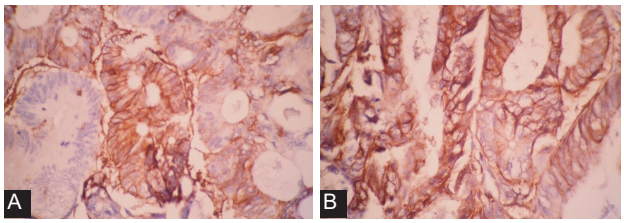

Figure 1. Immunohistochemical staining of the CD44 expression in a primary colorectal adenocarcinoma (×200), (A); with 3+ expression in more than 75%, (B): with +3 expression in 50-75%.

Statistical analysis

Finally, the collected data was entered into SPSS software (version 26). Data was reported as means ± standard deviation (SD) or frequency (percentage). Independent samples t test was conducted to compare the mean of quantitative data. Likewise, chi-squared test was employed to compare qualitative data in the positive and negative CD44 expression. Moreover, the Kaplan-Meier estimate was conducted to calculate the cumulative survival rate and draw the survival curve. In addition, Cox proportional-hazards regression was performed to determine the prognostic value of variables related to survival. The significance level of less than 0.05 was considered in all analysis.

Results

Out of 90 CRC patients in the present study, 61.1% and 38.9% were respectively male and female with the mean age of 60.80 ± 14.22 years. The positive and negative CD44 expression was observed in 42 (46.7%) and 48 (53.3%) cases, respectively. The frequency of CD44 expression was higher in patients undergoing chemotherapy ($P=0.001$); however, none of the patients' other demographic and clinicopathological factors had a significant association with the CD44 expression ($P>0.05$). In addition, the positive CD44 expression was reported in 78.6% and 21.4% of survived and non-survived patients, respectively ($P=0.144$; Table 1).

It should be noted that the positive CD44 expression was 33 and 8 among survived and non-survived patients, respectively while its negative expression was 31 and 17, accordingly. Comparing the expression of this gene with the patients' chemotherapy status according to the patient's status indicated that the highest frequency of gene non-

Table 1. Patients' demographic and clinicopathological factors based on the CD44 expression

Variables		Total (n=90)	CD44 expression		*P* value
			Positive (n=42)	Negative (n=48)	
Gender	Male	55 (61.1%)	26 (61.9%)	29 (60.4%)	0.855
	Female	35 (38.9%)	16 (38.1%)	19 (39.6%)	
Age (y)	<60	38 (42.2%)	18 (42.9%)	20 (41.7%)	0.909
	≥60	52 (57.8%)	24 (57.1%)	28 (58.3%)	
Family history		25 (27.8%)	12 (28.6%)	13 (27.1%)	0.924
Tumor location	Colon	56 (62.2%)	25 (59.5%)	31 (64.6%)	0.621
	Recto sigmoid	34 (37.8%)	17 (40.5%)	17 (35.4%)	
Tumor size (cm)	<5 cm	37 (41.1%)	18 (42.9%)	19 (39.6%)	0.924
	≥ 5cm	53 (58.9%)	24 (57.1%)	29 (60.4%)	
Histologic type	Typical adenocarcinomas	78 (86.7%)	37 (88.1%)	41 (85.4%)	0.443
	Mucinous adenocarcinoma	11 (12.2%)	4 (9.5%)	7 (14.6%)	
	Signet ring cell carcinoma	1 (1.1%)	1 (2.4%)	0 (0.0%)	
Histologic grade	Well differentiated	34 (37.8%)	18 (42.8%)	16 (33.3%)	0.516
	Intermediately differentiated	31 (34.4%)	12 (28.6%)	19 (39.6%)	
	Poorly differentiated	25 (27.8%)	12 (28.6%)	13 (27.1%)	
Stage	Early stage (I, II)	27 (30.0%)	15 (35.7%)	12 (25.0%)	0.268
	Advanced stage (III- IV)	63 (70.0%)	27 (64.3%)	36 (75.0%)	
Tumor budding	Low	64 (71.1%)	29 (69.1%)	35 (72.9%)	0.838
	High	26 (28.9%)	13 (30.9%)	13 (27.1%)	
Chemotherapy regimen	No concurrent chemotherapy	64 (71.1%)	22 (52.4%)	42 (87.5%)	0.001
	With concurrent chemotherapy	26 (28.9%)	20 (47.6%)	6 (12.5%)	
Metastasis		17 (18.9%)	6 (14.3%)	11 (22.9%)	0.297
Recurrence		9 (10%)	5 (11.9%)	4 (8.3%)	0.483
Outcome	Survived	64 (71.1%)	33 (78.6%)	31 (64.6%)	0.144
	Non-survived	26 (28.9%)	9 (21.4%)	17 (35.4%)	

expression in both survived and non-survived patients was found in patients without concurrent chemotherapy (P<0.05). In fact, it can be stated that the chance of the positive CD44 expression can be higher in patients undergoing chemotherapy (Figure 2).

The CD44 expression in the early stage with the mean of 4.56 ± 1.68 was slightly higher than its expression in the advanced stage with the mean of 4.38 ± 1.71 ($P=0.669$; Figure 3).

The evaluation of the factors related to the increased risk of mortality in CRC patients revealed that metastasis significantly increased the chance of mortality (HR = 7.639; $P=0.005$); however, other demographic and clinicopathological factors did not have a significant role in increasing the patients' risk of mortality ($P>0.05$). In addition, the positive CD44 expression reduced the risk of mortality to a lower and non-significant level (HR = 0.597; $P=0.452$; Table 2). In other words, the survival rate was higher in patients with a higher CD44 expression; however, no significant association was found between the CD44 expression and patients' survival rate (Figure 4).

Discussion

The results of the present study revealed that the positive CD44 expression was observed in 46.7% of cases such that the percentage of the positive CD44 expression was 78.6% and 21.4% in the survived and non-survived patients, respectively. Mashita et al reported the high CD44 expression ratios as an independent prognostic factor in CRC (11). However, hypoxia-inducible factor could up-regulate the CD44 variants (CD44v6 and v7/8) under a hypoxic condition (12). Moreover, CD44 variants were found to regulate reactive oxygen species (ROS) defense by stabilizing the xCT and promoting tumor growth (13). In fact, the mentioned studies indicated that CD44s and CD44v play a distinct role in both tumor initiation and progression.

Although standard CD44, v2, v3, v6 and v9 expressions have been reported for CRC patients (14), the prognostic role of CD44v and CD44s in CRC patients is still

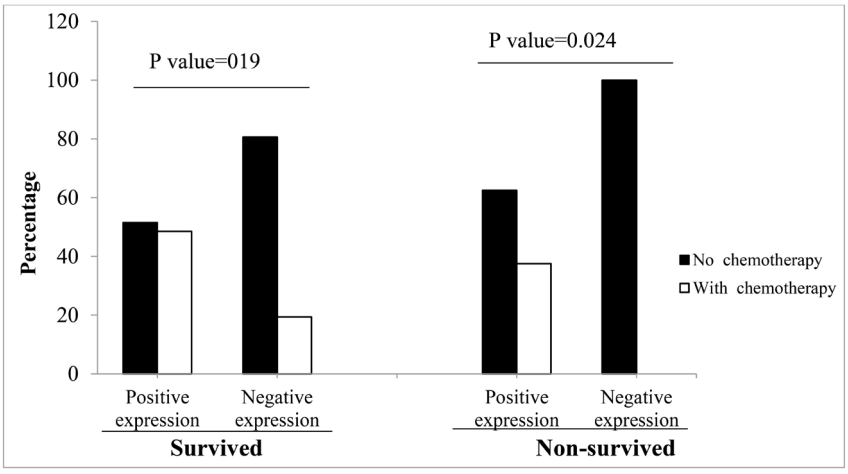

Figure 2. Comparison of the frequency of the CD44 expression according to patients' chemotherapy status in survived and non-survived groups.

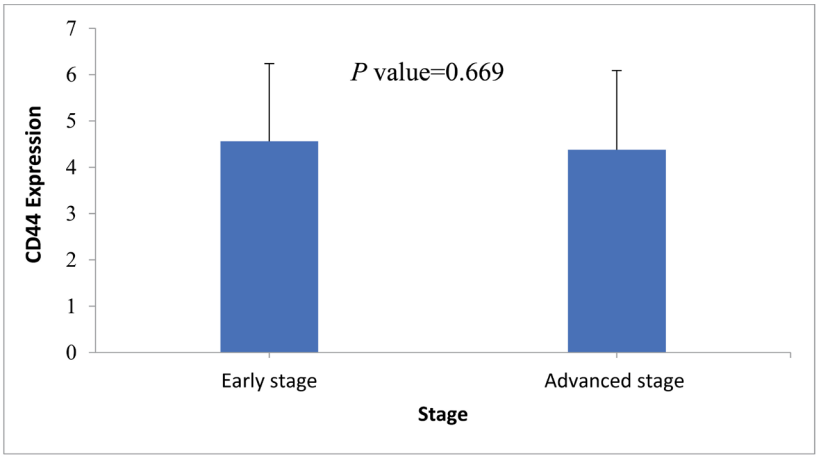

Figure 3. The mean of the CD44 expression according to disease stage.

Table 2. Investigation of the role of CD44 expression and other demographic and clinicopathological factors in the CRC patients' five-year survival rate

Variables	Survived (n=64)	Non-survived (n=26)	HR (95% CI)	*P* value
Gender				
Male	38 (59.4%)	17 (65.4%)	Reference	0.641
Female	26 (40.6%)	9 (34.6%)	0.592 (0.165-2.125)	
Age (y)				
<60	27 (42.2%)	11 (42.3%)	Reference	
≥60	37 (57.8%)	15 (57.7%)	1.117 (0.293-3.325)	0.984
Family history	15 (23.4%)	10 (38.5%)	1.405 (0.410-4.811)	0.588
Tumor location				
Colon	41 (64.1%)	15 (57.7%)	Reference	
Recto sigmoid	23 (35.9%)	11 (42.3%)	1.224 (0.379-3.954)	0.735
Tumor size (cm)				
<5	25 (43.1%)	9 (34.6%)	Reference	
≥ 5	33 (56.9%)	17 (65.4%)	1.309 (0.399-4.294)	0.657
Histologic type*				
Typical adenocarcinomas	56 (87.5%)	22 (84.6%)	Reference	
Mucinous adenocarcinoma	7 (10.9%)	4 (15.4%)	1.154 (0.239-5.580)	0.697
Histologic grade				
Well differentiated	24 (37.5%)	10 (38.5%)	Reference	0.247
Intermediately differentiated	22 (34.4%)	9 (34.6%)	0.435 (0.106-1.779)	
Poorly differentiated	18 (28.1%)	7 (26.9%)	0.546 (0.144-2.076)	0.374
Stage				
Early stage (I, II)	45 (70.3%)	10 (38.5%)	Reference	
Advanced stage (III- IV)	19 (29.7%)	16 (61.5%)	3.241 (0.322-4.566)	0.318
Tumor budding				
Low	48 (75%)	16 (61.5%)	Reference	
High	16 (25%)	10 (38.5%)	1.867 (0.572-6.088)	0.301
Chemotherapy regimen				
No concurrent chemotherapy	42 (65.6%)	22 (84.6%)	Reference	
With concurrent chemotherapy	22 (34.4%)	4 (15.4%)	0.494 (0.051-4.744)	0.574
Metastasis	2 (3.1%)	15 (57.7%)	7.639 (1.872-11.172)	0.005
Recurrence	6 (9.4%)	3 (11.5%)	1.059 (0.122-9.216)	0.959
Positive CD44 expression	33 (51.6%)	9 (34.6%)	0.597 (0.156-2.285)	0.452

*One patient had signet ring cell carcinoma and was survived.

controversial.

It is worth mentioning that although different CD44 variants were neither evaluated nor compared with healthy tissues in our study, generally the CD44 expression in CRC patients was approximately 50% that can be considered significant.

In addition, according to the results of this study, the CD44 expression in early stages was more than its expression in advanced stages although this difference was not statistically significant. Furthermore, other factors including gender, age, family history, tumor location, tumor size, and metastasis and also tumor recurrence were not significantly associated with the CD44 expression. In this regard, the results of the study by Hong et al indicated the higher CD44 expression in the primary tumor as compared with metastatic lymph nodes and advanced tumor stage (15). This result was in contrast with the findings of our study, as we did not find a significant

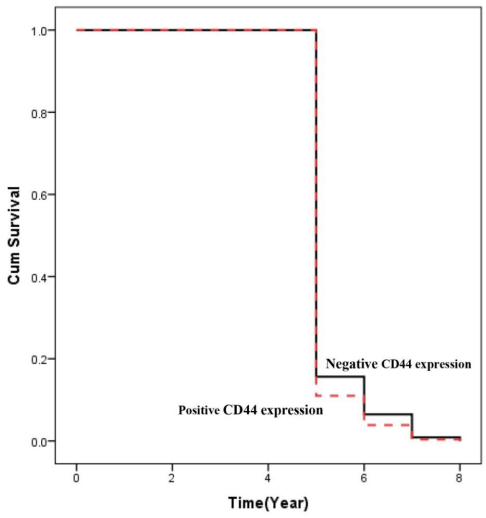

Figure 4. The overall survival rate as a function of the survival length for CD44 expressions.

difference between the CD44 expression and the tumor progression stages.

Various studies have revealed that CD44 plays a significant role in two stages of the invasive cascade including binding to the extracellular matrix and motility of cancer cells. Moreover, in metastasis, circulating tumor cells must attach to the endothelium, while, according to experimental models, is conducted by increasing the CD44 expression on the surface of tumor cells. The CD44 gene reacts with several molecules including collagen, fibronectin and laminin. The mentioned interaction has a crucial role in cancer progression by increasing cell proliferation, migration, and metastasis (16). Studies have revealed that silencing of the CD44 gene destroys the proliferation of cancer cells and reduces the migration and metastasis of cancer cells (17).

In addition, the results of the study by Hu et al, indicated that the level of CD44 gene expression in patients with lung cancer had no significant association with age, gender, smoking history, and cancer type. Although the mentioned study was conducted on lung cancer and our study was conducted on CRC (18), the obtained findings were identical.

The remarkable finding of this study was that the highest frequency percentage of gene non-expression was significantly found in patients without concurrent chemotherapy in both groups of survived and non-survived patients. In fact, it can be stated that the chance of positive CD44 expression was higher in patients undergoing chemotherapy.

Resistance to chemotherapy and radiotherapy is one of the features of cancer cells and is caused by the protective mechanisms against stress that is induced by reactive oxygen species. The defense against reactive oxygen species is enhanced by CD44 in cancer stem cells and consequently leads to the promotion of tumor growth, metastasis and chemical resistance. Hence, CD44 can be regarded as an acceptable marker for cancer progression and a diagnostic marker for certain cancer cells and can be expected to have numerous applications for examining medical treatments for the CD44 marker (19). Actually, the interaction-taking place between CD44 and hyaluronan accounts for tumor metastasis, development and chemoresistance phenotype expression. The cytotoxic effect of chemotherapy medications is impeded by the CD44 overexpression in different cancers.

In this regard, many studies have reported that the CD44 molecule can induce chemical resistance in many cancers (20).

In examining the factors related to CRC patients' risk of mortality or survival rate, it was shown that the survival rate was significantly lower in patients with metastasis; however, other demographic and clinicopathological factors did not play a significant role in increasing the risk of mortality or decreasing the survival rate. Regarding the association between the positive CD44 expression and the survival rate, it was also indicated that the risk of mortality was slightly lower in patients with the positive CD44 expression although no significant association was found between the CD44 expression and the survival rate (HR=0.597; P=0.452).

Contrary to the results of the present study, a meta-analysis study showed that the CD44 overexpression was significantly associated with worse overall survival (HR=1.32). Moreover, they evaluated different CD44 isoforms and reported that the high CD44v2 and CD44v6 expression had a significant association with the deterioration of the overall survival. Furthermore, they stated that the association between the CRC patients' CD44 expression and the survival rate was more obvious in stages I-IV (21).

Hong et al, evaluated that the CD44 expression in CRC and showed the association of the lower CD44 expression with the short disease-free survival and the increased tumor recurrence (15).

In addition, the results of the study by Qu et al, indicated the significant association of the low- CD44 expression with the decreased disease-free survival and overall survival in stage II and III CRC (22).

Meanwhile, Asao et al, reported the CD44s expression loss as a sensitive marker for lymph node metastasis in CRC (23). In addition, the CD44s expression was introduced as a prognostic factor for the overall survival in a multivariate analysis of CRC cases (24). The lack of any association between the CD44s expression and the patient outcome was reported in another study (25).

Therefore, some studies reported that factors such as genetic background and environmental factors in different regions, disease recurrence, CD44 gene expression level, and positivity of stromal CD44 gene expression could be significantly associated with cancer patients' overall survival (8,17).

It is noteworthy that to put in a nutshell, it can be stated that as the association of the increased CD44 expression with tumor progression and survival of patients with all types of cancers is still not confirmed, it is suggested to evaluate each variant of this gene and compare it with healthy tissues in future studies. Moreover, considering that CD44 binds to hyaluronan through the regions coded by exon 2 and 5, and the interaction between hyaluronan and CD44 leads to cancer cell proliferation, migration, and invasion, it is suggested to study and investigate the polymorphisms of exon 2, 5.

Conclusion

According to the results of the present study, more than 40% of the CD44 expression was positive in CRC patients, and the percentage of expression in the survived patients was higher than its percentage in non-survived ones. Moreover, none of the factors such as gender, age, family

history, tumor location, tumor size, tumor progression stage, metastasis, and tumor recurrence had any significant association with the CD44 expression. Furthermore, the chance of positive CD44 expression was higher in patients undergoing chemotherapy (survived or non-survived). In addition, the chance of survival was higher in the positive CD44 expression although no significant association was found between the CD44 expression and the patients' survival rate, and it was only revealed that the chance of survival was significantly lower in patients with metastasis.

Limitations of the study

The small sample size, retrospective nature of the study, non-evaluation of different CD44 variants and non-comparison of the CD44 expression in healthy tissues can be regarded as weaknesses of this study.

Authors' contribution

Conceptualization: MHS.
Methodology: MHS.
Validation: MHS.
Formal analysis: MM.
Investigation: MM.
Resources: MM.
Data curation: MM.
Writing—original draft preparation: MM.
Writing—review and editing: MS.
Visualization: MM.
Supervision: MM.
Project administration: MHS.
Funding acquisition: MHS.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of Isfahan University of Medical Sciences approved this study (Ethical code#IR.MUI.MED.REC.1399.567). Written informed consent was taken from all participants before any intervention at hospital admission. This study was extracted from pathology residency thesis of Maryam Malekmohammad at this university (thesis#399521). Besides, ethical issues (including plagiarism, data fabrication and double publication) have been completely observed by the authors.

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