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# Overexpression of CD44 as a predictor of metastatic potential in patients with colorectal cancer

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## Abstract

**Objective:** To correlate CD44 expression with the clinicopathological characteristics of patients with colorectal carcinoma (CRC).

**Materials and methods.** This study included 90 patients with CRC who underwent curative surgical resection. Standard histopathological techniques and immunohistochemistry analysis was used to investigate CD44 expression. Semi–quantitative scoring was used to categorize CD44 expression levels. Patients' clinicopathological characteristics were retrospectively examined.

**Results.** Overexpression of CD44 was found in 46.7% of all patients with CRC. Patients with right colon cancer had the highest CD44 expression (54,6%). Overexpression of CD44 was present in 69.6% of patients with metastatic lesions in visceral organs. We found statistically significant differences between CD44 overexpression and the presence of visceral metastases ($p = 0.015$), different T categories ($p = 0.011$), N status ($p = 0.006$), and G differentiation ($p = 0.011$). Our results showed that the disease stage has the greatest effect on CD44 overexpression ($p < 0.001$).

**Conclusion.** Overexpression of CD44 could be a reliable predictor of metastatic potential and poor prognosis in patients with CRC.

**Keywords:** colorectal cancer, stem cells, CD44

## Introduction

After lung and breast cancer, CRC is the third most common cancer worldwide and the second most common cause of cancer–related death after lung cancer. CRC has the third highest incidence rate and the third highest mortality rate among men, behind lung and prostate cancers. After breast and lung cancer, it has the second–highest incidence rate and the third–highest mortality rate among women [1]. Age is the most significant risk factor for developing CRC; over 40–year–olds account for 99 percent of CRC cases. The aging of the population in Europe is gradually increasing the incidence of CRC; however, lifestyle, diet, and environmental factors also have a significant impact. Family history is the most common risk factor for CRC after age [2]. Most CRC cases are brought on by dysplastic adenomatous polyps that have already developed. There are a few steps in the cancerogenesis process: repair of DNA, simultaneous activation of oncogenes, and inactivation of various genes that inhibit tumor growth. Colorectal epithelial cells grow selectively, the normal epithelium becomes an adenomatous polyp, and invasive CRC occurs as a result of these processes [3]. Disruption of protective mechanisms like APC (adenomatous polyposis coli), p53, and transforming growth factor (TGF–), as well as induction of oncogenic pathways like Ras, are required for progression from adenoma to cancer and metastatic disease simultaneously [4]. Conventional tumorogenesis models assume that all cells in a tumor population are capable of initiating or propagating tumors. The newfound Cancer Stem Cells (CSC) model indicates that only a small fraction of the tumor population can propagate tumor. In this hypothesis, the usefulness of existing diagnosis and therapy is called into question to suggest that a CSC model could be an efficient method for creating novel, strong diagnostic, treatment, and monitoring strategies [5]. Approximately 90% of colorectal cancer patients die as a result of metastatic spread of the primary tumor. Therefore, there is an idea to study new markers that can predict the type of malignancy and metastatic potential of colorectal cancer [6]. This study aimed to assess the CD44 expression and to correlate it with clinicopathological characteristics of patients with CRC.

## Materials and methods

### Subjects

Ninety patients with CRC who underwent curative surgical resection at the University Clinic for Digestive Surgery in Skopje, North Macedonia, between 2012 and 2017 were included in our study. The patient had not received any specific oncological treatment, radiotherapy, or chemotherapy preoperatively. The following clinical and pathological features were examined: age, sex, tumor location, T stage, N status, G differentiation, and presence of distant metastasis. Patients' clinicopathologic characteristics were retrospectively reviewed. Each patient signed an informed consent form to archive biomaterials. The study protocol was conducted according to the ethical guidelines of the Declaration of Helsinki, 1975, and was approved by the Ethics Committee of

the Faculty of Medicine, Skopje, North Macedonia on 25 May 2016 (03–2039/5).

### Pathohistological and immunohistochemical analysis

Postoperative material was processed grossly according to the protocol for colon cancer preparation and tissue samples for histological analysis were fixed in 10% neutral formalin for 18–24 hours. This material is treated with various alcohols and xylenes and molded into paraffin blocks. Paraffin blocks cut into 5–micron tissue samples were applied to the subject's spectacle lenses and routinely stained with hematoxylin–eosin. Microscopic analysis was performed using an optical microscope (Olympus). Histological analyses were performed to determine cancer histology and grade, local invasiveness, lymph node status, vascular invasion, distant metastasis, and stage. Tumor tissue samples were analyzed using immunohistochemical monoclonal antibodies. We used a monoclonal anti–CD44 mouse antibody (Novocastra, UK clone DF 1485, diluted 1:50) as the primary antibody (*Tabl. 1*). Antibodies were visualized using EnVision (Dako Denmark), a reference kit for antibody detection, using a modified method of avidin–biotin–immunoperoxidase complex. The technique involves pretreatment of tissue on the DAKO PT Link with saline at pH according to the manufacturer's specifications, application of a primary antibody, biotinylated secondary antibody, and reaction of an avidin–biotin–peroxidase complex. included. Progression of color reaction with diaminobenzidine tetrachloride (DAB). A control system was used to exclude non–specific staining. Her second sample from the same tissue applied to the same glass and stained with the same procedure but without the primary antibody, was used as a negative control. Fabrics recommended by the manufacturer were used as positive controls for each stain. Histopathological and immunohistochemical analyses were performed at the Institute of Pathology, Faculty of Medicine, Skopje.

### Scoring of CD44

In each case examined five prominent areas for each antibody were analyzed at high magnification (x10) from the edge and center of the tumor. CD44 expression levels were classified semi–quantitatively. Low positivity was defined as positive immunoreactivity in <50% of tumor glands, and positivity was defined as positive immunoreactivity in ≥50% of tumor glands [7]. CD44–positive glands were defined by cell membrane positivity. Samples showing high and low positivity for CD44 are shown in *Fig. 1 and 2*, respectively. The staining intensity of the used markers was not analyzed.

### Statistics

Statistical analysis was performed using the SPSS statistical package. Statistical analysis was performed using the student's t–test, Kolmogorov–Smirnov test, Mann–Whitney U test, one–way analysis of variance (ANOVA), Kruskal–Wallis test, and multiple regression analysis (multiple correlation coefficient – R). Statistical significance was defined as $p<0.05$.

## Results

The study population consisted of 90 patients, 52 (57.8%) male and 38 (42.2%) female. The mean age at surgery was 63.3 years (range: 17–91 years). Tumor distribution by anatomic location was as follows: left colon in 40 cases (44.5%), right colon in 22 cases (24.4%), and rectum in 28 cases (31.1%). The distribution of clinical stages at diagnosis was as follows: 13 (14.45%) stage I, 25 (27.8%) stage II, 39 (43.3%) stage III, and stage IV 13 (14.45%) cases. Twenty–three or (25.6%) of

Table 1. **Characteristics of immunohistochemical studies of the stem cell marker CD44**

| Antibody | Manufacturer | Clone | Dilution | Signal | Control |
|---|---|---|---|---|---|
| CD44 | Novocastra, UK | DF1485 | 1:50 | Membrane | Tonsylae |

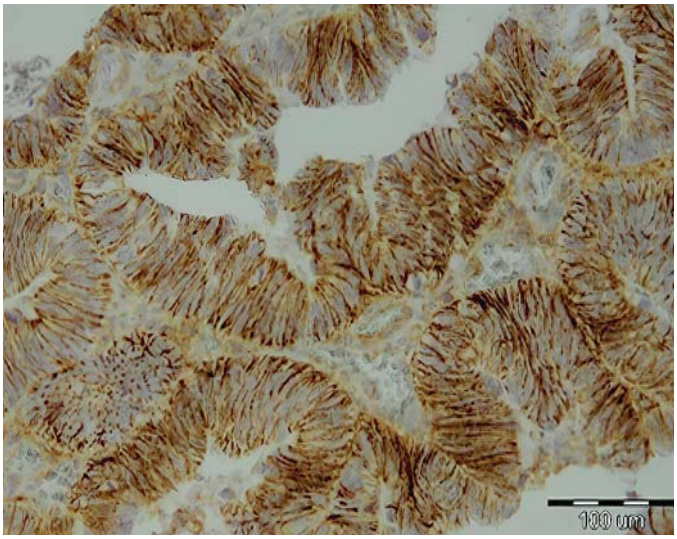


*Fig. 1.*
*Overexpression of CD44.*

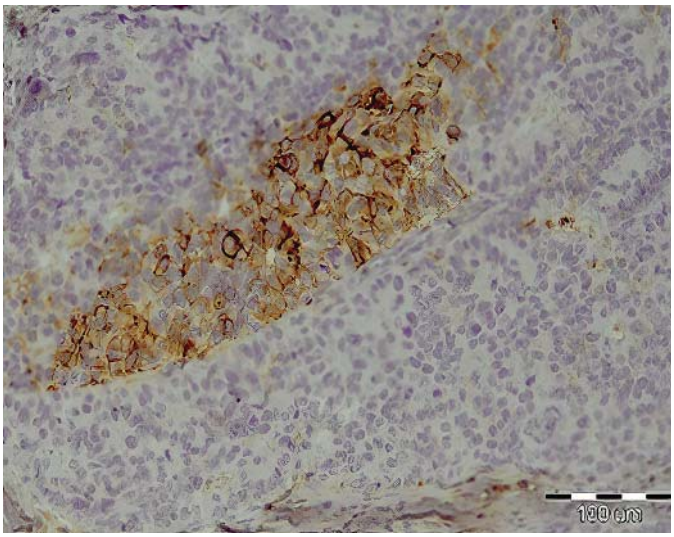


*Fig. 2.*
*Low expression of CD44.*

Table 2. **Clinicopathological characteristics of patients**

| Clinicopathological characteristics | Number of patients n (%) |
|---|---|
| Total number of patients | 90 (100%) |
| Age (means) year | 63,3 ± 12,4 |
| Gender | |
| Male | 52 (57,8) |
| Female | 38 (42,2) |
| Tumor location | |
| Left colon | 40 (44,5) |
| Right colon | 22 (24,4) |
| Rectum | 28 (31,1) |
| Stage of disease | |
| I | 13 (14,45) |
| II | 25 (27,8) |
| III | 39 (43,3) |
| IV | 13 (14,45) |
| T-category | |
| T1 | 5 (5,6) |
| T2 | 12 (13,3) |
| T3 | 45 (50) |
| T4 | 28 (31,1) |
| Nodal status | |
| N0 | 41 (45,6) |
| N1 | 24 (26,7) |
| N2 | 25 (27,8) |
| G-Differentiation | |
| G1 | 6 (6,7) |
| G2 | 72 (80) |
| G3 | 2 (13,3) |
| Distant metastases | |
| MS+ | 23 (25,6) |
| MS– | 67 (74,4) |

the patients with colorectal cancer had distant metastases at the time of diagnosis. Patient clinicopathologic features are shown in T*abl. 2*.

### Relationship between clinicopathological parameters and CD44 expression

Overexpression of CD44 was found in 42 cases (46.7%). The highest CD44 overexpression is in patients with right colon cancer (54.6%). Statistical analysis showed no significant differences concerning tumor localization and CD44 expression. Our study found an association between the presence of distant metastases and overexpression of CD44 (p=0.0153). The results of our study showed that stage IV had the highest expression of CD44 (92.3%). Our results showed that there was a statistically significant difference in the expression of CD44 in patients with different T categories, N status, and G differentiation. The relationship between clinicopathological parameters and CD44 expression is shown in *Tabl. 3*.

### Correlations between CD44 and independent variables

Results of multiple regression analysis showed that all independent variables together influenced CD44 expression (F = 10.24, p = 0.000001). Among independent variables, disease stage has the greatest impact on CD44 expression (t = –6,749 p = 0.000000).

*Tabl. 4* shows the results.

## Discussion

The adhesion molecule CD44 is a receptor for hyaluronic acid, which is also used as a marker for CSC. CD44 is known to be involved in cell growth, differentiation, and survival. As an important cell adhesion molecule, CD44 plays an important role in cancer cell migration associated with xenograft tumor initiation and colony formation, as well as tumor stage, lymph node infiltration, prognosis, and survival. CD44 has a molecular weight of 85–200KDa. CD44 cells exhibit CSC properties, meaning a single cell can self–renew, differentiate, and form a xenograft tumor that resembles the initial lesion. CRC cells that have the CD44+ marker have high tumorigenicity, especially in combination with CD133+ cells, where CD44+ cells cannot form new tumors. Also, CD44 can be used in conjunction with the stem cell marker CD166. A study done in immunodeficient mice showed that CD44+CD166+ cancer cells have a greater ability to develop a tumor compared to CD44+CD166–, CD44–CD166+, or CD44–CD166– cells. Using the combination of these markers plays an important role in the identification of colonic CSC [8].

Many diseases have high expression of CD44. It has been found in cancers and, also in inflammatory and autoimmune diseases. To find a precise link between CD44 with specific cancers, the scientists studied CD44 isoforms and their correlation with certain types of CSC and inflammatory immune responses. High expression of CD44 is present in many malignancies, chronic inflammatory responses, and autoimmune dysfunctions. For example, CD44v6 is associated with the aggressiveness of human non–Hodgkin lymphomas [9].

CD44 expression is high in human CRC [10]. Ishimoto and collaborators investigated the resistance to oxidative stress in CSC in intestinal tumors. The study showed that human gastrointestinal cancer cells with high expression of CD44 have an increased capacity to synthesize reduced glutathione and a better defense against reactive oxidative radicals, thus avoiding apoptosis and allowing themselves a longer life span [11].

In our study, we found overexpression of CD44 in 46, 7% of patients with CRC. These results are similar to the results presented in the study of Yan B, and results presented in a meta–analysis of Wang where was concluded that CD44 could be used to predict poor differentiation, lymph node metastasis, and distant metastasis [12, 13, 14].

The expression of CD44 was highest in patients with right colon cancer (54.6%). Statistical analysis showed no significant differences concerning tumor localization and CD44 overexpression. In the study of Lugli et al. A similar percent-

Table 3. **Relationship between clinicopathological parameters and CD44 expression**

| Clinicopathological parameter | CD 44 expression n (%) | | |
|---|---|---|---|
| | low/none | high | p-value |
| Tumor location | | | |
| left colon | 60 | 40 | 0,499 |
| right colon | 45,4 | 54,6 | |
| rectum | 50 | 50 | |
| Stage of disease | | | |
| I | 100 | 0 | 0,00001 |
| II | 92 | 8 | |
| III | 28,2 | 71,8 | |
| IV | 7,7 | 92,3 | |
| T-category | | | |
| T1 | 80 | 20 | 0,0119 |
| T2 | 91,7 | 8,3 | |
| T3 | 48,9 | 51,1 | |
| T4 | 39,3 | 60,7 | |
| Nodal status | | | |
| N0 | 68,3 | 31,7 | 0,0066 |
| N1 | 54,2 | 45,8 | |
| N2 | 28 | 72 | |
| G-Differentiation | | | |
| G1 | 83,3 | 16,7 | 0,0115 |
| G2 | 56,9 | 43,1 | |
| G3 | 16,7 | 83,3 | |
| Distant metastases | | | |
| MS+ | 30,4 | 69,6 | 0,0153 |
| MS– | 61,2 | 38,8 | |

age of overexpression of CD44 but in left colon carcinoma [8].

In our study, we found a strong association between the presence of distant metastases and overexpression of CD44 ($p < 0.00107$). Similar results are shown in the conducted meta–analysis of Wang where was concluded that CD44 overexpression has a significant correlation with distant metastasis ($P = 0.044$) [15]. 51.1% of patients in T3 category, have overexpression of CD44 and 60.7% of patients in T4 category, showed overexpression of CD44. The results showed a significant correlation between overexpression of CD44 and T category ($p < 0, 0119$). These results are similar to recently published data where it was found, that a strong statistically significant relationship between overexpressed CD44 in the primary colorectal carcinoma cell membrane and tumor grading [12, 16]. In our study, we found that CD44 overexpression was significantly higher in patients with N2 lymph node status than in patients with N0 and N1. In addition, we also found significant differences in CD44 expression in patients depending on G differentiation. Through multiple regression analysis, statistically significant correlations were detected between CD44 expression (dependent variable) and the following independent variables: T stage, N status, G differentiation, and the presence of distant metastases. The disease stage has the greatest statistical impact on CD44 overexpression ($p=0.00001$) [13]. No statistically significant correlations were found between CD44 expression (dependent variable) and independent variables: sex, age, tumor location, stage, nodal status, G differentiation, and presence of distant metastatic lesions (MS+). From the independent variables, we found a statistically significant correlation only between the expression of CD44 (the dependent variable) and the T category (partial correlation = 0.587, $P < 0.0000$). The results are similar to those presented in the study of Bhavikatti where was found that CD44 overexpression has a strong association with histopathological differentiation [16].

The results of our study showed that CD44 overexpression has the highest positive correlation with the stage of disease and we found significant differences in the overexpression of CD44 between patients with and without metastatic lesions, different T categories, N status, and G differentiation [13, 17, 18].

The limitation of the study is the small number of examined patients with CRC and the retrospective nature of the study. Larger prospective studies are needed to confirm the current evidence and the clinical utility of CD44 in CRC patients. The identification of the CD44 cancer stem cell marker and its implementation in routine clinical practice will allow the selection of subgroups of patients with high malignancy who are likely to develop metastases or local recurrence. It also offers the opportunity to develop more effective targeted therapies.

## Conclusion

Overexpression of CD44 could be a reliable predictor for metastatic potential and poor prognosis in patients with CRC.

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**Competing interests.** The authors have no conflict of interest.

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