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The role of *CCR5* polymorphism in colorectal cancer and liver metastasis in the Tunisian population

Marwa Weslati[1,2], Rahma Boughriba[3], Donia Ounissi[4], Meriam Hazgui[5] and Sonia Marghali[1,*]

[1]*Laboratory of Molecular Genetics Immunology and Biotechnology (LR99ES12), Faculty of Sciences of Tunis, University of Tunis El Manar*, Tunis, Tunisia

[2]*Colorectal Cancer Research Laboratory UR12SP14, Mongi Slim Hospital*, La Marsa, Tunisia

[3]*Laboratory of Genetics Immunology and Human Pathology (LR05ES05), Faculty of Sciences of Tunis, University of Tunis El Manar*, Tunis, Tunisia

[4]*Laboratory of Neurophysiology, Cellular Physiopathology and Biomolecule Valorization (LR18ES03), Faculty of Sciences of Tunis, University of Tunis El Manar*, Tunis, Tunisia

[5]*Laboratory of Mycology, Pathologies and Biomarkers (LR16ES05), Faculty of Sciences of Tunis, University of Tunis El Manar*, Tunis, Tunisia

***Corresponding author**: sonia.marghali@fst.utm.tn

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Abstract: Chemokines and their receptors are involved in cancer initiation and progression, including colorectal cancer (CRC) and liver metastasis formation. Our aim was to elucidate C-C chemokine receptor type 5 (*CCR5*) gene polymorphism (*CCR5Δ32*) impact on CRC and colorectal cancer liver metastases (CRLM) occurrence risk. We analyzed the *CCR5* gene mutational status in 108 primary CRC cases, 35 CRLM and 248 healthy individuals, and evaluated CCR5 expression in healthy tissue and tumors. Rare allele "Δ32" was more frequent in controls (7.2% vs 2.8% in CRC). All 35 metastases had wild-type *CCR5*. Our analysis showed that *CCR5* wild type has a significant risk of 2.73-fold (95% CI=1.22-7.31) to cause CRC while Δ32 reduced the risks 0.36-fold (95% CI=0.13-0.82). For CRC, *CCR5* correlated with left-sided tumors and liver metastases (P=0.040 and P= 0.039 respectively). As for CRLM, no correlation was found. Immunohistochemical profile analysis of CCR5 revealed a significant association with the male gender (P=0.049) and non-mucinous carcinomas (P< 0.001) in primary CRC. CCR5 expression revealed an association with the degree of tumor differentiation for both CRC and CRLM (P < 0.001). *CCR5Δ32* might be a protective factor against CRC development and dissemination.

Keywords: *CCR5Δ32*; colorectal cancer; liver metastasis; chemokines; Tunisian cohort

INTRODUCTION

CRC is the third most common cancer in the world and second in terms of mortality; it is responsible for 935000 deaths and 1.9 million new cases in 2020, and is growing steadily, affecting both genders [1]. Numerous deaths caused by solid malignant tumors are due to disseminated metastases in secondary organs. In this context, 40-50 % of patients with CRC mainly develop liver metastasis, less frequently in the lungs and more rarely in the brain [2,3]. The cancer process depends on a series of acquired or transmitted genetic alterations targeting oncogenes, tumor suppressor and repair genes [4]. Furthermore, the persistence of an initiated tumor cell, its *in situ* proliferation and metastatic dissemination requires its escape from the antitumor immunity surveillance system [5].

The CRC carcinogenesis model follows a multistep sequence, and the interactions between tumor cells and stromal components (especially immune cells) represent a very promising area of research to improve its prognosis and establish more effective personalized therapies [6].The protumor microenvironment is developed when the mesenchymal, immune cells and their precursors are recruited to the primary and/or secondary tumor site and undergo a phenotypic change to promote tumor growth and migration.

In 2001, the involvement of chemokines in organ-specific homing of breast cancer cells was highlighted for the first time [7]. A number of secreted cytokines/chemokines and receptors have been reported to be involved in the tumor-stroma interactions, particularly lymphocyte-mediated recruitment such as tumor-associated macrophage (TAM) to the tumor stromal microenvironment and tumor promotion [8,9]. In addition, it has been shown that many cancers, including CRC, are characterized by abnormal production of chemokines and/or aberrant expression of their receptors as a result of mutational events. This imbalance stimulates growth factor release to promote angiogenesis and tumor cell adhesion, migration and invasion [10,11].

Chemokines are diverse, selective and complex ligands. To date, over 50 chemokines and 18 receptors have been identified. Several ligands, also known as CC chemokine ligands (CCL), including CCL3, CCL4, CCL5, CCL8 but also CCL7 and CCL13, can bind to receptor CCR5 [12]. This receptor's mRNA was found in many human tissues, and it has been shown to be strongly expressed in the spleen and thymus, weakly in the ovaries and lungs, while at an intermediate rate in peripheral blood and small intestine; it can also be found in the epithelium, endothelium, fibroblasts, leukocytes, peripheral blood mononuclear cells and specifically on macrophages and peripheral T lymphocytes [13].

A major consequence of the role of coreceptors in human immunodeficiency virus (HIV) infection was the discovery of a mutant *CCR5* (rs333) that has a "32 base pair" (bp) deletion and results in the formation of a truncated protein at the third extracellular domain, disabling it from migrating to the cell surface and leading to CCR5 receptor deficiency [13]. In 2008, an HIV-positive patient became HIV-negative after transplantation of bone marrow cells from a homozygous donor for "Δ32"', and has since had an undetectable viral load [13]. Among chemokine receptors, CCR5 and its ligands have been the subject of many studies concerning different types of neoplasms, such as pancreatic, lung, colorectal, breast, ovarian, and prostate cancers, given its involvement in promoting tumor growth and metastasis [14-16].

Studies examining *CCR5Δ32* mutational status in CRC and CRLM are few and some are inconclusive [17-19]. Furthermore, an *in vitro* study revealed that *CCR5* knock out (KO) mice (*CCR5*$^{-/-}$) have slow-growing local tumors and a better response to vaccines against cancer [20]. The same was observed by other researchers, where they suggest the use of CCR5 antagonists as an anti-CRC treatment to dampen tumor progression and metastases formation [21-23]. Targeting the immune system through chemokines and chemokine receptors represents a strong approach in establishing more specific and personalized cancer therapies [6,24].

The aim of this study was to elucidate the impact of Δ32 polymorphism in primary and metastatic sporadic colorectal cancer patients. Our work is one of the few assessments to compare this mutation in both primary and secondary organs, CRC and CRLM, respectively, while focusing on the mutational status of *CCR5* rather than on the effects of blocking or repressing receptor activity using different chemicals.

MATERIALS AND METHODS

Patients and tumor features

This study protocol was approved by the Ethics Committee of Mongi Slim Hospital, La Marsa, Tunisia. We performed a retrospective analysis of 108 patients with primary CRC and 35 liver metastases. The control group comprised 248 randomly selected healthy individuals. The patients' collected clinicopathological features included age, sex, histological type, tumor location, differentiation and tumor invasion.

DNA extraction

Genomic DNA was extracted from frozen and/or paraffin-embedded tissue blocks using the PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. As regards the 248 healthy blood donors, genomic DNA was extracted from peripheral blood using the Wizard Genomic Purification Kit (Promega, Madison, WI, USA). The concentration of DNA samples was assessed using the Qubit dsDNA HS (high sensitivity) Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

Polymerase chain reaction amplification

Tumor DNA was amplified by the polymerase chain reaction (PCR) using the following primers for *CCR5Δ32* as follows: F: CTGTGTTTGCGTCTCTCCCA, R: CCTCTTCTTCTCATTTCGACA. PCR amplification was performed in a total volume of 25 µL of PCR mixture containing: 100 ng of each patient's DNA, H_2O, 10x PCR buffer (Promega, Madison, WI, USA), 10 µM of dNTPs (PureLink, Invitrogen, CA, USA), 50 µM of $MgCl_2$ (Promega, Madison, WI, USA), 1 µM of each primer (Invitrogen, Carlsbad, CA, USA) and 1 U of Taq DNA polymerase (Biomatik, Wilmington, De, USA). The PCR program was as follows: 94 °C for 3 min, and 33 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s, and a final extension step at 72°C for 5 min.

Evaluation of CCR5Δ32 amplification product by on-chip electrophoresis

DNA 1000 Chip (Agilent Technologies, Santa Clara, CA, USA) were loaded with samples as recommended by the manufacturer. Once the appropriate well was filled with the gel-dye mix, a 1-mL syringe was used to apply pressure. Next, a marker solution and DNA ladder were added. One µL of the PCR products was added to the 12 sample wells. After vortexing, the chip was placed in an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Fragment analysis was carried out using Agilent software. The 222-bp band represented the wild allele. If the band at 190 bp was visible, the cases were considered mutated and therefore carried the Δ32 deletion.

CCR5 immunohistochemistry

From formalin-fixed paraffin-embedded tissue for both primary CRC and liver metastasis, sections of 5-µm thickness were cut, deparaffinized and rehydrated. Antigen retrieval was performed using citrate buffer (pH = 9) (Cell Marque, Rocklin, CA, USA) at decreasing temperatures. All the samples were then exposed to peroxidase block (Novolink Polymer Detection Kit; Leica Biosystems, Newcastle, UK) for 5 min, then incubated overnight at 4°C with primary antibody to CCR5 (1:50, Monoclonal Mouse IgG2B clone, R&D Systems, Minneapolis, MN, USA). After washing with Tris washing buffer, the sections were incubated with a post-primary block for 30 min, a streptavidin enzyme complex for 30 min, diaminobenzidine as a chromogen for 10 min and hematoxylin counterstain for 5 min.

Statistical analysis

Statistical analysis was performed using SPSS software V20 (SPSS, Inc, Chicago, IL, USA). Associations between *CCR5* gene status and the different clinicopathological variables were assessed using the chi-square ($\chi 2$) test. The odds ratio (OR) was obtained by logistic regression analysis. A significance value of $P<0.05$ was considered statistically significant.

RESULTS

Patients and Tumor features

In primary tumors, the sex ratio was 1.16, with 50 women and 58 men, and the mean age was 61.02 years ranging from 17 to 94 years. For CRLM patients, male predominance was noted with a sex ratio of 1.69 (22 men and 13 women), and the overall mean age was 56.5 years, ranging from 26 to 77 years.

Histologically, we identified 28 tumors in the colon and 80 in the rectum for sporadic CRC cases. As regards CRC liver metastases, 15 had primary colon cancer and 20 originated from the rectum. For primary CRC, non-mucinous carcinoma (NMC) represented 73% and mucinous carcinoma (MC) 27% of cases. Regarding metastases, 74% were NMC and 26% were MC (Fig. 1, Table 1, Table 3).

Tumor grading was done according to the WHO criteria (World Health Organization Classification of Tumors of the Digestive System, 4th Edition) [25]. CRC samples were divided into 68 well-differentiated, 29 moderately differentiated and 11 poorly differentiated cases. As regards the colorectal liver metastases, we had 10 well-differentiated, 19 moderately differentiated and 6 poorly differentiated cases (Fig. 1, Table 1, Table 3). Tumor pathological classification was conducted according to the international TNM staging system based on the eighth edition of the American Joint Committee on Cancer (AJCC, 8th edition) [26]. This was conducted on 108 specimens taken from primary tumors and

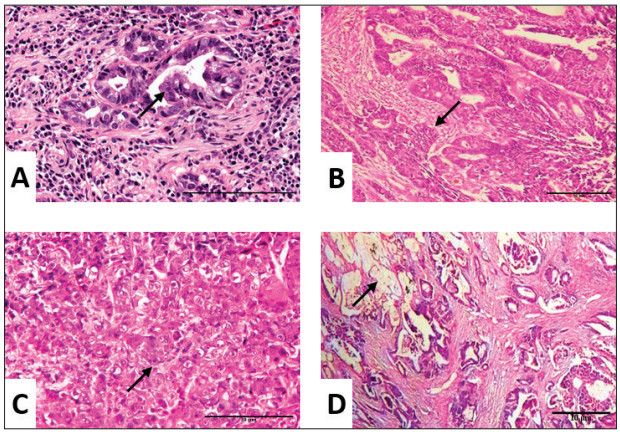

Fig. 1. Micrographs of colorectal carcinoma formalin-fixed and paraffin-embedded tissues counterstained with hematoxylin and eosin (scale bar=10 μm). Well-differentiated carcinoma where glands are surrounded by a dense fibrous stroma (**A**); moderately differentiated carcinoma with irregular confluent glands in a fibrous stroma (**B**); poorly differentiated carcinoma with no identifiable glands, only cancer cells presence (**C**); mucinous carcinoma where glands are surrounded by mucus (**D**). A magnification ×400; B and C magnification ×250; D magnification ×100.

35 from metastases, distributed into 60 CRC cases in primary stages (stages I and II), 48 CRC cases and 35 CRLM in advanced stages (stages III and IV).

CCR5Δ32 frequency

CCR5 gene analysis by PCR was performed on 108 CRC then compared with 248 control cases, showing that 96.2% (104/108) were homozygous for the wild allele (222 bp), 1.9% (2/108) were heterozygous (222 bp + 190 bp) and 1.9% (2/108) carried the Δ32 allele in the homozygous state (190 bp). For the control group, PCR analysis revealed that 86.3% (214/248) had the *CCR5* gene in its wild form, 12.9% (32/248) were heterozygous and 0.8% (2/248) were homozygous (Table 2). However, all liver metastasis cases had a wild homozygous genotype (222 bp). In CRC cases, the statistical analysis of the molecular results revealed correlation with tumor location (P=0.040) and metastases (P=0.039), with 7.1% of the heterozygous genotype detected in the right colon and none on the left side. As for the metastases, only 1% of heterozygous patients did not develop CRLM compared to patients who did (14.3%) (Table 1), and since all CRLM patients had a wild type *CCR5*, no correlation was found with the different clinicopathological parameters.

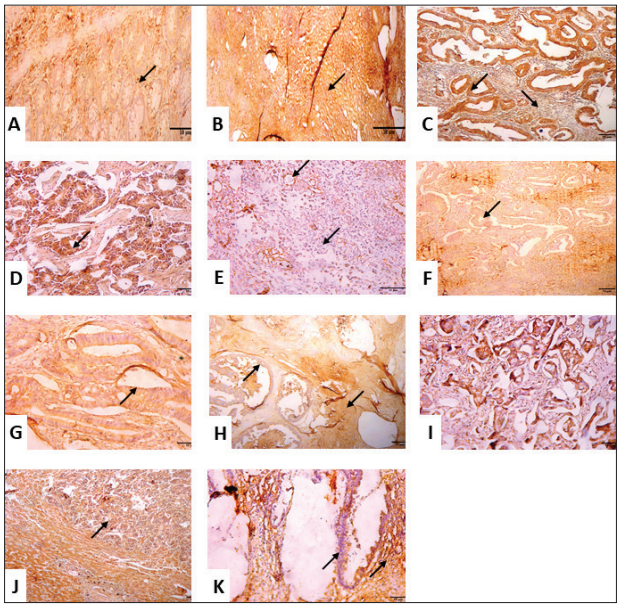

Fig. 2. Immunohistochemical analysis of CCR5-staining cells in CRC and CRLM tissues (scale bar=10 μm). Cytoplasmic moderate to weak positivity with anti-CCR5 in the glandular structures and the lamina propria of healthy colorectal tissue (**A**); intense positivity in healthy liver tissue (**B**); intense membrane-cytoplasmic staining of cancerous cells with an attenuated signal at the stroma in well-differentiated CRC (**C**); moderate cytoplasmic positivity in moderately differentiated CRC (**D**); weak to absent tumor cell staining, with vascular basal membrane positivity in poorly differentiated CRC (**E**); exclusive cytoplasmic staining for the (Δ32/Δ32) homozygous CRC patients (**F**); discontinuous membrane staining for the heterozygous patients (wt/Δ32) with moderate to intense cytoplasmic CCR5 expression (**G**); intense membranous and stromal positive staining with anti-CCR5 in well-differentiated CRLM (**H**); moderate cytoplasmic positivity in moderately differentiated CRLM (**I**); moderate to poor positivity in poorly differentiated CRLM (**J**); heterogeneous and discontinuous positivity of glandular structures, with negativity in the mucus and positivity in the fibroinflammatory stroma with anti- CCR5 in mucinous carcinoma (**K**). A and B magnification ×100; C, F and H-K magnification ×250; D, E and G magnification ×400.

Analysis of the odds ratio for CRC development showed that *CCR5* wild type had a significant risk of 2.73-fold (95% CI=1.22-7.31) to cause CRC, while the mutated gene Δ32 reduced risks 0.36-fold (95% CI=0.13-0.82) (Table 2).

CCR5 immunohistochemistry expression

To study the CCR5 expression pattern, immunohistochemistry (IHC) was performed on all 108 cases of primary CRC and 35 CRC liver metastases. IHC slides were assessed by two pathologists with no

Table 1. Clinicopathological features of primary colorectal cancer in function of CCR5 allelic distribution and IHC expression.

Clinicopathological features	CCR5 Genotype				CCR5 IHC Expression			
	wt/wt	wt/Δ32	Δ32/Δ32	P value	Intense	Moderated	Poor	P value
	n=104	n=2	n=2		n=69	n=27	n=12	
	(%)	(%)	(%)		(%)	(%)	(%)	
Age (years)								
≥60 (n=64)	63 (98.4)	0 (0)	1 (1.6)	0.217	46 (71.9)	13 (20.3)	5 (7.8)	0.106
<60 (n=44)	41 (93.2)	2 (4.5)	1 (2.3)		23 (52.3)	14 (13.8)	7 (15.9)	
Sex								
Female (n=50)	48 (96)	1 (2)	1 (2)	0.989	27 (54)	18 (36)	5 (10)	**0.049**
Male (n=58)	56 (35.7)	1 (1.7)	1 (1.7)		42 (72.4)	9 (15.5)	7 (12.1)	
Tumor location								
Right colon (n=28)	26 (92.9)	2 (7.1)	0 (0)		18 (64.3)	7 (25)	3 (10.7)	0.997
Left colon (n=80)	78 (97.5)	0 (0)	2 (2.5)	**0.04**	51 (63.7)	20 (25)	9 (11.2)	
Histological type								
NMC (n=75)	71 (94.7)	2 (2.7)	2 (2.7)	0.401	53 (70.7)	21 (28)	1 (1.3)	**< 0.001**
MC (n=33)	33 (100)	0 (0)	0 (0)		16 (48.5)	6 (18.2)	11 (33.3)	
Differentiation								
Well (n=68)	64 (94.1)	2 (2.9)	2 (2.9)		68 (100)	0 (0)	0 (0)	
Moderate (n=29)	29 (100)	0 (0)	0 (0)	0.655	1 (3.4)	27 (93.1)	1 (3.4)	**< 0.001**
Poor (n=11)	11 (100)	0 (0)	0 (0)		0 (0)	0 (0)	11 (100)	
Stages								
I, II (n=11)	10 (90.9)	0 (0)	1 (9.1)	0.155	7 (63.6)	4 (36.4)	0 (0)	0.369
II, IV (n=97)	94 (96.9)	2 (2.1)	1 (1)		62 (63.9)	23 (23.7)	12 (12.4)	
Lymph node metastasis								
No (n=61)	58 (95.1)	1 (1.6)	2 (3.3)	0.450	39 (63.9)	15 (24.6)	7 (11.5)	0.987
Yes (n=47)	46 (97.9)	1 (2.1)	0 (0)		30 (63.8)	12 (25.5)	5 (10.6)	
Liver metastasis								
No (n=101)	98 (97)	1 (1)	2 (2)	**0.039**	63 (62.4)	26 (25.7)	12 (12.9)	0.42
Yes (n=7)	6 (85.7)	1 (14.3)	0 (0)		6 (85.7)	0 (0)	1 (14.3)	

wt/wt refers to *CCR5* wild type genotype.
wt/Δ32 refers to *CCR5* heterozygote genotype.
Δ32/Δ32 refers to *CCR5* mutated homozygous genotype.
OR-odds ratio; CI-confidence interval

Table 2. *CCR5* genotype and Δ32 frequencies in CRC patients and the control group.

Genotype/ Allele	Controls n (%)	Patients n (%)	P value	OR (95% CI)
wt/wt	214 (86.3)	104 (96.2)	-	1.00 (Reference)
wt/Δ32	32 (12.9)	2 (1.9)	**0.005**	0.13 [0.02-0.43]
Δ32/Δ32	2 (0.8)	2 (1.9)	0.473	2.06 [0.24-17.34]
Δ32	36/496 (7.2)	6/216 (2.8)	**0.024**	0.36 [0.13-0.82]

wt/wt refers to *CCR5* wild type genotype.
wt/Δ32 refers to *CCR5* heterozygote genotype.
Δ32/Δ32 refers to *CCR5* mutated homozygous genotype.
OR-odds ratio; CI-confidence interval

knowledge of either the patients' clinical or pathological characteristics. Based on the staining intensity, the tumors were graded into three groups as follows: 1 – strong with membrane-cytoplasmic staining; 2 – intermediate; 3 – weak. The expression intensity and distribution depended on the histological type: mucinous carcinoma (MC) versus non-mucinous (NMC), the differentiation degree and *CCR5* mutational status in colorectal and liver healthy tissue; cytoplasmic expression with stromal distribution was observed in fibroblasts, lymphocytes and in the liver parenchyma (Fig. 2A and B).

For CRC cases harboring wild *CCR5*, the membrane signal disappeared, and the cytoplasmic intensity decreased based on the decreasing differentiation degree, ranging from well-differentiated (Fig. 2C) to moderate (Fig. 2D) and poorly differentiated (Fig. 2E). IHC staining for patients carrying the Δ32 allele showed an exclusive cytoplasmic expression for the homozygous ones (Δ32/Δ32) since the truncated receptor does not reach the membrane (Fig. 2F).

A discontinuous membrane staining was observed in the two heterozygous patients (wt/Δ32) with moderate to intense cytoplasmic signal (Fig. 2G). Similarly, the liver metastasis cases showed the same profile as the CRC samples with wild-form receptor as the signal, and cytoplasmic intensity varied depending on the differentiation degree from well (Fig. 2H), to moderate (Fig. 2I), and poorly differentiated (Fig. 2J). Regarding MC for both CRC and LM specimens, CCR5 expression was cytoplasmic, moderate to poor, affecting enterocytes while absent in the mucus-secreting cells (Fig. 2K). Note that those samples had wild-type *CCR5* gene.

Statistical analysis of the CCR5 IHC profile displayed a significant association with male gender (P=0.049) and non-mucinous carcinomas (P<0.001) in primary CRC. It also revealed an association with tumor differentiation degree for both CRC and CRLM (P<0.001) (Table 1, Table 3).

Table 3. Clinicopathological features of liver metastases and CCR5 expression

Clinicopathological features	CCR5 IHC Expression			
	Intense n=10 (%)	Moderate n=19 (%)	Poor n=6 (%)	P-value
Age (years)				
≥60 (n=16)	6 (37.5)	6 (37.5)	4 (25)	0.181
<60 (n=19)	4 (21.1)	13 (68.4)	2 (10.5)	
Sex				
Female (n=13)	4 (30.8)	8 (61.5)	1 (7.7)	0.519
Male (n=22)	6 (23.7)	11 (50)	5 (22.7)	
Tumor location				
Right colon (n=16)	4 (25)	9 (56.2)	3 (18.8)	0.906
Left colon (n=19)	6 (31.6)	10 (52.6)	3 (15.8)	
Histological type				
NMC (n=26)	8 (30.8)	13 (50)	5 (19.2)	0.68
MC (n=9)	2 (22.2)	6 (66.7)	1 (11.1)	
Differentiation				
Well (n=10)	10 (100)	0 (0)	0 (0)	
Moderate (n=19)	0 (0)	19 (100)	0 (0)	**≤ 0.001**
Poor (n=6)	0 (0)	0 (0)	6 (100)	

IHC – Immunohistochemistry

DISCUSSION

Colorectal carcinogenesis implicates different immune cells and effectors such chemokines and their receptors in mediating the recruitment of lymphocytes and especially TAM in the tumor stromal microenvironment [8,9], and can be characterized by the production of abnormal chemokines and/or aberrant expression of their receptors following mutational events, so that understanding their involvement in tumor growth and metastatic progression helps establish more effective therapies [24]. In this context, our study of the Tunisian cohort aimed to shed light on the impact of *CCR5*Δ32 deletion on CRC development and local/distant progression and its repercussions on chemokine activities (CCL3, CCL4 and CCL5).

In its wild form, CCR5 is expressed by diverse adenocarcinoma and immune system cells and its activation by a specific chemokine can trigger different signaling pathways [27]. Cancer cell-derived chemokines appear to have contradictory roles by either promoting or suppressing cancer progression, depending on the infiltrating cell type and immune effect potency [28-30]. In fact, several reports associate CCR5/CCL5 intermediate or strong expression to extended CRC patient survival as well as tumor regression [31,32], but other studies show that this chemokine receptor significantly contributes to tumor growth through its main actors CCL5, CCL4 and CCL3 [11,16,19,29], and that neutralization of this receptor's chemokines can reduce tumor invasion in colorectal [21,23], breast [33,34] and lung cancers [35,36]. For CRC treatment, CCR5 is widely studied, given its involvement in cancer progression and metastasis, and is a frequently inhibited using specific molecules to hinder the interaction between mesenchymal stem cells (MSC)/CRC cells and to effectively treat CRC advanced stages [37,38].

The current study assessed the frequency of Δ32 deletion in 108 patients with primary CRC and 35 CRC liver metastases, compared with 248 controls. The Δ32 allele was found in 7.2% of the control subjects, compared to 2.8% in CRC patients. Our frequencies are close to those of Aoki et al. in breast cancer, where Δ32 frequency was 7.77% in controls versus 3.47% in patients [39]. Given the rarity of such a mutational event, many studies did not reveal any association with the clinicopathological features, such as CRC meta-analyses where frequencies of the mutated allele in studied cases displayed no correlation [17,18]. In this context, our study pinpoints correlations with both left-sided CRC and metastases. Based on the literature, reports hypothesize

an equal distribution regarding location; one study described a lower percentage of rectal cancers [31]. As for liver metastases, our molecular study reports the absence of the deletion in all 35 cases. However, one study reported that in spite of the limited cohort size and concurrency of Δ32, heterozygote patients had a lower frequency of synchronous metastasis than those harboring wild-type *CCR5* [19].

Genetic profile may affect the expression and/or function of both chemokines and their receptors, causing heterogeneous and often controversial preliminary results depending on the primary cancer site [40-49]. In this context, the impact and the results can be conflicting even in the same type of neoplasms. Some studies indicate that Δ32 polymorphism is associated with cancer risk, such as prostate [45], breast [49,50] and gallbladder cancers [51]; however, other studies have shown that there is no significant association between the mutation and cancer risk in prostate [43,52], breast [39,46,47] and lung cancers [48]. In our study, we report that mutation Δ32 reduced the risks of CRC 0.36-fold (95% CI=0.13-0.82).

The second part of our work focused on CCR5 immunohistochemical expression. In healthy tissue, staining was low to moderate and exclusively cytoplasmic, involving glandular cells, fibroblasts, lymphocytes, and liver parenchyma. Regarding CRC tissue, the expression was observed in both stroma and cancerous cells. Our statistical study highlights a significant association between intense expression and male gender (P=0.049), non-mucinous histological type (P<0.001) and differentiation where the intensity depended on the degree of decreasing differentiation ranging from well to moderately and poorly differentiated cases (P<0.001). Furthermore, we show that CCR5 immunoexpression also varies depending on mutational status.

In cases where *CCR5* was truncated, we found a profile similar to that reported in the literature, with an exclusively cytoplasmic expression of the receptor in homozygous patients (Δ32/Δ32). Moreover, a weak and discontinuous membranous staining was observed in the two heterozygous patients (wt/Δ32) with a moderate to intense cytoplasmic signal. Even though no correlation is described in the literature regarding CCR5 expression, gender and NM carcinoma, our results are similar to those describing the discontinuous signal when the gene is mutated [19,53].

In CRLM, the staining was intense and membranous-cytoplasmic in 100% of the well-differentiated cases, moderate in 100% of the moderately differentiated and weak in all the poorly differentiated ones (P<0.001). Suarez-Carmona et al. reported a globally heightened expression compared to primary tumors along with a "patchy" distribution of the signal [19].

Regarding mucinous adenocarcinomas, the protein exclusively exhibits cytoplasmic staining, depending on the tumor cells, whether they are enterocytes or mucus-secreting. Indeed, purely mucus-secreting and independent cells show no positivity, contrasting with positive stromal staining due to its fibro-inflammatory composition.

Not much data are reported for this histological CRC type due to the lack of expression of chemokine receptor in this subtype.

Reports about CCR5 have been conflicting. Many highlight a significant correlation with both CD8 + T-lymphocytes infiltrating the tumor margins and the absence of metastases, and its expression is considered an independent favorable prognostic factor [31]. Moreover, high CCL5 levels, being major contributors in CTL chemoattraction, is an indicator of prolonged survival [32].

Several studies similar to ours and in different cancer types (colorectal, breast, lung) have demonstrated that overexpression of a functional CCR5 is heavily involved in chemotaxis and immune cells' recruitment to the tumor dissemination site, highlighting a chemokine-receptors protumor effect [22,53]. They demonstrate through *in vitro* or *in vivo* experiments that targeting CCR5 via siRNA (second RNA interference) or an FDA (Food and Drug Administration)-approved antagonist (maraviroc), hinders the receptor's biological activity and can be an effective therapy for treating CRC and liver metastasis [21,23,38]. The chemokine-receptor complex molecular interactions seem to be in favor of the immune system and consequently tumor cell elimination [30,31], but CCL5 [16,23], CCL3 [10,15,29] and CCL4 [11,14,15] overexpression, by different cells as well as tumors cells in the tumor microenvironment, exhibit protumor properties [30].

Thus, with its paradoxical features, non-mutated CCR5 signaling prompts cancer cells' homing, helping them to survive by increasing their DNA repair and boosting the proinflammatory prometastatic immune phenotype [16,53]. All the protumor properties could be canceled or reversed in the case of Δ32 presence. Indeed, a dysfunctional/repressed CCR5 receptor would decrease macrophage recruitment during tumor development, thereby hindering the angiogenesis process, tumor growth and tumor migration, and is therefore detrimental to neoplasm development.

CONCLUSION

In conclusion, CCR5 has been a target in many cancer studies, given its complex implication in both antitumor immunity and various carcinogenesis processes. However, reports are sometimes conflicting, especially when it comes to alterations affecting its biological activity. In this context, our results suggest that *CCR5Δ32* may be a protective factor against colorectal cancer development and liver metastasis formation. Moreover, immune-mediated cancer treatments are very promising therapeutic approaches, especially for patients with late-stage and metastatic cancer, where the common procedures such as surgery, radiation therapy and chemotherapy mostly fail to provide long-term benefit. This also emphasizes the need to carry out studies targeting, in addition to CCR5, its chemokines such CCL5, CCL4 and CCL3, their effectors and their targets, to help develop more effective and less invasive therapies.

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Author contribution: MW collected the data/samples, conducted the analysis and wrote the manuscript. RB performed the statistical analysis and interpretation. DO and MH were involved in performing the experiments and revising the manuscript. SM provided overall direction and final revision.

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