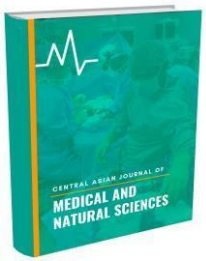

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Immunological Investigation of CD133 in Samples of Iraqi Colorectal Cancer Patients

Maryam A. Nemat*[1], Farooq I. Mohammed[2], Shilan K. Jabbar[3]

1. Department of Biology, College of Science, University of Kirkuk, Iraq
2. Department of Criminal Evidence, College of Science, University of Kirkuk, Iraq
3. Department of Biology, College of Education for Women, University of Kirkuk, Iraq

* Correspondence: maryam0abdulrazak@gmail.com

Abstract: Colorectal cancer (CRC) is a disease that occurs when cells in the colon or rectum proliferate out of control. The biomarker CD133 has been found on the surface of CSCs in colorectal cancer. The overexpression of CD133 has been linked to a poor prognosis, decreased overall survival, and therapy resistance in colorectal cancer and a number of other tumor types. This study aimed to investigate the expression intensity of CD133 in colorectal cancer patients and evaluate the relationship between this marker and the clinic-pathological characteristics. Expression of CD133 was studied by using immunohistochemical test in paraffin blocks of colorectal cancer and normal tissues in patients who were referred to Kirkuk General Hospital, Azadi Teaching Hospital, and GIT & Hepatology Teaching Hospital. CD133 expression was detected in 32% of colorectal cancer cases, with varying intensities (25% strong, 50% moderate, 25% weak). Expression was predominantly observed in adenocarcinomas and in tumors located in the colon. CD133-positive cases were more frequent among males and older age groups. Associations were noted between CD133 expression and tumor grade, stage, and site. These findings suggest a potential link between CD133 and colorectal cancer progression. This study highlights the potential role of CD133 as a cancer stem cell marker in colorectal cancer. CD133 expression was associated with specific clinicopathological features, indicating its involvement in tumor progression and treatment resistance. The findings suggest that CD133 could serve as a prognostic indicator and a potential therapeutic target.

Keywords: CD133, Colorectal Cancer, Cancer Stem cells, IHC

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1. Introduction

Colorectal cancer (CRC) is a disease that begins when cells in the colon or rectum spread uncontrollably [1]. Colorectal cancer (CRC) is a common type of malignant neoplasms. It placed between second and fourth in the globe in terms of incidence, depending on the location, gender, and type of the cancer [2]. The term "colorectal cancer" describes a slow-growing cancer of the colon or rectum that starts as a polyp or tissue growth [3].

In Iraq, colorectal cancer (CRC) has the second-highest incidence rate for both genders during 2022, there were 2,871 cases of CRC accounting for 7.3% out of 39,068 newly diagnosed cancer cases including 1869 colon tumors, 887 rectum tumors and 115 rectosigmoid tumors [4]. In the United States, CRC ranks third in terms of both cancer-related diagnoses and cancer-related deaths for both men and women and it is the biggest cause of mortality for men under 50 years old. There were an estimated 153,020 cases of CRC in the United States in 2023, including 106,970 colon tumors and 46,050 rectal tumors [1]. It is widely acknowledged that colorectal cancer (CRC) primarily affects the elderly.

The incidence rate of CRC has decreased in many regions of the world for individuals over 50, but it has grown annually by 1% to 3% for those under 50, a condition known as early onset CRC. In the United States, people born in the 1990s had a twice as high chance of developing CRC as people born in the 1950s [5]. The majority of colorectal cancers (CRCs) are sporadic tumors (about 70%) and 30% of all colorectal cancers are hereditary [3]. There are also multiple factors that implicated in the development of CRC, it has been shown that those with a history of cancer, colon polyps, inflammatory bowel disease, diabetes mellitus, or cholecystectomy—or their relatives—are more likely to develop CRC. Lifestyle factors also play an important role in the etiology of colorectal cancer. Research demonstrates that incorrect food habits, alcohol intake, cigarette smoking, being overweight or obese, and physical inactivity all raise the risk of colorectal cancer [2].

Colorectal cancer is diagnosed in three common ways: asymptomatic results from routine screening; symptoms such as anemia, weight loss, or change in bowel habits that require further testing; or emergently, with perforation or obstruction [6]. Cancer staging describes the overall appearance of the tumors. It can include invasion, spread to local lymph nodes, distant metastasis, or tumor size. Cancer grading describes how the tumor's cells and tissue appear at a microscopic level [7]. Cancer stem cells (CSCs) are a specific type of abnormal cells with the ability to self-renew and differentiate that are present in the vast majority of malignancies. They share certain traits with stem cells and contribute to the heterogeneous generations of cancer cells which form the tumor [8].

Recent research has discovered and isolated cancer stem cells (CSCs), recognized as a primary factor in resistance to oncological treatments and contributing to both local and distant recurrence [9]. Cancer stem cells (CSCs) can defend themselves against chemical or genetic damage, much like adult stem cells do. Chemotherapy and radiation therapy are the most often utilized cancer treatments. Specialized proteins found in cancer stem cell membranes protect the cells from chemotherapy by preventing drug molecules from entering the cell. CSCs are able to resist the effects of radiation therapy because they possess a specific enzyme that protects against reactive oxygen species caused by radiation. Furthermore, CSCs have increased DNA repair activity, which reduces apoptotic events [10].

CD133 was one of the first immunomarkers to be identified as unique to CSC. This immunomarker, also called prominin-1 (PROM1), is a transmembrane surface glycoprotein that weighs 120 kDa and is involved in a variety of cell processes, including apoptosis, autophagy, cell metabolism, carcinogenesis, and resistance to chemotherapy and radiation. CD133 overexpression is one of the most significant features of stem cells in colorectal cancer [11]. In order to gain a better understanding of how CD133 is modulated in both normal and cancer stem cells, numerous molecular processes have been studied. Research using normal and cancer stem cell lines has shown that cells in the G1/G0 phase of the cell cycle exhibit lower CD133 antibody reactivity than cells in the G2/M phase, indicating a degree of cell cycle dependence associated with CD133 expression [12]. In colorectal cancer, CSCs and CD133 expression are linked to several pathways, including WNT, TGF-β, Notch, and Hedgehog signaling. It has been discovered that all these pathways are activated in CD133-positive cells which are play an important role in growth and development of colorectal cancer, growth and maintenance of cancer stem cells, angiogenesis, CSC differentiation, stem cell self-renewal, and metastatic activity. CD133 also contributes to the invasiveness of tumors by cell-cell and the cell-matrix interactions [13].

The presence of CD133+ cells increase resistance to anti-cancer treatment. This increased resistance is associated with a reduced response to treatment, which results in a shorter survival rate and a worse prognosis for cancer patients. Additionally, CD133+ cells are associated with enhanced tumor metastasis and proliferative potential. In cancer, CD133+ generally has a significant prognostic impact. Presence of CD133+ cells has been associated to decreased overall survival rate [10]. The marker CD133 is frequently used to identify and separate colorectal cancer stem cells. Also, it has been studied in order to gain a better knowledge of the traits and roles of cancer stem cells in colorectal cancer [13].

2. Materials and Methods

2.1 Patients and Samples

The 50 paraffin-embedded colon and rectum samples along with normal tissues paraffin-embedded blocks were selected from Kirkuk General Hospital, Azadi Teaching Hospital in Kirkuk and GIT & Hepatology Teaching Hospital in Baghdad. The samples were analysed under the supervision of a qualified pathologist to assess variations in disease parameters, including gender, age, tumor site, histological type, tumor grade, pathological stage, and CD133 expression in colorectal cancer stem cells. All tissue cut sections from both tumor and healthy colorectal tissues were stained immunohistochemically.

2.2 Immunohistochemistry staining procedure

Sections of cut tissue with 5 micrometers thick were prepared and stained by hematoxylin and eosin (H&E). Paraffin-embedded tissue blocks were sectioned into 3µm slices using a microtome and placed on positively charged slides. The slides underwent deparaffinization in a hot air oven at 60°C for 30 minutes, followed by immersion in xylene and sequential rehydration in graded ethanol concentrations before being transferred to distilled water. Antigen retrieval was performed by incubating slides in citrate buffer (EnVision FLEX Target Retrieval Solution, Low pH (50x)) with pH 6 at 97°C for 20 minutes in a water bath, followed by cooling at room temperature and then were placed in container containing wash buffer (EnVision FLEX Wash Buffer (20x)) for 3 minutes. Tissue sections were then encircled with a liquid blocker pen, and endogenous peroxidase activity was inhibited using a hydrogen peroxide block (EnVision FLEX Peroxidase-Blocking Reagent). After that, the slides were washed by distilled water and placed into wash buffer for 5 minutes. Diluted primary antibody at a ratio 1:50 for CD133 (Elabscience PROM1 Polyclonal Antibody) was applied and incubated for 30 minutes at room temperature, followed by washing with distilled water and then they were placed into wash buffer for 5 minutes. A secondary HRP-labeled antibody (EnVision FLEX /HRP) was then added and incubated for another 30 minutes. The slides then were washed by distilled water and placed into wash buffer for 5 minutes.

To visualize antigen-antibody binding, DAB chromogen substrate (one drop of EnVision FLEX DAB+ Chromogen per 1 mL EnVision FLEX Substrate Buffer) was applied for 10 minutes at room temperature, followed by counter staining with hematoxylin (EnVision FLEX Hematoxylin) for 2 minutes. The slides were then washed, dehydrated in graded ethanol, mounted using DPX mounting medium, and left to dry. Stained slides were examined under a light microscope (Lieca) by a qualified pathologist at 10X and 40X magnifications. Staining intensity was assessed semi quantitatively based on membrane-bound brown staining, categorized as negative, weak, moderate, or strong, and used for statistical analysis and conclusion drawing. Positive expression of CD133 was determined by brown coloration on the cell membranes and the intensity of expression was measured by the intensity of brown staining.

3. Results and Discussion

The study was involved both 28 men (56%) and 22 women (44%) with age range from 25-85 years old. Age between 61 and 70 were the higher percent of patients with colorectal cancer (28%) followed by 24% to patients in age range 51-60. The above mentioned results showed similar to the results obtained by [14] which may suggest age relating correlation of these markers and could be used as a possible indicator in this age. This may be due to weak immune system and simultaneous diseases in elder ages group.

In terms of tumor location of the collected samples, the highest percentage was in the colon 56% (28 patients) than the rectum 30% (15 patients) and the recto-sigmoid 14 % (seven patients). Tumor type in 48 patients was adenocarcinoma (96%) and in 2 patients was mucinous adenocarcinoma (4%) which are all similar to the results obtained by [4]. Regarding the grade of colorectal cancer patients, the highest percentage was moderate (33 samples with 66%) and lowest percentage was well (seven samples with 14%), as

shown in Figure 1. These results showed similar to the results obtained by [14] in Iraqi patients.

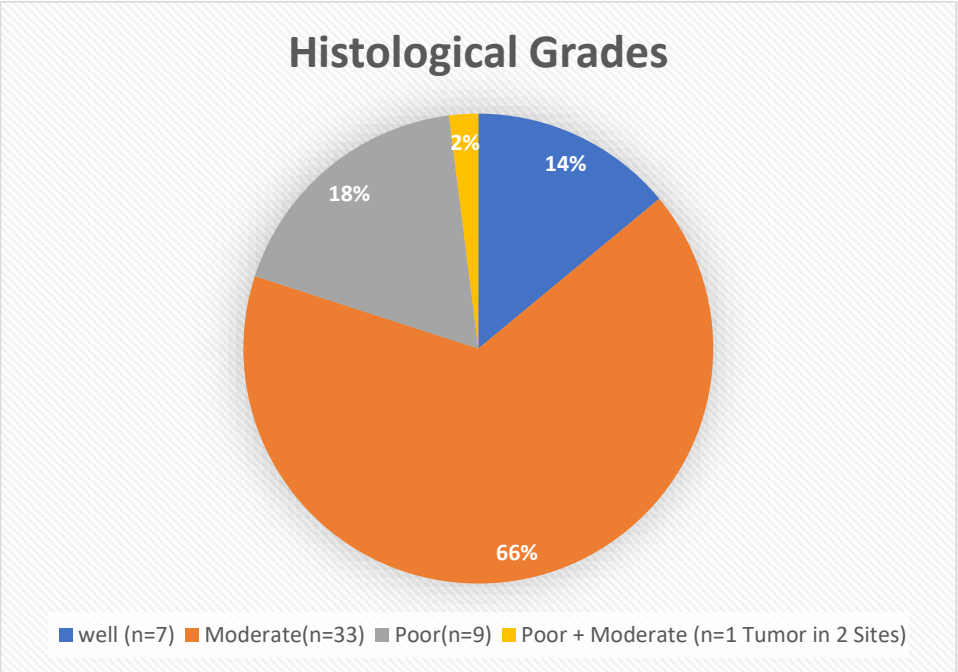

Figure 1. Distribution of Colorectal Cancer Patients According To Histological Grades.

In terms of TNM stages of 45 colorectal cancer patients (the condition of 5 cases was unknown because they were colonoscopy specimens), the highest percentage in T (Tumor) stage was T3 (28 patients with 62.2%) and lowest percentage was T4 (four patients with 8.8%). In addition, 19 patients (42.2%) had lymph node metastasis (N Stage). Also, 42 (93.3%) and 3 (6.6%) patients were in M0 and M1a (Metastasis Stage) respectively. The above results showed similar to the results obtained by [15]. Out of 50 patients, only 20 individuals were successfully contacted and responded to the questionnaire, which was based on various risk factors. Among these 20 patients, 17 (85%) were newly diagnosed cases, while two (10%) were relapse cases, one (5%) was exhibited treatment resistance, and one (5%) was presented with both relapse and resistance. Regarding patient outcomes, four patients (20%) were dead, and 16 (80%) were alive at the time of data collection [15], [16].

The analysis of risk factors was revealed that six patients (30%) were classified as overweight. Geographically, seven patients (35%) were from rural areas, and 13 patients (65%) were from urban areas. Lifestyle factors were included alcohol use in two patients (10%), smoking in six patients (30%), and regular physical activity in seven patients (35%). Associated medical conditions and syndromes were identified in this cohort were included FAB syndrome in one patient (5%), inflammatory bowel disease (ulcerative colitis) in one patient (5%), diabetes in seven patients (35%), colorectal polyps in four patients (20%), and inherited conditions in eight patients (40%). Additionally, three patients (15%) had received previous treatment for certain cancers, all of whom were the same individuals identified in the relapse cases. These findings were provided a comprehensive overview of the clinical, demographic, and lifestyle characteristics of the cohort, highlighting the predominance of new diagnoses and the influence of comorbidities and risk factors. However, the inability to contact the remaining 30 patients represents a limitation that may affect the overall representativeness and generalizability of the data.

The expression of CD133 was evaluated in a cohort of 50 colorectal cancer patients using Immunohistochemical (IHC) test. CD133 positivity was identified in 16 patients (32%), with expression intensity categorized as strong in four cases (25%), moderate in eight cases (50%), and weak in four cases (25%). The expression pattern of CD133-positive cases was focal in eight patients (50%) and diffuse in eight patients (50%). CD133 negativity was observed in 34 patients (68%).

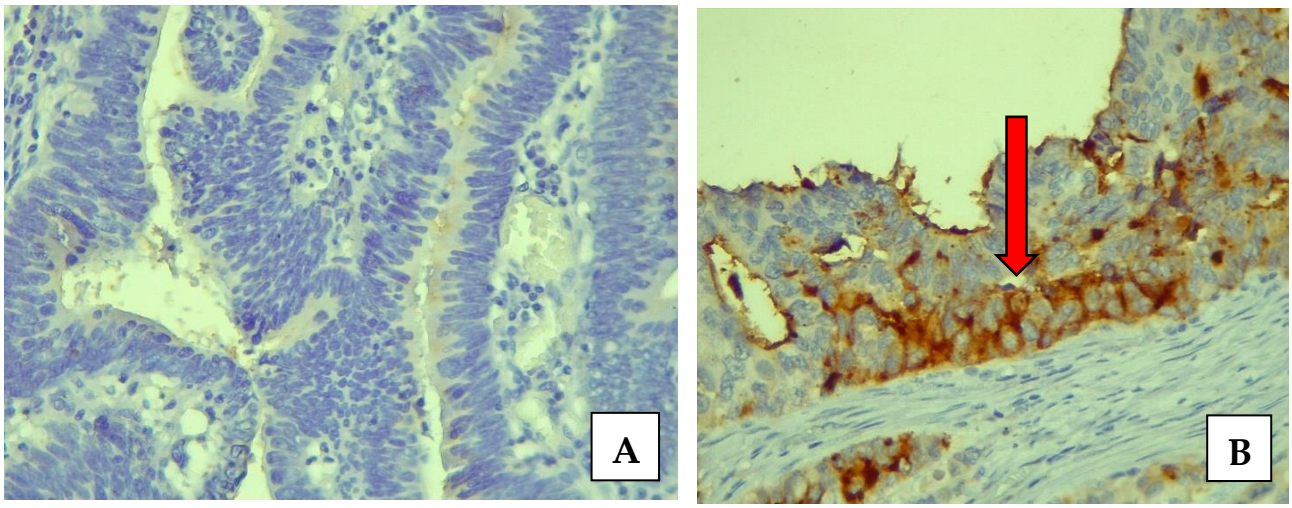

Figure 2. (A) Section of Negative IHC for CD133 (B) Section of Positive IHC for CD133, Power 400X.

Among the 16 patients with positive CD133 expression, males were represented the majority with ten cases (62.5%), while females were accounted for six cases (37.5%), as shown in Table 1. This was indicated a higher prevalence of CD133 positivity in males.

Table 1. Distribution of Positive CD133 Patients according to Gender.

Gender	Strong		Moderate		Week		Total	
	No.	%	No.	%	No.	%	No.	%
Male	3	18.8	4	25	3	18.8	10	62.5
Female	1	6.2	4	25	1	6.2	6	37.5

The highest percentage of CD133 positivity was observed in the colon accounting for ten cases (62.5%), followed by the rectum with four cases (25%), and the rectosigmoid region with two cases (12.5%). These findings were suggested that CD133 expression was predominantly associated with the colon due to higher incidence of cancer in the colon in this study than the rectum and the rectosigmoid region. The colon has a significantly larger surface area than the rectum, providing a greater opportunity for malignant transformations to occur. Since the colon is primarily responsible for absorbing water and nutrients, stool remains in this region for a longer duration, increasing the exposure of colonic epithelial cells to carcinogens and pro-inflammatory agents (American Cancer Society, 2018). As shown in Table 2:

Table 2. Distribution of Positive CD133 Patients according to Tumor Sites.

Tumor Sites	Strong		Moderate		Week		Total	
	No.	%	No.	%	No.	%	No.	%
Colon	1	6.2	6	37.5	3	18.8	10	62.5
Rectum	2	12.5	2	12.5	0	0	4	25
Rectosigmoid	1	6.2	0	0	1	6.2	2	12.5

As shown in Table 3, the highest percentage of CD133 positivity was observed in the both 41–50 and 61–70 age groups (each five cases with 31.3%), followed by the 51–60 age group (three cases with 18.8%). The lowest percentage was observed in the 71–80 age group (only one case with 6.3%). These results were suggested that CD133 positivity was mostly shown in fifties age group and the elderly people.

Table 3. Distribution of Positive CD133 Patients according to Age Groups.

Age	Strong		Moderate		Week		Total	
	No.	%	No.	%	No.	%	No.	%
31-40	1	6.2	0	0	1	6.2	2	12.5
41-50	1	6.2	4	25	0	0	5	31.2
51-60	1	6.2	1	6.2	1	6.2	3	18.7
61-70	1	6.2	3	18.7	1	6.2	5	31.2
71-80	0	0	0	0	1	6.2	1	6.2

As shown in Table 4, the majority of CD133-positive cases were identified as adenocarcinoma accounting for 15 cases (93.8%), while mucinous adenocarcinoma was observed in only one case (6.2%). This was indicated a strong association between CD133 positivity and adenocarcinoma due to higher incidence of the adenocarcinoma in this study than the mucinous adenocarcinoma.

Table 4. Distribution of Positive CD133 Patients according to Histological Types.

Histological Types	Strong		Moderate		Week		Total	
	No.	%	No.	%	No.	%	No.	%
Adenocarcinoma	4	25	7	43.8	4	25	15	93.8
Mucinous Adenocarcinoma	0	0	1	6.2	0	0	1	6.2

4. Conclusion

Based on our findings, this study provides further evidence for the involvement of CD133 as a potential cancer stem cell marker in colorectal cancer (CRC). The immunohistochemical analysis revealed a positive expression of CD133 in 32% of the examined cases, with varied intensity and distribution, predominantly in adenocarcinomas and in tumors located in the colon. The findings suggest that CD133 expression may be associated with specific clinicopathological features, including tumor site, histological type, and patient age. The presence of CD133-positive cells highlights their possible role in tumor progression, therapy resistance, and unfavorable prognostic outcomes in CRC. These results support the hypothesis that CD133 could serve not only as a diagnostic and prognostic biomarker but also as a potential therapeutic target in colorectal cancer management. However, further large-scale studies are recommended to validate these observations and explore the molecular mechanisms underlying CD133-mediated tumorigenesis and treatment resistance in CRC.

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