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Co-expression of Stem Cell Markers CD133 and CD44 as Predictors of Metastatic Potential of Colorectal Carcinoma

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ABSTRACT

Objective: One of the most prevalent cancers in the world is colorectal carcinoma (CRC). Aggressive cancer forms and a poor prognosis are linked to cancer stem cell (CSC) markers. The study aimed to determine whether the co-expression of the CSC markers CD133 and CD44 could predict an increased risk of metastasis in colorectal cancer.

Material and Methods: Our study included 90 patients with CRC. All patients were divided into two subgroups: Metastatic CRC and non-metastatic CRC. Initially, tumor samples were examined using conventional histological techniques, and then immunohistochemical analysis with monoclonal antibodies against CD133 and CD44 markers was performed.

Results: High co-expression of CD133 and CD44 was observed in 71.4% of patients with metastatic disease, compared to 37.9% in patients without distant metastases. Discordant expression of both markers was found in 8% of the subgroup with metastatic CRC and 13.4% of the subset without metastatic CRC. Statistical analyses showed a significant association of increased expression of CD133 and CD44 with the disease stage, T- category, and N- nodal status. With multiple regression analysis, the stage of disease was singled out as the factor with the greatest and statistically significant influence on the expression of CD133 ($p<0.0001$) and CD44 ($p<0.0001$).

Conclusion: Co-expression of CD133 and CD44 plays an essential role in predicting the metastatic form of CRC. Both stem cell markers can be implemented in standard pathohistological diagnostics and can be useful markers for pre-therapeutic oncology screening.

Keywords: Colorectal carcinoma, stem cells, CD133+, CD44+, metastatic potential

INTRODUCTION

Colorectal cancer (CRC), with about 10% of all cancer cases, is the second most common cause of cancer-related deaths globally and the third most common type of cancer overall. The majority of cases affect individuals 50 years of age and older. Several lifestyle factors, including a sedentary lifestyle, obesity, smoking, excessive alcohol use, a high intake of processed meats, and a poor intake of fruits and vegetables, may increase the risk of CRC (1). The most frequent risk factor CRC, after advancing age, is family history. Less than 5% of cases of CRC are caused by the two most prevalent familial cancer syndromes, hereditary nonpolyposis and familial adenomatous polyposis (2). Most cases of CRC occur as a result of pre-existing dysplastic adenomatous polyps. The process of carcinogenesis includes a few steps: inactivation of various genes that suppress tumor growth, repair of DNA and simultaneous activation of oncogenes. These processes lead to selective growth of colorectal epithelial cells, then transformation of normal epithelium to adenomatous polyps, eventually leading to invasive CRC (3). Progression from adenoma to cancer and metastatic disease requires simultaneous disruption of protective mechanisms, including adenomatous polyposis coli, p53, and transforming growth factor β, as well as induction of oncogenic pathways, such as renin-angiotensin-system (4,5). Traditional tumorigenesis models imply that every cell in the tumor population can initiate and propagate tumors. The recently discovered cancer stem cells (CSCs) model indicates that only a tiny percentage of cells can spread malignancies. This hypothesis raises the question of the effectiveness of current diagnosis and therapy, suggesting

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that the CSCs model can be used to rationally develop new and robust diagnostic, therapeutic, and monitoring strategies (6,7). About 90% of patients with CRC die as a result of metastatic dissemination of the primary tumor, which implies researching new markers that can predict the metastatic potential in colorectal carcinoma (8). This study aimed to ascertain if CD133 and CD44 CSC markers might indicate an increased risk of CRC metastasis by performing immunohistochemistry examination of surgical material taken from individuals with the disease.

MATERIAL and METHODS

Subjects

This retrospective cohort study included ninety (n=90) patients who underwent surgical treatment at the University Clinic for Digestive Surgery in Skopje, North Macedonia, with a primary clinical diagnosis of CRC. Patients were divided into two groups: metastatic CRC and non-metastatic CRC. Before surgery, the patients were not treated with chemotherapy, radiation therapy, or immunotherapy. The clinical and pathological features included as covariates were age, gender, histological grade, and disease stage. The Ethical Committee of the Medical Faculty in Skopje, North Macedonia (number: 03-2039/5) approved the study protocol on 25 May 2016.

Pathohistological and Immunohistochemical Analysis

Macroscopically processed postoperative material and tissue samples for histological investigation were preserved in 10% neutral formalin for 18 to 24 hours as part of the dissection routine for colorectal cancers. The material was molded into paraffin blocks following a series of xylene and alcohol processing steps. Hematoxylin-eosin staining was performed on slides after applying paraffin blocks cut from 5-micron tissue samples. Light microscopy (Olympus) was used for investigation. The histological type and grade of the cancer and its local invasiveness, lymph nodal status, vascular invasion, distant metastases, and disease stage were all ascertained using histological investigation. Representative samples of tumor tissue were immunohistochemically analyzed using monoclonal antibodies against CD133 and CD44. The primary antibodies used were anti-CD133 rabbit monoclonal antibody (Miltenyi, Germany; clone AC133, dilution 1:11) and anti-CD44 mouse monoclonal antibody (Novocastra, UK; clone DF1485, dilution 1:50). For antibody visualization, a modified Avidin-Biotin Immunoperoxidase Complex method was employed, utilizing the EnVision detection system (Dako, Denmark). To ensure specificity and exclude nonspecific staining, an internal control system was implemented. Negative control samples consisted of identical tissue sections processed in the same staining chamber using the same protocol, but without the application of the primary antibody. Positive controls provided by the manufacturer were used for each antibody staining procedure. Tissue pretreatment was performed in the DAKO PT Link system, with buffers of appropriate pH values according to the manufacturer's recommendations. The staining protocol included the application of primary antibodies, followed by secondary antibodies conjugated with biotin, and the avidin-biotin peroxidase complex reaction. The chromogenic detection was performed using 3,3'-diaminobenzidine tetrachloride. Pathohistological and immunohistochemical analyses were performed at the Institute of Pathology, Medical Faculty in Skopje.

Scoring of CD133 and CD44 Expression

For each antibody, five visual fields at medium magnification (10×) were analyzed, including both peripheral and central regions of the tumor tissue in each case. The expression levels of CD133 and CD44 were semi-quantitatively classified as low when more than 50% of tumor glands or cells were negative, and high when more than 50% were positive. CD133 positivity was defined by apical-luminal staining of glandular epithelium or intraglandular cellular debris, while CD44 positivity was defined by membranous staining of tumor cells. The intensity of staining was not evaluated for either marker. CD44 and CD133 expression was defined with scoring systems that assessed the percentage of positive cells and a histochemical or overall score. CD44 and CD133 expression levels are the median values of our series of staining.

Statistical Analysis

The software SPSS was used for the statistical analysis of the obtained data. The following tests were used: Spearman's rank-order correlation, the Student's t-test, the Kolmogorov-Smirnov test, the Shapiro-Wilk W test, the Mann-Whitney U test, the One-Way Analysis of Variance (ANOVA), the Kruskal-Wallis test, and multiple regression analysis (multiple correlation coefficient, or R). The threshold for statistical significance was set at $p<0.05$.

RESULTS

Patient Clinicopathological Parameters

The patients' clinicopathological parameters are compiled in Table 1. Of the 90 patients in the study, 53 were between 50 and 70 years, and 27 were older than 71 years. There were 52 cases involving males and 38 involving females. The left colon (44.5%) had the highest tumor localization, followed by the rectum (31.1%) and the right colon (24.4%). There were no statistically significant variations in disease location between male and female patients (Mann-Whitney U test, $Z=1.578$, $p=0.11$). Age groups and illness location did not significantly differ (Kruskal-Wallis ANOVA: $H=5.796$, $p=0.1219$). Nonetheless, at more advanced stages of the disease, especially in patients with metastatic colorectal cancer, elevated expression of CD133 and CD44 was noted.

CD133 and CD44 Co-expression and Its Correlation with Metastasis

Compared to 37.9% of patients without metastatic disease, 71.4% of patients with metastatic disease had high levels of CD133 and CD44 co-expression. 13.4% of patients with non-metastatic colorectal cancer, and 8% of patients with metastatic colorectal cancer, had a discordant expression of both markers. According to statistical analysis, the illness stage, T-category, and N-nodal status strongly correlated with significant co-expression of CD133/CD44. By multiple regression analysis, the illness stage was found to be the most critical factor impacting CD133 ($p<0.0001$) and CD44 ($p<0.0001$) expression. Stage III and IV tumors showed the highest expression levels, which were associated with a higher risk of metastasis. Tables 2 and 3 present the respective findings.

Statistical Correlation of CD133/CD44 Expression

• **Tumor stage:** High expression of CD133 was significantly associated with stage III (79.5%) and stage IV (84.6%) tumors compared to stage I (0%) and stage II (4%) ($p<0.0001$). A similar trend was observed for CD44.

• **T-category:** CD133 expression was significantly higher in T3 (51.1%) and T4 (64.3%) tumors compared to T1 (20%) and T2 (8.3%) tumors ($p<0.01$). A similar distribution was noted for CD44 ($p<0.05$).

• **Nodal status:** High CD133 and CD44 expression correlated significantly with N2 status ($p<0.05$ for CD133; $p<0.01$ for CD44).

• **Tumor differentiation (G grade):** Significant differences in CD133 ($p<0.05$) and CD44 ($p<0.05$) expression were found across different differentiation grades.

Frequency of High and low Co-expression of CD133/CD44 in Subgroups of Patients According to Stage of Disease

According to results from multiple regression analysis, which suggest that the stage of the disease is the most significant factor in the expression of CD133 and CD44, we performed an analysis of differences in the co-expression of CD133/CD44 among subgroups of patients according to the stage of disease

Table 1. Patients' clinicopathological parameters

Variables	Number of patients
Age (years)	
<30	1
31-50	9
50-70	53
>71	27
Sex	
Male	52
Female	38
Localization	
Right colon	22
Left colon	40
Rectum	28
T-category	
T1	5
T2	12
T3	45
T4	28
Nodal status	
No	41
N1	24
N2	25
Tumor status	
G1	6
G2	72
G3	12

Table 2. Correlation between CD133 and independent factors using multiple regression analysis

Independent variables	**R=0.71 R^2=0.51 F=9.27 p=0.000001**				
	Beta in	**Partial correl.**	**Tolerance**	**t (80)**	**p-level**
Sex	0.0064	0.0085	0.8515	0.076	0.939152
Age	-0.0781	-0.0484	0.1886	-0.433	0.665494
Age groups	0.1529	0.0939	0.1866	0.844	0.400973
Localization	-0.0347	-0.0467	0.8871	-0.418	0.676953
Stage	-0.6884	0.5850	0.5374	-6.451	**0.000001**
T-category	0.0343	0.0391	0.6401	0.350	0.726851
Nodal status	-0.0740	-0.0953	0.8195	-0.856	0.394240
G differentiation	-0.1106	-0.1442	0.8505	-1.303	0.196046
Distant metastasis +	0.0908	0.1109	0.7398	0.998	0.321036

and presence of distant metastasis. The first subgroup consisted of patients at stages I and II, and the second subgroup consisted of patients at stages III and IV of the disease. The results showed statistically significant differences between subgroups of patients (Pearson's chi-square: χ^2=55.36; df=1; p<0.00001). The results are shown in Figure 1.

DISCUSSION

CEA and CA 19-9 are widely used markers in gastrointestinal malignancies, but their low sensitivity and specificity limit their prognostic value in CRC. CD133 and CD44 have recently been identified as key markers for colorectal CSCs, with their high co-expression linked to poor prognosis. A meta-analysis by Chen et al. (9) identified CD133 as a significant prognostic marker in CRC, correlating with advanced tumor stage, nodal involvement, and vascular invasion. Jing et al. (10) found CD44 to be an independent prognostic factor, more reliable for predicting hepatic metastases and survival than CD133. Khelwatty et al. (11) reported that while CD133 alone predicts poor survival, combining CD133 and CD44 enhances risk stratification. Tsunekuni et al. (12) observed that patients expressing both markers had significantly shorter survival, suggesting their combined analysis could aid treatment decisions and follow-up strategies. Another study suggested that CD133 expression may be related to sensitivity to radiotherapy or chemotherapy in colorectal cancer. Still, the presence of CD133-positive cancer cells alone cannot support the concept of CSCs in colorectal cancer. In the same study, other molecules, such as CD44, have been proposed as additional putative markers for CSCs in CRC (13). Our study identified an association between distant metastases and high expression of the cell markers CD133 and CD44, consistent with literature. Recent data showed a high correlation between CD133/CD44 co-expression and liver metastases in patients with colorectal carcinoma (14). In this study, CD44 expression was identified as an independent marker associated with patient survival. It was a more precise prognostic marker for liver metastases, metastatic disease, and survival than CD133. Horst et al. (15) demonstrated that CD133 expression does not correlate with CD44 and that

Table 3. Multiple regression analysis-correlation between CD44 and independent variables

Dependent variables	**R=0.73 R^2=0.53 F=10.24 p=0.000001**				
	Beta in	**Partial correl.**	**Tolerance**	**t (80)**	**p-level**
Sex	0.0902	0.1213	0.8515	1.092	0.277807
Age	-0.1129	-0.0718	0.1886	-0.643	0.521513
Age groups	0.1781	0.1121	0.1866	1.009	0.315861
Localization	0.1184	0.1614	0.8872	1.463	0.147422
Stage	-0.6749	0.5874	0.5374	-6.493	**0.000000**
T-category	0.0937	0.1093	0.6401	0.983	0.328228
Nodal status	-0.1397	-0.1824	0.8196	-1.659	0.100917
G differentiation	-0.1628	-0.2151	0.8505	-1.971	0.052225
Distant metastasis +	0.0732	0.0920	0.7398	0.826	0.410866

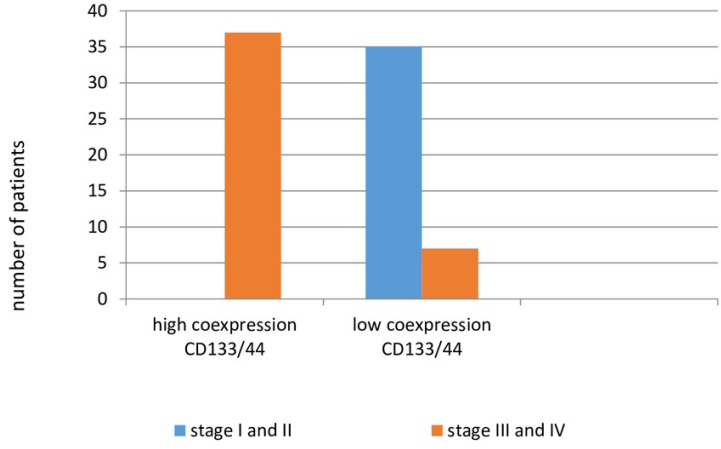

Figure 1. Frequency of high and low co-expression of CD133/CD44 in groups of patients according to stage of disease.

CD133 is the best individual marker for poor survival prognosis. In contrast, combined analysis of both markers may have greater prognostic power in patients with colorectal carcinoma. In another study, CD133 expression was observed in 69% of patients with metastatic disease, while increased expression of CD44 was observed in 61% of patients (16). In our study, high expression of both markers was detected in 69% of patients. Statistical analysis in our study showed a significant association of increased expression of CD133 and CD44 with disease stage. High expression of CD133 was found in tumors at stage III (79.5%) and stage IV (84.6%), compared to tumors at stage I (0%) and stage II (4%). A similar immunohistochemical expression distribution was observed for CD44, with 71.8% high expression in stage III tumors and 92.3% in stage IV tumors; compared to stage I (0%) and stage II (8%) tumors. These results correlate with findings from recent studies (13,17). Regarding the variations in CD133 and CD44 immunohistochemical expression in tumors belonging to distinct T categories, our analysis revealed that CD133 was significantly more expressed in T3 (51.1% high expression) and T4 (64.3% high expression) tumors than in T1 (20%) and T2 (8.3%) tumors. Compared to T1 (20%) and T2 (8.3%), tumors, CD44 expression was found to be similarly distributed in T3 (51.1%) and T4 (60.7%) cancers. In a meta-analysis of CD133 as a colorectal CSC marker, Chen et al. (9) discovered a substantial association in patients with colorectal cancer. According to their research, in patients with colorectal cancer, elevated CD133 expression was substantially linked to worse clinical outcomes and specific clinicopathological characteristics, including T3 and T4 categories, N category, and vascular invasion. In contrast to the findings of Chen et al. (9) and our findings, we did not find a favorable association between higher T-category (T3) and high CD133 expression in our earlier study (15). Their study only compared T2 and T3 tumors, unlike ours, which included T1 and T4 tumors. Their study followed patients for 10 years post-surgery and demonstrated significantly lower survival rates correlated with high CD133 expression. Regarding differences in CD133 and CD44 expression in tumors with different nodal statuses, our study showed significantly higher CD133 expression in tumors with N2 nodal status, than in others (N0 and N1). The statistical significance was greater for CD44 in tumors with N2 nodal status than in N0 and N1. CD133/CD44 showed the highest co-expression frequency in tumors with N2 nodal status compared to N0 and N1 status. In our study, the expression and co-expression of the examined markers were strongly correlated with high nodal status (N2). This supports established gold standards in colorectal surgery, such as complete mesocolic excision (CME) and total mesorectal excision (TME). In N1-2 tumors, complete loss of CD44 expression was predominant than its high expression in the N0 category. Differences may arise from their study fusing N1 and N2 statuses into a single category, while our study considered N0, N1, and N2, separate groups. The results are similar to previously published data (18). Our study showed a significant difference in the expression of CD133 and CD44 across tumors with different differentiation grades (G). Given the small size of G1 (6 samples) and G3 (12 samples), statistical analysis between individual groups was not performed. However, in the G1 group, low CD133 expression predominated (83.3%), while in G2, the difference was less significant (54.2% low expression vs. 45.8% high expression), and in G3, high CD133 expression was predominant (75%). A similar distribution was observed for CD44 expression. Our study found that in patients with metastatic disease (stages III and IV), CD44 and CD133 expression was increased compared to patients without metastases (stages I and II) (11,13).

Study Limitations

Our study had several limitations that should be acknowledged. The first limitation is the study's small sample size, single-center setting, and retrospective design. The second limitation was that some subgroups of patients (those with G1 tumors) are underrepresented, which affects statistical robustness. This is the result of the lack of prior specific case selection, as the cohort was composed of a consecutive case series. The decision to include consecutive cases was necessitated by the limited time frame allocated for data collection, which restricted the possibility of applying more selective inclusion criteria. Another limitation of the study is the absence of disease-free subjects and results on overall survival outcomes, which limits determining its true prognostic utility. These were the result of the financial limitations of the study. Thus, a larger prospective study is needed to establish the true prognostic value of both markers as predictors of the metastatic potential of colorectal carcinoma.

CONCLUSION

Our findings support the notion that CD133 and CD44 are helpful prognostic indicators, strongly correlated with the course of the disease and survival in individuals with colorectal cancer. We conclude that higher or lower CD44 expression is linked to higher or lower CD133 expression, but not vice versa. Low expression of CD44 does not always indicate low expression of CD133. Early diagnosis, patient monitoring, and tailored therapy selection may benefit from understanding the molecular pathways behind colorectal CSCs and identifying particular markers like CD44 and CD133. Analysis of stem cell markers may change conventional treatment approaches and help lower the risk of CRC metastases and local recurrence. The following essential findings from this investigation led to the conclusion:

• Elevated CD133/CD44 co-expression was considerably more prevalent in the group with metastatic disease than in the group without metastatic disease.

• Stages III and IV carcinomas had higher CD133/CD44 co-expression levels than stages I and II.

• In the individual analysis of CSC markers, high vs. low carcinoma expression incidence, varied significantly according to the tumor T-category, nodal status, and differentiation grade.

• The N status analysis supports the gold standards for TME CME, which are already established in colorectal surgery.

• A cut-off value for identifying CSC marker expression must be established to produce more trustworthy results.

• These markers should be correlated with other parameters impacting cancer aggressiveness and chemoresistance to provide future opportunities for broader applicability and a better understanding of CSCs. The objective is to include them as a pre-treatment oncological screening tool, a standard prognostic marker, and a commonly used pathohistological diagnostic tool.

Ethics

Ethics Committee Approval: The Ethical Committee of the Medical Faculty in Skopje, North Macedonia (number: 03-2039/5) approved the study protocol on 25 May 2016.

Informed Consent: Retrospective study.

Footnotes

Author Contributions

Concept - O.K., I.K., R.J.; Design - O.K., I.K., R.J.; Materials - O.K., I.K., R.J.; Data Collection or Processing - O.K., I.K., R.J.; Analysis or Interpretation - O.K., I.K., R.J.; Literature Search - I.K.; Critical Review - O.K., I.K., R.J.; Writing - I.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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