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ORIGINAL ARTICLE

High expression of CD133 – stem cell marker for prediction of clinically agressive type of colorectal cancer

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ABSTRACT:

Background: Colorectal cancer (CRC) is one of the most common malignancies in the world. The cancer stem cell (CSC) markers are associated with aggressive cancer types and poor prognosis. The objective of the study was to evaluate the CD133 expression and to correlate it with clinicopathological features in patients with CRC.

Material and Methods: Our study included ninety patients with CRC who underwent curative surgical resection from 2012 to 2017 at the University Clinic for Digestive Surgery, Skopje, North Macedonia. Tumor samples were first analyzed with standard histopathological methods and then the CD133 expression was investigated immunohistochemically. The level of expression of CD133 was classified semiquantitatively. Low positivity was defined as positive immunoreactivity in <50% of tumor glands, and high positivity was defined as positive immunoreactivity in ≥50% of tumor glands. Furthermore, clinicopathological features of patients were retrospectively reviewed.

Results: High expression of CD133 was found in 47.8% of patients' CRC samples. In 69.6% of patients with metastatic lesions in visceral organs we found high expression of CD133. We found statistically significant differences in the expression of CD133 between patients with and without visceral metastatic lesions (P = 0.0153), between patients with a different T category (P = 0.0119), N status (P = 0.0066) and grade (G) (P = 0.0115). Our results showed that the stage of disease has the greatest impact on expression of CD133 (P < 0.00001).

Conclusion: High expression of CD133 is a useful marker for prediction of the clinically aggressive type of CRC and can be routinely implemented in standard pathohistological diagnostics.

KEYWORDS: CD133, colorectal cancer, immunohistochemistry, stem cells

ABBREVIATIONS

ANOVA – ONE-way analysis of variance
APC – adenomatous polyposis coli
CRC – colorectal cancer
CSC – Cancer Stem Cells
DAB – diaminobenzidine
TGF-β – transforming growth factor β

INTRODUCTION

In the Western world, colorectal cancer (CRC) is the second most common cancer in women after breast cancer, and it is the third most common cancer in men after lung cancer and prostatic cancer [1]. The most significant risk factor for development of CRC is age – 99% of CRC cases are people aged over 40 years. In Europe, the incidence of CRC is gradually increasing due to population aging, but lifestyle, diet and environmental factors have also great impact on the incidence of CRC. After age, family history is the most common risk factor of CRC [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, repairment of DNA and simultaneous activation of oncogenes. These processes lead to selective growth of colorectal epithelial cells, then transformation of normal epithelium to adenomatous polyp and finally to invasive CRC [3]. Progression from adenoma to cancer and metastatic disease requires simultaneous disruption of the protective mechanisms, including APC (adenomatous polyposis coli), p53 and transforming growth factor β (TGF-β), and induction of oncogenic pathways such as Ras [4, 5]. Traditional models of tumorigenesis imply that every cell in the tumor population is capable of tumor initiation and propagation. The newfound Cancer Stem Cells (CSCs) model indicates that only a small fraction of tumor population possesses the capability for tumor propagation [6]. This hypothesis raises the question of the effectiveness of current diagnostics and therapy, suggesting that the CSCs model can be used for rational development of new diagnostic, therapeutic and monitoring strategies [7]. About 90% of patients with CRC die as a result of metastatic dissemination of the primary tumor, which implies the idea of researching for new markers that will be able to predict the aggressive type and metastatic potential in CRC [8]. The aim of the study was to evaluate the CD133 expression and to correlate the CD133 expression with clinicopathological futures in patients with CRC.

MATERIALS AND METHODS

Subjects

The study included ninety (n = 90) patients with primary clinical diagnosis of CRC, who underwent curative surgical resection from 2012 to 2017 at the University Clinic for Digestive Surgery, Skopje, North Macedonia. Preoperatively, patients were not subjected to specific oncological, radiotherapeutic or chemotherapeutic treatment. The following clinical and pathological features were included: age, gender, tumour location, T stage, N status, G – differentiation

and presence of distant metastases. Clinicopathological futures of patients were retrospectively reviewed. Each patient signed an informed consent for archiving the biological material. The study protocol was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Ethical Committee of the Medical Faculty in Skopje, North Macedonia (03-2039/5) on 25 May 2016.

Pathohistological and immunohistochemical analysis

Postoperative material was macroscopically processed according to the protocol for dissection of CRCs, and tissue samples for histological analysis were fixed in 10% neutral formalin for 18–24 hours. The material was processed in a series of alcohol and xylene, followed by molding in paraffin blocks. Paraffin blocks cut in 5-micron tissue samples were applied to slides and routinely stained with hematoxylin-eosin [9]. Microscopic analysis was carried out with the light microscopy (Olympus). Histological analysis was performed in order to determine the histological type and grade of the cancer, local invasiveness, lymphonodal status, vascular invasion, distant metastasis, and stage of disease. Samples of tumor tissues were analyzed by immunohistochemical monoclonal antibodies. We used the anti-CD133 monoclonal rabbit antibody (Miltenyi, Germany clone AC133, DILUTION 1:11) as the primary antibody (Tab. I.). For visualization of the antibody, a modified method of Avidin-Biotin immunoperoxidase complex was used, with EnVision (Dako Denmark), which is the reference kit for detecting antibodies. This technique enrolls: pretreatment of the tissue in DAKO PT Link with saline with pH value according to the manufacturer's references, aapplication of the primary antibody, secondary antibody labeled with biotin and the potential reaction of the avidin-biotin peroxidase complex, with further development of colored reaction with diaminobenzidine tetrachloride (DAB). A control system was used to exclude the non-specific staining. As a negative control, the second sample was used from the same tissue, which was applied on the same slide and stained with the same procedure, but without application of the primary antibody. As a positive control for each of the stains, a recommended tissue from the manufacturer was used. Pathohistological and immunohistochemical analyses were conducted at the Institute of Pathology, Medical Faculty in Skopje.

Scoring of CD133

For each antibody, five prominent areas were analyzed, with high magnification (x10) from the margins and from the central parts of the tumor in each investigated case. The level of expression of CD133 was classified semiquantitatively. Low positivity was defined as positive immunoreactivity in <50% of tumour glands, and high positivity was defined as positive immunoreactivity in ≥50% of tumour glands [10]. Positive glands of CD133 were defined by the presence of apical-luminal staining of the glandular epithelia or staining of the intraglandular cell debris. Specimens which demonstrated high and low positivity for CD133 are shown in Fig. 1., 2. respectively. The intensity of staining was not analyzed for the used marker.

Statistical analysis

Statistical analysis was performed with the Statistical package SPSS. We performed the statistical analysis by using: 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 coefficients – R). The statistical significance was defined as $P < 0.05$.

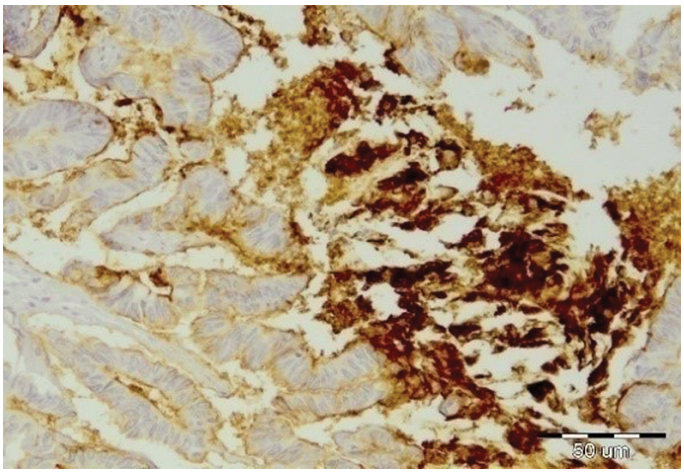

Fig. 1. High expression of CD133.

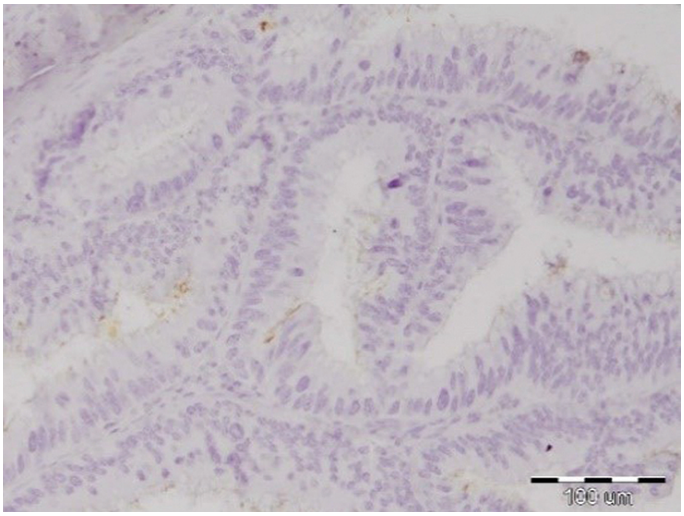

Fig. 2. Low expression of CD133.

RESULTS

The study population included total 90 patients, 52 (57.8%) males and 38 (42.2%) females. Mean age at the time of surgery was 63.3 years (range: 17–91 years). The distribution of tumors according to the anatomic location was as follows: left colon in 40 (44.5%), right colon in 22 (24.4%) and rectum in 28 (31.1%) cases. Clinical stage distribution at the time of diagnosis was as follows: stage I in 13 (14.45%) cases, stage II in 25 (27.8%) cases, stage III in 39 (43.3%) cases, stage IV in 13 (14.45%) cases. Among 90 colorectal carcinoma patients, twenty-three (25.6%) cases had distant metastases at the time of diagnosis. Clinicopathological characteristics of the patients are presented in Tab. II.

The Relationship of clinicopathological parameters and expression of CD133

High expression of CD133 was detected in 43 (47.8%) cases. The highest expression of CD133 was found in patients with rectal

Tab. I. Characteristics of immunohistochemical examination of stem cell marker CD133.

ANTIBODY	MANUFACTURER	CLONE	DILUTION	SIGNAL	CONTROL
CD133	Miltenyi, Germany	AC133	1:11	Cytoplasmatic	Bone marrow

carcinoma – 53.67%. The statistical analysis showed no significant differences according to tumour location and expression of CD133. In our study we found an association between the presence of distant metastasis and high expression of CD133 (P = 0.0153). The results of our study showed a high expression of CD133 in stage III (79.5%) and stage IV (84.6%). Our results showed statistically significant differences in the expression of CD133 in patients with different T category, N status and G – differentiation. The relationship of clinicopathological parameters and expression of CD133 are presented in Tab. III.

Correlation between CD133 and independent variables

The results of the multiple regression analysis showed that all independent variables together have impact on CD133 expression (F = 9.27; P = 0.000001). Among independent variables, the stage of disease had the greatest impact on CD133 expression (t = -6.451; P = 0.000001). The results are shown in Tab. IV.

DISCUSSION

So far, CEA and CA 19–9 have been the most commonly applied markers in gastrointestinal malignancies and elevated levels of both CEA and CA19–9 have also been suggested to be associated with poor prognosis in CRC. Low sensitivity and specificity reduce their utility as tumor markers, indicating the need for additional, more reliable markers for CRC [10–12].

CD133 is a cell surface marker, well known as prominin-1, that have recently been associated with colorectal cancer stem cells. The expression of high CD133 is promoted as useful to identify putative colorectal cancer stem cells and tumors with a poor prognosis. Many recent studies have shown association between expression of CD133 and clinical-pathological characteristics in patients with colorectal carcinoma and that high expression of CD133 can be associated with the presence of distant metastasis, thus CD133 can be a prognostic marker for aggressive CRC [13– 20]. In a meta-analysis conducted by Chen et al., CD133 was the most useful surface marker for CRC stem cells and had a major prognostic impact on CRC patients. This study showed that CD133 high expression was significantly associated with poorer clinical outcomes and some clinicopathological factors such as T3, and T4 category, N category and vascular invasion in CRC patients [21].

The study of Jing showed that CD133 was extremely co-expressed in colorectal cancer with hepatic metastases [22]. In the study of Horst, it was demonstrated that expression of CD133 was the best sole marker to predict low patient survival [23].

Nagata et al. found that the overall survival of patients who were positive for CD133 was significantly shorter than that of all other patients [24]. In another study, it was suggested that CD133 expression may be related to sensitivity to radiotherapy or chemotherapy in CRC, but the presence of CD133-positive cancer cells alone cannot support the concept of CSCs in CRC [25].

Tab. II. Clinicopathological characteristics of patients.

CLINICOPATHOLOGICAL CHARACTERISTICS	NUMBER OF PATIENTS N (%)
Total number of patients	90 (100%)
Age (mean + SD) 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	
No	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)

Recent studies indicate resistance to 5-fluorouracil and oxaliplatin treatment in tumor cells with higher expression of CD133 [25–28]. A study by Wang found a correlation between CD133 expression and lymphovascular invasion, degree of tumor differentiation, TNM stage and tumor regression in preoperative treatment that indicate the prognostic value of this marker in the prognosis of the disease [29].

In our study we found a high expression of CD133 in 43 cases (47.8%). These results are similar with the results in the study by Horst, where CD133 was the best single marker for poor prognosis of survival, with high expression detected in 69% of patients with metastatic disease [23].

Highest expression of CD 133 was found in 53 patients with rectal carcinoma (67%). Statistical analysis showed no significant differences regarding tumour location and expression of CD133.

Tab. III. The relationship of clinicopathological parameters and expression of CD133.

CLINICOPATHOLOGICAL PARAMETER	CD133 EXPRESSION N (%)		
	Low/non	High	p
Tumor location			
left colon	47.5	52.5	
right colon	40.9	59.1	0.675
rectum	53.6	46.4	
Stage of disease			
I	100	0	
II	96	4	0.00001
III	20.5	79.5	
IV	15.4	84.6	
T-category			
T1	80	20	
T2	91.7	8.3	0.0119
T3	48.9	51.1	
T4	35.7	64.3	
Nodal status			
No	65.8	34.2	
N1	54.2	45.8	0.0066
N2	28	72	
G-Differentiation			
G1	83.3	16.7	
G2	54.2	45.8	0.0115
G3	25	75	
Distant metastases			
MS+	30.4	69.6	
MS-	59.7	40.3	0.0153

Tab. IV. Correlation between CD133 and independent variables.

INDEPENDENT VARIABLES	R = 0,71 R2 = 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
Tumor location	-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
MS +	0.0908	0.1109	0.7398	0.998	0.321036

Our study found an association between the presence of distant metastases and high expression of CD133 ($P < 0.05$). Similar results were shown in the study by Jing [22]. We found a high expression of CD133 in stage III (79.5%) and in stage IV of disease (84.6%), and a significant correlation between high expression of CD133 and stage of disease ($P < 0.0001$). These results are similar with the published literature data [29]. Among patients with category T3, 51.1% had high expression of CD133 and among patients with category T4 – 64.3%. The results showed a significant correlation between high expression of CD133 and category T ($P < 0.0119$). These results are similar with the results of Chen et al.; in their meta-analysis they reviewed a correlation between poor prognosis and high expression of CD133 in category T3 and T4, while in the study by Horst, no significant correlation was found between high expression of CD133 and higher T category [21, 23]. In our study we found a significantly higher expression of CD133 in patients with N2 nodal status than in patients with N0 and N1. Also, we found significant differences in the expression of CD133 in patients according to G - differentiation. By multiple regression analysis we determined a statistically significant correlation between the expression of CD133 (dependent variable) and the following covariables: T – stage, N – status, G – differentiation and presence of distant metastasis (independent variables). The stage of disease has the greatest statistical impact on the expression of CD133 (partial correlation = 0.585, $P < 0.0001$). We did not find any statistically significant correlation between the expression of CD133 (dependent variable) and the following covariables: age, gender and tumour location.

The results of our study showed that CD133 expression has the highest positive correlation with the stage of disease and we found significant differences in expression of CD133 between patients with and without metastatic lesions, different T category, N status and G differentiation. From the obtained results we can conclude that CD133 stem cell marker may be a useful predictor for an aggressive type and metastatic potential in patients with CRC.

The limitation of the study is the small number of CRC cases that were examined and the retrospective nature of CRC. Larger prospective studies are required to validate the current findings and the clinical utility of CD133 in patients with CRC. Also, we recommended studies including multiple stem cell markers, such as CD44, EpCAM etc. The identification of CD133 cancer stem cell marker and its implementation in routine clinical practice will allow us to isolate a subgroup of patients with clinically aggressive disease form, high metastatic potential, and local recurrence. This will also create an opportunity to develop new efficient targeted therapies that yield tangible clinical benefits to patients with CRC.

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Competing interests: The authors declare that they have no competing interests.

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