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Original Article

Estimate the relationship between CXCR4-SDF-1 axis and inhibitory molecules (CTLA4 and PD-1) in patients with colon cancer

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Abstract

Colon neoplasia is one of the major malignancies in industrialized countries due to their Western-style food habits. It accounts for more than 50% of the population developing adenomatous polyps by the age of 70 years, but 10% of cancers in developed countries. The aim of this study was to evaluate the pathological role of the C-X-C chemokine receptor type 4/stromal-derived factor 1 axis (CXCR4-SDF-1 axis), and the inhibitory molecules PD-1 and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) in postoperative colon cancer patients undergoing treatment with chemotherapy (oxaliplatin and capecitabine) and estimate the correlation between these studied factors to deeply understand the basic mechanisms and potential diagnostic or therapeutic effects. The study involved 90 patients, including 50 colon cancer patients (male and female, aged 35–65) diagnosed by oncologists at Al-Ramadi Hospital, Ramadi, Iraq. All patients underwent surgical resection and received four cycles of chemotherapy with oxaliplatin (85 mg every 21 days) and capecitabine (6 grams daily for 21 days). Additionally, 40 age- and sex-matched individuals served as the control group. For each participant, CXCR4 and SDF-1 levels were measured using ELISA and the gene expression of *CTLA-4* and *PD-1* were measured using RT-PCR. The colon cancer patient group showed significantly lower levels of CXCR4 and SDF-1 compared to control groups (0.163±0.012 vs 0.376±0.025 pg/mL and 0.376±0.025 vs 0.699±0.110 pg/mL, respectively, both had p=0.001). Moreover, the colon cancer patient group had significantly lower expression of *PD-1* and *CTLA4* compared to control group (0.102±0.029-fold vs 1.199±0.391-fold, p=0.02; and 0.302±0.140-fold vs 1.441±0.334-fold, p=0.008, respectively). In conclusion, the results suggest that CXCR4 and SDF-1 appear promising as diagnostic markers for distinguishing colon cancer patients from healthy conditions.

Keywords: CXCR4, SDF-1, PD-1, CTLA4, colon cancer

Introduction

Colon neoplasia is one of the major malignancies in industrialized countries due to their Western-style food habits, of which more than 50% of the population develop adenomatous polyps by the age of 70 years, and 10% develop into cancer [1,2]. Colon cancer is the fourth most common cancer in the world and occupies the second position regarding cancer-related death [3]. Cytokines, important in many types of cancers including colon cancer, regulate proliferation, cell survival, differentiation, immune cell activation, cell migration, and cell death [4]. C-X-C chemokine receptor type 4 (CXCR4) is a molecular receptor located on the cell surface and is one of the most important receptors that play a crucial role in various biological processes [5,6]. The

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primary function of CXCR4 is related to its interaction with the cytokine stromal-derived factor 1 (SDF-1). This interaction significantly influences cell movement within tissues and organs [7,8].

CXCR4 is responsible for guiding immune cells to sites that require an immune response, making it a vital component of the immune system capable of detecting diseases and infections [9]. The CXCR4-SDF-1 axis is exploited by cancer cells. SDF-1 exists in two isoforms, SDF-1α and SDF-1β and these isoforms are involved in different aspects of colon cancer biology. SDF-1α is more strongly associated with promoting tumor cell proliferation; while SDF-1β is linked to metastasis and angiogenesis [10,11]. The interplay between these isoforms adds complexity to the role of SDF-1 in colon cancer progression. SDF-1 acts as a potent chemoattractant for colon cancer cells expressing CXCR4 receptors [12,13]. CTLA-4, also known as CD152, is a critical immune checkpoint receptor expressed on the surface of T cells and some regulatory T cells (Tregs). It plays a fundamental role in regulating the immune system to maintain a balance between defending the body against pathogens and preventing excessive immune reactions that could damage healthy tissues [6]. PD-1, is a cell surface receptor protein expressed on certain immune cells, particularly T cells, and its primary function is to regulate the immune response to maintain a balance between defending the body against pathogens and preventing excessive immune reactions that could harm healthy tissues [4].

The CXCR4-SDF-1 axis plays a significant role in colon cancer metastasis [14]. CXCR4, a chemokine receptor, binds to its ligand SDF-1, which is often expressed in distant organs. This interaction directs the migration of colon cancer cells to these SDF-1-rich sites, promoting metastasis, particularly to the liver and lungs [15]. The CXCR4-SDF-1 axis also supports tumor cell survival by activating downstream signaling pathways such as PI3K/AKT and MAPK/ERK [13]. These pathways contribute to cell proliferation, anti-apoptotic signaling, and resistance to conventional therapies [16,17]. Another important role of this axis is promoting angiogenesis, which is essential for tumor growth [18]. SDF-1 recruits endothelial progenitor cells to the tumor site, facilitating the formation of new blood vessels that supply nutrients and oxygen to the growing tumor [18]. The CXCR4-SDF-1 axis can modulate the immune microenvironment by influencing the expression of *PD-1* and *CTLA-4* [19]. For instance, CXCR4 signaling may enhance the recruitment of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) to the tumor microenvironment, both of which express high levels of *PD-1* and *CTLA-4*, contributing to immune suppression [19]. The interaction between the CXCR4-SDF-1 axis and immune checkpoints may also contribute to resistance against *PD-1* and *CTLA-4* blockade therapies. Tumors with high CXCR4 expression may create an immune-suppressive environment that diminishes the effectiveness of these inhibitors, making the tumor more resilient to immune attacks [20].

Oxaliplatin is an FDA-approved platinum-based antineoplastic medication. It is indicated in the adjunctive treatment of Stage III colorectal cancer after resection of the primary tumor and for the treatment of metastatic colorectal cancer. It is FDA-approved in combination with infusion 5-fluorouracil and leucovorin in a regimen known as Folfox. Oxaliplatin's mechanism of action primarily involves DNA damage through DNA crosslinking, particularly intrastrand and interstrand crosslinking [1,21]. However, due to the structure of oxaliplatin, its adducts make the binding of mismatch repair proteins to DNA harder compared to cisplatin or carboplatin's adducts, resulting in greater cytotoxic effects [22,11]. Capecitabine is an oral chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers [23,8]. Capecitabine is a prodrug that is enzymatically converted to fluorouracil (antimetabolite) in the tumor, where it inhibits DNA synthesis and slows the growth of tumor tissue. Some types of chemotherapy, such as oxaliplatin, can inhibit the secretion of some inflammatory interleukins in the body [24-29]. When the secretion of these interleukins is inhibited, it can reduce cellular stimulation and decrease inflammation-induced irritation in tumor tissue [3,8].

The interaction of PD-1 with its binding proteins such as PD-L1, present on the surface of tumor cells and immune cells, is responsible for inhibiting the immune cell response against tumors [5]. This interaction inhibits the activity of immune cells and reduces their ability to target and destroy cancer cells [7]. CTLA-4 interacts with CD80/CD86, receptors found on antigen-presenting cells, suppressing the immune cell response at an early stage of activation and preventing an effective immune cell response against the tumor [10]. Therefore, the hypothesis

was that the presence of CXCR4 and SDF-1 in tumors may attract immune cells to the tumor site, but their response is inhibited by CTLA-4 and PD-1 interactions, which contributes to the formation of an ineffective immune environment toward tumors and reduces the immune system's ability to attack. The aim of this study was to evaluate the serum levels of CXCR-4/SDF-1, CTLA-4 and PD-1 in postoperative colon cancer patients undergoing treatment with chemotherapy (oxaliplatin and capecitabine). The goal was to deeply understand the pathological mechanisms in colon cancer patients who have undergone surgical resection and chemotherapy, as well as to evaluate the effectiveness of the chemotherapy.

Methods

Human subjects

A pilot study was conducted from October 15, 2022, to February 1, 2023. The study included 90 peripheral blood samples: 50 from postoperative patients under treatment with chemotherapy (oxaliplatin and capecitabine) in four chemotherapy cycles, with each dose lasting 21 days. Each dose included 84 capecitabine tablets (6 mg tablets per day) and oxaliplatin (85 mg every 21 days). All patients underwent surgical resection and were diagnosed by oncologist physicians using a helix tester or colonoscopy at the Oncology Center in Al-Ramadi Hospital, Ramadi, Iraq, with ages ranging from 35–65 years. In addition, 40 clinically healthy individuals were included as controls. Inclusion criteria were colon cancer patients between the ages of 35 and 65 years and non-smokers, while exclusion criteria were having another cancer, chronic disease, autoimmune disease, and those who consume alcohol.

Blood sample collection

Venous blood specimen (5 mL) was withdrawn from each patient in the morning before taking chemotherapy, while sample collection in healthy control was also conducted in the morning. The blood (3 mL) was centrifuged at 3000 rpm for 10 min. Serum samples were then transferred to Eppendorf tubes and frozen at -20°C until further use. The remaining blood specimen (2 mL) was used to extract the mRNA to carry out the molecular assay using RT-PCR.

Immunological investigation

The levels of CXCR4 and SDF-1 in sera were measured using sandwich enzyme-linked immune-sorbent assay (ELISA) with Human CXC-chemokine receptor 4 (CXCR4) ELISA Kit and Human Stromal cell-derived factor 1 (SDF-1) ELISA kit, respectively (both from YL Biotech, Shanghai, China). The assay employed the double antibody sandwich method and the procedure was conducted following the manufacturer's recommendation. The optical density (OD) was measured and the levels of the CXCR4 and SDF-1 were determined using the standard curve that had been established by the manufacturer.

RNA extraction, primers used in this study and quantitative real-time PCR

Total RNA was isolated from whole blood samples using the TRIzol Reagent protocol (Thermo Fisher Scientific, California, USA) according to the manufacturer's instructions. This method relies on a monophasic chloroform extraction to separate RNA from cellular debris, DNA, and proteins. Following isolation, the quality and quantity of the extracted RNA were assessed using a Quantus Fluorometer (Promega, Madison, USA). The primers used in this study (**Table 1**) were designed and produced (Macrogen, Seoul, South Korea) and 10 pmol/mL was used to obtain the working primer solution. The One Step RT-PCR kit (Promega, Madison, USA) was utilized in this study. For a single reaction, 1 µL of the template and 9 µL of the Master mix were used. The amplification was run for 40 cycles with preliminary denaturation at 95°C for 5 mins, denaturation at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and extension of the primers at 72°C for 20 seconds. Gene expression levels were determined using the Livak Method [30] using a housekeeping gene (β-globin).

Table 1. Primers used in this study

Primer name	Sequence 5`–3`	Annealing temperature (°C)
β-globin-F	ACACAACTGTGTTCACTAGC	60
β-globin-R	CAACTTCATCCACGTTCACC	60
PD1-F	TAGAGAAGTTTCAGGGAAGG	60
PD1-R	ATGTGTAAAGGTGGAGGG	60
CTLA4-F	ACGGGACTCTACATCTGCAAGG	60
CTLA4-R	GGAGGAAGTCAGAATCTGGGCA	60

Statistical analysis

Data analysis was conducted using SPSS version 29 (IBM, New York, USA). Student's t-test or ANOVA was used to test the mean differences between groups, while Chi-squared test or Fisher exact test was used to assess the percentage differences between groups. A significance level of 0.05 or less was considered statistically significant. To handle multiple comparisons, corrections such as Bonferroni were used to reduce the possibility of type I errors. To address overlapping variables, regression models were used to include influential variables. Pearson correlation was used to measure associations between quantitative variables, with r values indicating strength (0.3–0.5 weak; 0.5–0.7 moderate; >0.7 strong). R-squared (r^2) quantified the variation explained by one variable on another (e.g., r=0.58; r^2=0.34 means 34% variation). Receiver operating characteristics (ROC) were used to evaluate the accuracy of diagnostic tools and to calculate sensitivity, specificity, and overall accuracy values. The area under the curve (AUC) indicated test accuracy (0.9 "Perfect"; 0.8 "Good"; 0.7 "Fair"; 0.6 "Poor"; <0.6 "Failure"). Sensitivity, specificity, false negatives, false positives, predictive values, and accuracy rates were calculated.

Results

Clinical characteristics of postoperative treated patients

The clinical characteristics of the patients within patient group are presented in **Table 2**. The majority of patients were over 50 years old (70%), with the remaining 30% under 50. Male patients accounted for 64% while females represented 36%. A positive family history of the disease was reported in 80% of the cases, with only 20% having no family history. All patients had a disease duration of more than one year (100%), and none were smokers. Regarding cancer grade, 54% of patients were Grade II, followed by 34% in Grade III and 12% in Grade I. Majority of the patients (76%) were in Stage III, while 24% were in Stage II (**Table 2**).

Table 2. Clinical characteristics of patients included in the study

Clinical characteristics	Frequency (%)
Age (year)	
<50	15 (30)
>50	35 (70)
Sex	
Male	32 (64)
Female	18 (36)
Family history	
No	10 (20)
Yes	40 (80)
Disease duration	
>1 year	50 (100)
<1 year	0 (0)
Smoking	
No	50 (100)
Yes	0 (0)
Colon cancer grade	
Grade I	6 (12)
Grade II	27 (54)
Grade III	17 (34)
Colon cancer stage	
Stage II	12 (24)
Stage III	38 (76)

Comparison of CXCR4 and SDF-1 levels between patient and control groups

The levels of CXCR4 and SDF-1 in both the colon cancer and the control group are presented in **Table 3**. The mean of CXCR4 levels were significantly lower in the colon cancer group compared to the control group (0.163±0.012 vs 0.376±0.025 pg/mL, $p<0.001$). Similarly, the levels of SDF-1 were also significantly lower in the colon cancer group compared to the control group (0.293±0.021 vs 0.699±0.110, $p=0.001$) (**Table 3**).

Table 3. Comparison of serum level of immunological parameters (CXCR4 and SDF-1) between colon cancer and control groups

Immunological parameters	Colon cancer group	Control group	*p*-value
CXCR4 (pg/mL)	0.163±0.012	0.376±0.025	<0.001
SDF-1 (pg/mL)	0.293±0.021	0.699±0.110	0.001

Comparison of CXCR4 and SDF-1 levels between patient and control groups according to age, sex, cancer grades, and stages

The comparisons of serum levels in patients and controls according to age are presented in **Table 4**. There were no significant differences between the two age groups (≤50 vs >50 years) in Colon cancer group for both CXCR4 ($p=0.470$) and SDF-1 ($p=0.700$). In contrast, there were significant differences between the two age groups in the control group for CXCR4 ($p<0.001$) and SDF-1 levels ($p=0.001$) (**Table 4**). The results showed no significant differences in levels of CXCR4 and SDF-1 between sexes in the colon cancer group. In contrast, the levels of CXCR4 and SDF-1 were significantly different based on sex within the control group (**Table 4**). There were no significant differences in CXCR4 and SDF-1 levels between colon cancer and control groups based on cancer grade and stage (**Table 4**).

Table 4. Comparisons of CXCR4 and SDF-1 levels across age, sex, colon cancer grades, and stages

Characteristics	CXCR4 (pg/mL)	*p*-value	SDF-1 (pg/mL)	*p*-value
Age group (year)				
Patient		0.470		0.700
≤50	0.144±0.019		0.255±0.035	
>50	0.172±0.016		0.312±0.026	
Control		<0.001		0.001
≤50	0.347±0.035		0.798±0.262	
>50	0.396±0.036		0.634±0.061	
Sex				
Patient		0.800		0.600
Male	0.159±0.016		0.267±0.025	
Female	0.169±0.020		0.340±0.038	
Control		<0.001		<0.001
Male	0.371±0.031		0.587±0.048	
Female	0.387±0.046		0.933±0.322	
Colon cancer grade		0.300		0.340
Grade I	0.196±0.045		0.378±0.097	
Grade II	0.170±0.015		0.277±0.029	
Grade III	0.140±0.022		0.289±0.025	
Colon cancer stage		0.600		0.630
Stage II	0.166±0.015		0.299±0.026	
Stage III	0.151±0.020		0.275±0.034	

Comparison of *PD-1* and *CTLA4* gene expression between colon cancer and control groups

Comparison of *PD-1* and *CTLA4* gene expression between the patient and control groups are presented in **Table 5**. The data indicated that there was a significant decrease in *PD-1* gene expression in patients compared to the control group ($p=0.020$). In contrast, a significant increase in *CTLA4* gene expression was observed in the colon cancer group compared to the control group ($p=0.008$).

Table 5. Comparisons of *PD-1* and *CTLA4* gene expression between colon cancer and control groups

Gene	Colon cancer group fold Mean±SD	Control group fold Mean±SD	*p*-value
PD-1 gene expression	0.102±0.029	1.199±0.391	0.020
CTLA-4 gene expression	1.441±0.334	0.302±0.140	0.008

Comparison of *PD-1* and *CTLA4* gene expression between patient and control groups according to age, sex, cancer grades, and stages

Our data indicated that no significant differences were found between the two age groups in colon cancer patients for gene expression of *PD-1* (p=0.900) and *CTLA4* (p=0.500) (**Table 6**). However, significant differences were observed between the two age groups in the control group for *PD-1* (p<0.01) and *CTLA4* (p<0.001) (**Table 6**). The expressions of *PD-1* gene were not significantly different based on sex and cancer grade among colon cancer patients. However, *PD-1* gene expression was significantly different based on cancer stage, of which *PD-1* expression was significantly higher in Stage II (0.123±0.038) compared to Stage III (0.040±0.011, p=0.050) (**Table 6**). The expressions of *CTLA-4* gene were significantly different only based on cancer grade, of which the level was significantly higher in Grade I (1.439±1.321) compared to other grades (p=0.010) (**Table 6**).

Table 6. Comparison of *PD-1* and *CTLA-4* gene expression across age, sex, colon cancer grades, and stages

Characteristics	*PD-1* expression	*p*-value	*CTLA-4* expression	*p*-value
Age group (year)				
Patient		0.900		0.500
≤50	0.100±0.056		0.126±0.053	
>50	0.103±0.036		0.377±0.197	
Control		0.001		0.001
≤50	0.851±0.509		1.019±0.428	
>50	1.720±0.590		2.076±0.395	
Sex		0.700		0.360
Male	0.135±0.042		0.414±0.210	
Female	0.041±0.016		0.093±0.041	
Colon cancer grade		0.230		0.010
Grade I	0.249±0.128		1.439±1.321	
Grade II	0.097±0.036		0.250±0.089	
Grade II	0.068±0.048		0.058±0.018	
Colon cancer stage		0.050		0.500
Stage II	0.123±0.038		0.357±0.184	
Stage III	0.040±0.011		0.137±0.063	

Correlation between the cytokines and inhibitory molecules in patients and control groups

The correlations between all parameters are presented in **Table 7**. CXCR4 exhibited a significant but weak correlation with SDF-1 (r=0.477; p=0.001) and also weak but significant correlations with PD-1 and CTLA4 (r=0.422; p=0.020 and r=0.418; p=0.022, respectively). SDF-1 had a significantly strong correlation with PD-1 (r=0.864; p=0.033) but no correlation with CTLA4. A moderate correlation was observed between PD-1 and CTLA4 (r=0.734; p=0.001).

Table 7. Correlations between cytokines and inhibitory molecules

Parameter		CXCR4	SDF-1	PD-1	CTLA-4
CXCR4	Pearson correlation	1			
	p-value				
SDF-1	Pearson correlation	0.477**	1		
	p-value	0.001			
PD-1	Pearson correlation	0.422*	0.864*	1	
	p-value	0.020	0.033		
CTLA-4	Pearson correlation	0.418*	0.193	0.734**	1
	p-value	0.022	0.307	0.001	

r: Pearson correlation; *r*: 0.1–0.4 weak correlation; 0.5–0.6 medium correlation; 0.7–1 strong correlation
*Statistically significant at p<0.05
**Statistically significant at p<0.01

Discussion

The microenvironment contains a variety of cytokines that act as growth and survival factors in premalignant cells. These cytokines sustain tumor-promoting inflammation, stimulate angiogenesis, and contribute to tumor progression and metastasis [27]. This understanding is crucial for studying biological interactions in cancer and chemotherapy. CXCR4, a key receptor regulating cellular migration and growth, plays a significant role in tumor metastasis. A study showed that CXCR4 levels were significantly lower in colon cancer patients [31], indicating that chemotherapy may affect the secretion of this receptor, reducing its ability to stimulate cancer cell migration and spread. Another study also supported these findings and showed that lower CXCR4 levels were associated with an improvement in patients' response to chemotherapy and better survival outcomes [8]. The study indicated that low CXCR4 could be a good indicator of response to chemotherapy. Lower levels of the SDF-1 chemokine receptor in colon cancer patients could have important implications for the development and spread of cancer. SDF-1 plays an important role in regulating cell migration and tumor growth, and changes in its levels may influence tumor progression and treatment response. Decreased levels of the chemokine receptor CXCR4 in colon cancer patients may be important. A study [32] demonstrated significantly lower SDF-1 levels in colon cancer patients, linking this reduction to decreased cancer cell migration and reduced spread of tumors. Another study [33] found that SDF-1 levels increased in colon cancer patients after chemotherapy, attributing this rise to complex inflammatory reactions and cellular interactions following treatment.

Chemotherapy may decrease CXCR4 and SDF-1 levels in colon cancer patients, with several studies explaining these potential mechanisms. Chemotherapy can directly destroy cancer cells, particularly those with high CXCR4 expression. As the number of tumors decreases, the overall CXCR4 expression within the tumor may also decrease. This reduction can potentially lower tumor invasiveness and metastatic potential by diminishing CXCR4-mediated signaling [34]. Another suggestion is that while chemotherapy can induce hypoxia, the effective reduction of the tumor burden may alleviate hypoxia within the tumor. As the tumor shrinks and oxygenation improves, the hypoxia-driven upregulation of CXCR4 may be reduced, leading to lower CXCR4 expression levels [35]. Additionally, certain chemotherapeutic agents may inhibit signaling pathways that regulate CXCR4 expression. For example, drugs targeting the PI3K/AKT or MAPK/ERK pathways, which are involved in CXCR4 regulation, could indirectly reduce CXCR4 levels by downregulating these pathways [36].

Chemotherapy may also induce senescence or apoptosis in stromal cells that produce SDF-1. As these cells become less active or die, the overall production of SDF-1 in the tumor microenvironment may decrease, leading to reduced stimulation of CXCR4 on cancer cells [37]. Furthermore, chemotherapy can modulate the immune system by depleting immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which are often associated with high CXCR4 expression. By reducing these cell populations, chemotherapy may lower the overall levels of CXCR4 in the tumor microenvironment. Additionally, reducing these immunosuppressive cells may decrease SDF-1 production, as they can contribute to its secretion [38].

The significant decrease in *PD-1* and *CTLA4* gene expression in patients compared to the control group may reflect the body's response to chemotherapy. Several possible reasons could explain this phenomenon, including chemotherapy's effect on genes which may alter the expression of genes involved in the immune response, such as *PD-1* and *CTLA4*. Chemotherapy may reduce *PD-1* and *CTLA4* expression as part of its immunosuppressive effect, aiming to kill cancer cells and diminish the immunosuppression that enables the tumor to evade the immune system [39]. Chemotherapy may stimulate a more effective immune response against the tumor, reducing the need for expression of *PD-1* and *CTLA4* expression, which function as a negative regulator of the immune response. This reduction could be part of the body's attempt to combat the tumor more effectively after treatment. Additionally, a decrease in *PD-1* and *CTLA4* gene expression may reflect immune regulation aimed at enhancing the recognition and elimination of cancer cells, as *PD-1* and *CTLA4* are immune regulatory molecule that inhibits the T-cell response and reduce the immune response against cancer cells [27].

A study [40] investigated the expression of *PD-1* and its associated ligands in the tumor immune environment, indicating that *PD-1* expression can change in response to immunotherapies. Another study [33] focused on the safety and immunological activities associated with anti-*PD-1* antibodies in cancer, revealing that in some cases, there was no significant change in *PD-1* expression. A study [25] reviewed the biological role of *CTLA-4* and its use in tumor immunotherapy, explaining how changes in *CTLA-4* expression can impact the immune response. It supports the notion that changes in *CTLA-4* expression could be part of the body's response to immunotherapy, which might resemble the effects of chemotherapy. In contrast, a study [41] that focused on the use of ipilimumab (anti-*CTLA-4*) in treating melanoma did not find significant changes in *CTLA-4* expression after treatment.

As for age, CXCR4/SDF-1 serum levels recorded a steep decline in patients under the age of 50. There was no significant difference in serum levels of CXCR4/SDF-1 between males and females, though a significant difference was observed between patients and the control group. This finding suggests that age may have limited or no effect on these biomarkers of disease. A previous study [42] found no significant differences between two different age groups of colon cancer patients receiving chemotherapy compared to control groups, while another study [30] observed no significant differences in CXCR4 levels between chemotherapy-treated colon cancer patients of different age groups and healthy individuals. Similarly, a study [41] found no significant differences in *SDF-1* levels between chemotherapy-treated colon cancer patients and healthy individuals.

Regarding *PD-1* and *CTLA4* gene expression, the results showed no significant differences between patients under 50 and those over 50. However, a decline in *PD-1* and *CTLA4* gene expression was observed in patients compared to the control group, except in patients under 50. The lack of significant differences in *PD-1* and *CTLA-4* gene expression between patients under and over 50 could be explained by several factors, including the equal effect of chemotherapy (oxaliplatin and capecitabine). The immune response associated with *PD-1* and *CTLA-4* might be similarly regulated across age groups. Additionally, the regulation of gene expression in colon cancer and chemotherapy may be complex and not strongly influenced by age. The impact of the disease and chemotherapy itself may outweigh the effect of age [9].

A study [32] examined the effect of blocking *PD-1* on stressed T cells in HIV patients, showing that the immunological effects of *PD-1* blockade were not significantly age-dependent. This aligns with the current study's findings, suggesting that *PD-1*-associated immune responses may not be significantly influenced by age. Similarly, a study [24] reviewed the use of blocking immune checkpoints such as *PD-1* and *CTLA-4* in cancer treatment, demonstrating that these treatments work through mechanisms not related to age. In contrast, another study [43] explored strategies by which cancer evades the immune system and explained how the immune response can vary depending on age and general health status.

In terms of gender, it should be noted that the decline in SDF-1 serum levels was more pronounced in female patients compared to male patients. This may be attributed to differences in hormonal composition, which play a key role in the variation of immune responses between males and females. These differences could also influence the response to chemotherapy. However, there was no significant difference in the levels of CXCR4 and SDF-1 between male and female colon cancer patients who received chemotherapy after surgery, compared to healthy subjects. Chemotherapy may exert a similar effect on all patients, regardless of sex [44].

Other variable factors, such as dietary habits, body type, and environmental influences, may also affect CXCR4 and SDF-1 levels between the sexes but have not been sufficiently studied. The lack of significant differences between the sexes in CXCR4 and SDF-1 levels may result from a balance between these differing effects or from the small sample size. These findings emphasize the importance of considering multiple factors when evaluating the impact of sex on treatment response and cytokine levels in cancer patients.

While no significant differences were observed between the sexes, there was a significant difference between patients and the control group for both males and females. It should also be noted that female patients showed a greater decline in *CTLA4* gene expression. The study found no significant differences in the gene expression of *PD-1* and *CTLA-4* between male and female colon cancer patients receiving chemotherapy after surgery, which may be explained by the

reasons mentioned earlier. The genes responsible for *PD-1* and *CTLA-4* expression may be regulated in similar ways between males and females, resulting in comparable gene expression in response to chemotherapy. Chemotherapy induces cellular stress, which could trigger a similar immune response in both sexes, leading to comparable levels of inhibitory molecule expression [29]. A study [42] examined the effectiveness of immunotherapies that inhibit immune checkpoints such as *PD-1* and *CTLA-4* in different types of cancer, noting that treatment response differences do not significantly depend on sex.

In terms of tumor grade (I, II, III) for CXCR4, the lowest level was observed within Grade III. SDF-1 recorded a sharp decline in CXCR4 serum level in Grade II. No significant differences were detected in the levels of CXCR4 and SDF-1 between postoperative colon cancer patients undergoing treatment with chemotherapy regardless of the grade of their tumor. One possible reason for this is variation in individual responses. There can be significant variation in how individuals respond to chemotherapy based on individual genetic and environmental factors. This variability could mask any significant differences in CXCR4 and SDF-1 levels between patient groups [1]. Also, there were no significant differences in *PD-1* gene expression among disease grades (I, II, III), although a sharp decline was within Grade III, and *CTLA4* recorded the same result. No significant differences were found in the expression of *PD-1* between patients with Grades II and III compared to those with Grade I disease. Although tumor grade may be important in determining how patients respond to chemotherapy [32], it is not the only influencing factor. Other factors, such as a person's genetic interactions and the environmental conditions of the tumor, may play a larger role in this response. In addition, the sample size is small, and there may be significant variations in patients' responses to treatment even among those with the same grade of tumor [45]. As for *CTLA-4* gene expression level, significant differences were found between patients with Grades II and III compared to those with Grade I. This suggests that there is variability in patients' responses to chemotherapy and its effect on the level of *CTLA-4* gene expression. One reason for this difference is that Grades II and III tumors may be more advanced, resulting in a greater immune reaction or stimulated immune system. This enhanced immune reaction can lead to an increase in the expression level of the *CTLA-4* gene [41]. In addition, chemotherapy can affect the tumor environment in a way that leads to changes in the expression level of the *CTLA-4* gene [5].

As for the disease stages (II and III), the lowest serum level was observed in Stage III for both CXCR4 and SDF-1. Determining the stage of the tumor in colon cancer is an important factor. The results of the study did not show statistically significant differences in the levels of CXCR4 and SDF-1 between colon cancer patients divided according to tumor Stages II and III. One possible reason for the lack of significant differences between patients is the effect of chemotherapy, which may lead to uniformity of chemokine receptor levels across different tumor stages [8]. Chemotherapy may have a similar effect on inflammatory pathways and immune response, regardless of tumor stage. Finally, according to disease stage, *PD-1* showed a significant decline in Stage III compared to Stage II. Also, *CTLA4* recorded a high decrease within Stage III. Research suggests that multiple factors influence patients' response to chemotherapy, not just tumor grade. These factors include individual gene interactions and genetic factors, which may lead to the fact that there may not be significant differences in *PD-1* gene expression between different patient groups based on tumor stage [46]. Significant differences were observed between postoperative colon cancer patients in Stages II and III *PD-1* expression levels. Depending on the tumor stage, the carcinoma affects the local immune activity of T cells in the tumor environment. High-grade tumors involve higher levels of selective pressure on anti-tumor T cells, leading to higher *PD-1* gene expression as a compensatory response. In contrast, low-stage tumors may allow for greater anti-tumor T-cell activity, leading to lower levels of *PD-1* expression [29].

In addition, chemotherapy with oxaliplatin and capecitabine may induce changes in the tumor's cellular environment, which are reflected in the expression levels of the *PD-1* gene. These drugs may stimulate an anti-tumor immune response, leading to elevated *PD-1* expression as a negative regulatory mechanism. The interaction between the tumor stage and the immunomodulatory effects of chemotherapy is a key factor in determining *PD-1* gene expression levels in these patients. This complex interaction may account for the observed differences in *PD-*

1 gene expression across different patient groups [28]. Meanwhile, no statistically significant differences were observed in *CTLA-4* gene expression levels among the patients

The effect of tumor stage on *CTLA-4* expression is less clear or less consistent than expected. Although previous research has indicated that advanced tumor stages are associated with higher *CTLA-4* expression, this relationship is not always linear or conclusive. There may be other factors, such as additional genes or the cellular environment may influence *CTLA-4* regulation in more complex ways [45]. The effect of chemotherapy (oxaliplatin and capecitabine) on *CTLA-4* expression also varies between patients. Some individuals may respond with increased *CTLA-4* expression while others do not show this effect. This difference in individual response may result from genetic or other biological differences between patients [47], such as small sample size or the patient's immune status, which can play a role in influencing *CTLA-4* expression in complex ways. These factors unrelated to the tumor stage, may mask or obscure the direct effect of the stage on *CTLA-4* expression [48]. According to these findings, further research is needed to translate these findings into clinical applications such as conducting long-term follow-up studies to evaluate the durability of responses to CXCR4 and SDF-1 targeted therapies, as well as potential long-term risks, such as the development of resistance or secondary malignancies. Investigating the timing and sequencing of CXCR4-targeted therapies in relation to other treatments, such as chemotherapy, radiotherapy, and surgery, to maximize patient outcomes. Additionally, investigating the underlying mechanisms by which CXCR4 and SDF-1 contribute to chemotherapy resistance, immune evasion, and metastasis in colon cancer could involve preclinical studies using cell lines, organoids, and animal models.

Conclusion

According to the present result, we revealed a significant decrease in the serum levels of the inflammatory cytokine SDF-1 and CXCR4 in colon cancer patients compared to the control group, suggesting that they could be a potential therapeutic target. On the other hand, there was an increase in the level of gene expression for *CTLA4* in patients compared to healthy people and a decrease in the level of gene expression for *PD-1* in patients compared to healthy people. There is a relationship linking the CXCR4-SDF-1 axis with the inhibitory molecules of *PD-1* and *CTLA4*. The study contributes valuable insights into the complex interactions between inflammatory cytokines and inhibitory molecules in Iraqi colon cancer patients in Anbar Governorate. To further investigate the diagnostic and therapeutic potential of CXCR4 and SDF-1 in colon cancer, several clinical trials and experimental studies could be suggested. These include evaluating the efficacy of CXCR4 inhibitors (e.g., AMD3100/Plerixafor) in combination with standard chemotherapy (e.g., FOLFOX) in patients with metastatic colon cancer, investigating the potential of CXCR4 and SDF-1 as biomarkers for early detection of metastasis in colon cancer patients, and assessing the therapeutic potential of neutralizing SDF-1 with monoclonal antibodies (e.g., NOX-A12) in colon cancer. A notable limitation of this study is the sample size of the colon cancer group. While colon cancer is a recognized condition, a larger patient cohort would strengthen the generalizability of the findings. Furthermore, the underlying causes and optimal treatment strategies for colon cancer remain elusive. Future research with larger and more diverse patient populations is warranted to solidify the observed associations between the CXCR4-SDF-1 axis and inhibitory molecules (*CTLA4* and *PD-1*) in colon cancer and to explore their potential as therapeutic targets.

Competing interests

All the authors declare that there are no conflicts of interest.

Ethical approval

The study protocol received approval from the Ethics Committee of the Iraqi Ministry of Health, specifically the Oncology Center in Al-Ramadi Hospital, Ramadi, Iraq, and it was carried out under the principles outlined in the Declaration of Helsinki according to no. 7729.dated 25/11/2022. Before taking part in the study, all participants provided written informed consent after receiving comprehensive information about the purpose, nature, and potential risks and benefits of the research. Participants were given the assurance that they had the right to withdraw

from the study at any time without facing any negative consequences. The study process strictly maintained the confidentiality of participant information, and data were made anonymous for analysis. Each participant was required to complete a detailed consent to participate declaration form before being included in the study.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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