Immunohistochemical Expression of CD44 as Cancer Stem Cells Marker in a Sample of Iraqi Patients with Colorectal Carcinoma

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ABSTRACT

Colorectal cancer is one of the most frequent malignancies in the world, and it is also one of the most deadly. Distant metastases and recurrence frequently cause patient death. Cancer stem cells (CSCs) play a crucial role in colorectal cancer spread and relapse. CSCs are a type of cancer cell that can self-renew, divide indefinitely, and differentiate in multiple directions. CD44,CD133, CD24, EpCAM, LGR5, and ALDH are some cell surface markers used to identify colorectal CSCs. They are highlytumorigenic, chemoresistant, and radioresistant, making them important in colorectal cancer metastasis and recurrence as wellas disease-free survival. The current study included 60 Iraqi male patients. They were assigned into three groups. Group I consisted of 20 newly diagnosed colorectal cancer patients, group II consisted of 20 relapsed patients who were treated withchemotherapy and were cured, but the tumor relapsed, and group III consisted of 20 patients who demonstrated resistance to chemotherapy treatment. All clinicopathological markers were analyzed, and they demonstrated a higher tumor grade in group II compared to the other groups and a difference in age between groups. CD44 expression was higher in groups II and III than in group I, with some Immunohistochemical slides showing no typical colorectal cancer cells but positive for CD44, indicating the existence of cancer stem cells. Similar results were reported in the resistance group. The findings could be explained by the fact that strong CD44 expression indicates a large number of cancer stem cells, which are important in tumor relapse and treatment resistance. CD44 may be used as a biomarker for cancer stem cells as a findings of these results, allowing for their detection and aiding in the process of tailoring therapy for these cells within the tumor mass, minimizing tumor relapse and chemotherapy resistance.

Keywords: CD44, Cancer stem cells, Colorectal carcinoma, Tumor relapsing.

1. Introduction

Colorectal cancer (CRC), the third-deadliest cancer in the United States, is a disease that arises from epithelial cells lining the colon or rectum of the gastrointestinal tract, most commonly as a result of Wnt signaling pathway alterations that increase signaling activity. The mutations can be inherited or acquired, and they are most likely to arise in the stem cells of the intestinal crypt [1]. In all colorectal tumors, the Adenomatous Polyposis Coli (APC) gene which encodes the APC protein, is the most often changed. The catenin protein is prevented from accumulating by the APC protein. Catenin accumulates in large amounts and translocates to the nucleus, where it binds to DNA and activates proto-oncogene transcription in the absence of APC. These genes are involved in stem cell renewal and differentiation in general, but they can cause cancer if they are overexpressed [2]. Colorectal cancer (CRC) is one of the most common cancers globally, with a 5-year relative survival rate of roughly 10% for both metastatic rectal and colon cancer. Although early detection, prevention, and customized medicine have increased the response rate to traditional treatments, therapy resistance and metastasis remain the leading reasons for CRC death. [3].

Over the last 15 years, evidence has accumulated that suggests cancers, including CRC, are stem-cell disorders. Early detection and treatment are crucial for a better prognosis. Patients with early-stage CRC have a five-year survival rate of more than 90%, compared to only 11% for those with locally advanced or metastatic disease [4] . Furthermore, patients with metastatic CRC have a median lifespan of only two years, despite several therapeutic alternatives including as surgical resection, chemoradiation, monoclonal antibodies to tumor growth factors, and liver-directed therapy for metastatic cancer [5]. Unfortunately, only a small percentage of metastases respond to these treatments, and even fewer are cured, underlining our lack of understanding of the molecular mechanisms underlying this most lethal stage of CRC [6].

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The CD44 gene encodes a hyaluronic acid receptor. CD44 is a protein with a molecular weight of 85–200 kDa that is found throughout the body. It's a transmembrane glycoprotein that controls cell-cell communication, adhesion, and migration. When compared to $CD44^-$ cells, $CD44^+$ colon cancer cells (Isolated from a colorectal cancer patient and discovered in the G0 and G1 phases) exhibit aggressive proliferation, high colony formation, insensitivity to apoptosis, and resistance to chemo- and radiotherapies. Short hairpin RNA (shRNA) knockdown of CD44 causes a decrease in cell proliferation, migration, and invasion, but not apoptosis [7, 8].

Aims of the study

To achieve the above aim, the following objectives were carried out:

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1. Detection of the emerging cells of tumor (Cancer stem cells) in colorectal cancer clinical samples.
2. Determination of surface CD markers for production drugs targeting.
3. Immunohistochemical detection of cancer stem cells in colorectal cancer clinical samples in newly diagnosed relapsed and resistant patients.

2. Materials and Methods

Samples Collection

The study included 60 male patients ranging in age from 30 to60 years. The samples were gathered in partnership with Dr.Majid Al-Dari's clinic from the Histology Unit of the Medical City Hospital, Department of Education Laboratories, Ministry of Health and Environment. Sections of sampleswere taken from the Raji Al-hadithy laboratories. Group I contained 20 samples from patients newly diagnosed with colorectal cancer, Group II included samples from relapsed patients who were subjected to chemotherapy treatment, and the tumor relapsed, and Group III included 20 samples from patients who had developed resistance to chemotherapy.

Immunohistochemistry Protocol

- The tissue sections were determined by drawing a circle

around them with a pap pen.

- Enough drops of hydrogen peroxide block were added to cover the sections, incubated for 5–10 minutes, and washedthree times in TBS washing.
- Protein block was applied and incubated for 5-–10 minutes at room temperature to block nonspecific background staining and washed three times in TBS washing.
- Diluted primary antibody at a ratio (1/100 for CD44) was added and incubated for 30 min.
- Sections were washed three times in TBS and incubated for 30 minutes in a humidified chamber at room temperature.
- Sections were washed three times in TBS, secondary antibody conjugated with HRP was applied and incubatedfor 30 min in a humidified chamber at room temperature.
- Chromogen substrate (DAP) was added and incubated for 5 minutes at room temperature. Sections were washed withdistilled water and TBS wash.
- Hematoxylin was added for 1-3 min at room temperature and washed in distilled water and Tap water wash.
- Dehydration: Sections were dehydrated by immersing the slide sequentially in
- 70 % ethanol for 2 minutes.
- 80% ethanol for 2 minutes.
- 100% ethanol for 7 minutes.
- Xylene for 3-5 minutes [9].

1. The slides were dehydrated before being mounted with DPX and covered with coverslips. The slides were examined under a light microscope at 10X, 20X, and 40X magnifications. The results were compared to the positive control, which was defined by the kit's brochure. The intensity and proportion of staining were used to determine positivity in a semi-quantitative manner. When the cell membrane was stained with brown color, a scale wascalculated for CD44.
2. Score 0 (negative): (none of the cells revealed positivity forthe marker)
3. Score 1 (weak positive (1+): number of positive cells represent 10% or less of total (few scattered ≤10%)
4. Score 2 (moderate positive (2+): the positive cells 11≤30%.
5. Score 3(strong positive (3+): the positive cells31 ≤50 %.
6. Score 4 (very strong (4+): the positive cells more than 50%.

Statistical Analysis

The SPSS program version 24 was used to analyze the data, and the results were expressed using simple statistical metrics like mean and standard deviation. ANOVA was used to determineto mean differences, which were then tested using either the LSD or Duncan test. $p \leq 0.05$ was deemed to be an acceptablethreshold of significance [10].

3. Results and Discussion

Table 1 shows the results of the clinicopathological investigation. When comparing the results between groups, they show a wide range of age, grade, and tumor site, with group I having the highest tumor grade. The resistant group has a larger percentage of CD44 expression than the other groups, indicating the presence of most cancer stem cells. This is explained by the ability of these cells (CSCs) to withstand chemotherapy through several methods, [11, 12] including their having to specific receptors on their surface showing their ability to introduce the chemotherapy inside the cells and degrade it via specific enzymes [13].

In this study, all the slides obtained from the groupsof patients were subjected to immunohistochemical (IHC) staining and the CD44 marker expression was measured andcompared for each of the three groups of the study, and then analyzed microscopically. The percentage of CD marker was measured for five fields for each slide. The positive tumor cells stained with DAB stain reveal a brownish color indicatingthe positivity of CD44 marker expression, while the deep orlight brownish color is the way of measuring the approximate intensity or the percentage of the marker expression within the IHC stained tumor mass slides. The accurately expressed cancer stem cells within the tumor mass were evaluated for CD

Table 1: Clinicopathological parameters of patients with colorectal carcinoma.

Clinical pathological parameters	Group I (No. 20 newly diagnosed)	Group II (No. 20 relapsed)	Group III (No. 20 resistant)
Age	44.4 ± 13.9	55.7±12.3	38.7±12.7
Grade	high	high	low

Tumor location	30 % rectum 70 % colon	23% rectum 77% colon	15%rectum85% colon
Type	Adenocarcinoma 70% Mucoidcarcinoma 30%	Adenocarcinoma 88% Mucoidcarcinoma 12%	Adenocarcinoma 65% Mucoidcarcinoma 35%
CD44 Expression	15%	33%	44%
Note: Data are Mean ± SD.			

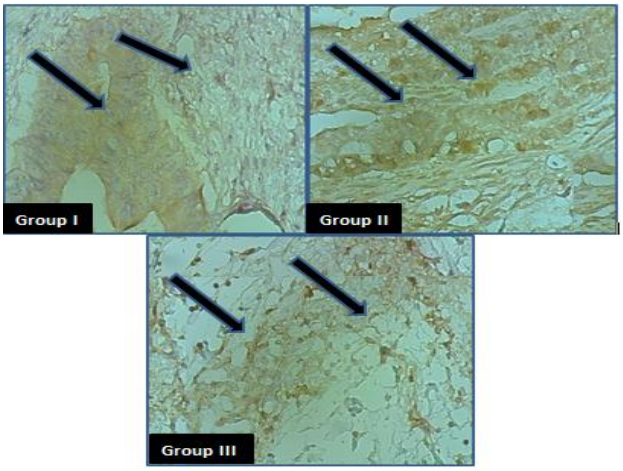

Figure 1: Immunohistochemical expression of CSCs in each group of the study, 40X.

marker through calculating the positive CD44 marker cells andthen comparing their numbers to the total tumor cells in the field The technique for Clinical Pathology is accredited by the American Society for Clinical Pathology. It involves dividing the number of positive cells for (ASCP) the specified marker in five selected microscopic fields by the total number of positive and negative cells (Figure1) [14, 15]. Positive CD44 cells within the tumor mass of group I appear brownish colored, indicating their positivity to the CD44 marker and calculated as cancer stem cells within the tumor mass, whereas negative CD44 cells appear deep blue to violet-colored, indicating their reactivity to each measured CD marker and calculated as cancer stem cells within the tumor mass. Wang et al. [7], Li et al. [16] of positivity for each CD within each group are shown in Figure The results show significant differences between groups. Group I (newly diagnosed patients)

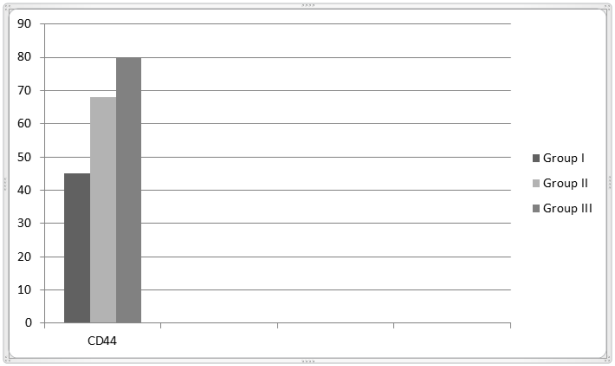

Figure 2: Percentage of immunohistochemical expression of CD44for the groups I (newly diagnosed patients), II (relapsed patients), andIII (resistant patients) showing significant differences between groups,(data are mean±SD).

demonstrates a moderate expression to CD44 marker as compared to other groups, while the most highly expressed CD marker is shown in the resistant group compared to the other groups. The CD marker is expression is higher in Group II (relapsed patients) than in Group I (newly diagnosed patients), with a significant difference between the two groups. These findings corroborate each other [15, 17, 18]. The possible explanation is that the resistant group possesses a high concentration of cancer stemcells within the tumor mass. As a result, the patients of these samples showed a history of resistance to the chemotherapy without any responses through and at the end of the recurrenttherapy sessions courses.

4. Conclusion

Cancer stem cells can be the major cause of colorectal cancerresistance to treatment and tumor relapse. CD44 marker canbe targeted as a surface marker for cancer stem cells. The immunohistochemical expression of cancer stem cells marker CD44 was the highest in the resistant patient group, reaching 80%. Immunohistochemical analysis can be efficiently used for the detection of cancer stem cells in colorectal cancer. The detection of cancer stem cells in colorectal cancer clinical samples is very important and can be used to prevent the relapsing of tumors in patients after treatment and in resistance of tumors to treatments. Tumors targeting therapy strategies could be easily and readily achieved via the early diagnosis ofcancer stem cells within the tumors since these types of cells possess different mechanisms for resistance to chemotherapy.

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