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Original Article

Evaluating the Association between *CCR5delta32* Polymorphism (rs333) and the Risk of Breast Cancer in a Cohort of Iranian Population

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Abstract

Background: CC chemokine receptor 5 (*CCR5*) is introduced as an immune response modulator. The activity of CCR5 influences breast tumour development in a p53-dependent manner. This study aimed to investigate the frequency of *CCR5delta32* and its association with the risk of breast cancer in 1038 blood samples in North East of Iran.

Methods: In this case-control study, we genotyped 570 control samples and 468 breast cancer patients by a gel electrophoresis-based gap-polymerase chain reaction (gap-PCR) method Mashhad, Iran. The data were analyzed using the SPSS software.

Results: Of 570 controls included, 542 (95.09%) had *CCR5delta32* $^{wild/wild\ (W/W)}$ genotype, 28 samples (4.91%) had *CCR5delta32* $^{wild/deletion\ (W/D)}$ genotype and none of them were *CCR5delta32* $^{deletion/deletion\ (D/D)}$ genotype (0%). While 428 samples of patients (91.45%) had *CCR5delta32* $^{W/W}$ genotype, 40 samples (8.55%) had *CCR5delta32* $^{W/D}$ and *CCR5delta32* $^{D/D}$ homozygous was nil (0%) amongst cases. All samples were in the Hardy–Weinberg equilibrium ($P>0.05$). According to the allele frequency, D allele, as a risky allele, in the cases was more than the control samples (0.0427 vs 0.0245, respectively) (P=0.0206). Hence, W/D genotype may confer a risk effect (OR=1.77, CI: 1.09-2.90; P=0.0206) compared with WW genotype between case and control groups.

Conclusion: There is a statistically significant association between *CCR5W/D* and breast cancer risk. CCR5 may be regarded as a target for the prevention of breast cancer in certain conditions such as interaction with p53 variants, which remains to be further investigated.

Keywords: *CCR5D32*; p53 pathway; Breast carcinoma; Immunogenetics; Metastasis

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Introduction

One of the most prevalent cancers in women is breast cancer, which increases the mortality rate in women worldwide (1, 2). Breast cancer is introduced as the most frequent malignancy in Iranian women (3, 4). Based on the previous studies, genetic and environmental factors are complicated in the risk as well as the expansion of breast cancer (5-9). A functional correlation exists between cancer and inflammation receptors. The inflammatory responses to the breast cancer tumor site proliferation and development of cancer cells are also involved in metastatic breast cancer (10, 11). In addition, inflammatory factors such as chemokine-like chemokine receptor (CCR5), which affect the chemotactic factors for inflammatory cells, have important roles in leukocyte trafficking, chemotaxis, angiogenesis, lymphocyte development, inflammatory processes, tumor development and metastasis (12).

Chemokines are small soluble molecules secreted by cells, which are greatly well-known for their ability to influence cancer cell development *via* inflammation and to motivate cellular migration, mostly by leukocyte recruitment in inflammation (13, 14). Moreover, different types of tumour cells express chemokine and chemokine receptors (15). In addition, high levels of CCR5 are great co-receptors for infection of macrophages and T lymphocytes via the macrophage-tropic strains of HIV-1; which their expression has been identified in cancer tissues. The CCR5 may be notably involved in the metastasis and proliferation of various cancers like breast cancer (16, 17).

Inadequate and potent *CCR5* expression is especially related to the non-metastatic progression of breast cancer. In addition, the local production of the Chemokine (C-C motif) ligand 5 (CCL5) is also significant in the development of breast tumour and associated with a poor prognosis of breast cancer (16, 17). CCR5 also results in the growth of breast cancer stem cells. Overexpression of the CCR5 cells indicates reduced p-γH2AX-Histone H2AX phosphorylation proteins, which alarms DNA damage and consequently leads to the enhancement of DNA repair (18). The deficiency of *CCR5* affects the apoptotic cell death of melanoma via the prohibition of nuclear factor kappa light chain enhancer of activated B cells (NF-*κ*B) and upregulation of IL-1Ra (19). Moreover, the *CCR5* expression has an important role in the subpopulation of breast cancer cell lines and affects the functional response to CCL5.

Furthermore, oncogene transformation enforces the expression of CCR5, and the subpopulation of cells, which express functional CCR5 shows enhanced the invading characteristic of the cancer cells. The role of CCR5 in breast tumour cell growth (mostly in luminal breast cancer), suggests it affects the p53 of the MCF-7 cells. Moreover, *CCR5* blockade increases the proliferation of MCF-7 cell line in the attendance of p53 but does not affect the proliferation in the xenografts carrying a p53 mutation (20). Additionally, Vicriviroc or Maraviroc, known as the CCR5 antagonists, block the function of CCR5 HIV coreceptor and reduce metastasis of basal breast tumour, but does not affect the cell viability/proliferation in vitro. Maraviroc decreases pulmonary expansion of breast cancer *i*n vivo. Moreover, the antagonists of CCR5 can be used as an adjuvant combination therapy to decrease the possibility of metastatic breast cancer in patients with certain conditions (21). Therefore, prevention of CCR5 via an antagonist decreases the leukocyte influence and tumor growth and subsequently reduces the subcutaneous transfusion of the cells in vivo (22). In addition, a decrease in CCR5 acts as a suppressive receptor in cancer metastasis. The deficiency of *CCR5* increases the delay of tumor growth, and the inhibitors of CCR5 prevent a rise in the cancer cells in cancers like breast cancer (23, 24). *CCR5delta32* (*CCR5D32*) has been found in the *CCR5* gene, located on chromosome *3p21.3*. *CCR5D32* induces a strong resistance in homozygote persons against the HIV-1 infection. Moreover, studies have been done on the distribution of *CCR5D32* in various populations (25). The effect of *CCR5D32* poly-

morphism is dependent on the type of cancer; although, other studies have not reported the same association (26, 27).

This mutation was not present in Africans as well as most Asian people, but it was detectible in African-Americans, which the reason was explained as an ethnic combination (28, 29). The roles of the CCR5 receptor of chemokine CCL5 remain unclear in breast cancer progression and there is a controversy between articles. These variations might be due to the low statistical power, small sample size, clinical heterogeneity, ethnic difference, as well as the confounding effects that have not been taken into account.

In our study, we removed these limitations and tried to investigate the relation between *CCR5D32* and the risks of breast cancer with an appropriate sample size in an Iranian population.

Materials and Methods

Study population and blood collection

In this case–control study, 468 clinically confirmed sporadic breast cancer patients were recruited in Mashhad, Iran. After taking informed consent, 10 ml peripheral blood sample was collected from 1038 individuals (468 cases and the 570 controls). The healthy female individuals were selected among women in the North East of Iran.

The genomic DNA extraction and genotyping

The pure genomic DNA was extracted from 1038 blood samples by the salting-out technique protocol (30, 31). The PCR was performed to identify the genotype of the cases and healthy controls by primers, which flank the 32- base pairs (bp) deletion. The reaction volume (10 µl) was composed of 5-10 ng of the genomic DNA template, 0.1 µM of each primer, 3 µl Master mix PCR mixture (2X) include 1.5 mM MgCl and 0.5 U of Taq polymerase in 2x reaction buffer (from 0.5U/µl) and 4 µl distilled water in the total reaction of 10 µl. The 5' to 3' sequence of common forward and reverse primers were as the following; forward primer sequence was 5' AGG TCT TCA TTACAC CTG CAG C 3', and reverse primer was 5' CTT CTC ATT TCG ACACCG AAG C 3' (Table 1) (32). Genotypes were detected according to the final size of PCR products, in which 169 bp and 137 bp products were related to wild-type and *CCR5D32* genotypes, respectively (Fig. 1).

Table 1: Primer sequences that used in this study

Primers	*Sequence 5'-3'*	*Length*	*Products*		
			$CCR5^{W/W}$	*CCR5D32*	
				$CCR5^{D/D}$	$CCR5^{W/D}$
Forward	AGG TCT TCA TTACAC CTG CAG C	22 bp		137 bp	169 & 137 bp
Reverse	CTT CTC ATT TCG ACACCG AAG C	22 bp	169 bp		

Wild: W; Deletion: D; Bp: basepairs.

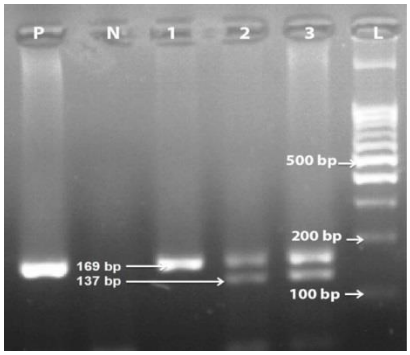

Fig. 1: The gel electrophoresis of PCR amplified DNA. Lane 1: wild type ($CCR5^{Wild/Wild}$); lanes 2 and 3: heterozygous genotype ($CCR5^{Wild/\Delta 32}$). **P**: Positive control sample; **N**: Negative control sample; **L**: 100 bp DNA size marker

The reaction was performed in an Applied Biosystems PCR (Life Technologies). Under the following thermal conditions including first denaturation at 95 °C for 10 min; 33 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and also 72 °C for 30 sec; and last elongation at 72 °C for 5 minutes. The electrophoresis of PCR products (5-7 µl) was done in 3% agarose gel (Invitrogen), staining with DNA Green Viewer (Pars Tous; Iran) (Consort, Germany), and then they were visualized under UV light using SYNGENE U: genius gel documentation system.

The collected information was analyzed using the SPSS statistics software ver. 12 (Chicago, IL, USA). In addition, differences in the allele and the genotype frequencies of CCR5D32 variant between cases and healthy samples were evaluated using gene count and also the $\chi 2$ test. Moreover, Odd ratios (OR) with 95% confidence interval (CI) using logistic regression test was calculated. In this study, for all analyses, the cut-off values of $P \leq 0.05$ were considered to be significant.

Ethical approval

Our study was conducted in accordance with the Declaration of Helsinki after being permitted via the local ethics committee of Mashhad University of Medical Sciences, Iran (Ethical code: IR.MUMS.fm.REC.1394.399). In this line, written informed consent has been received from all cases and controls.

Results

Demographic data of the patients and controls including age, living place, habits and situation, family history of cancer and clinical information were collected in a questionnaire (Table 2). Of 570 controls included, 542 (95.09%) had *CCR5D32* $^{wild/wild\ (W/W)}$ genotype, 28 samples (4.91%) had *CCR5D32* $^{wild/deletion\ (W/D)}$ genotype and none of them were *CCR5D32* $^{deletion/deletion\ (D/D)}$ genotype (0%). While 428 samples of patients (91.45%) had *CCR5D32* $^{W/W}$ genotype, 40 samples (8.55%) had *CCR5D32* $^{W/D}$ and *CCR5D32* $^{D/D}$ homozygous was nil (0%) amongst cases. All samples were in the Hardy–Weinberg equilibrium. According to the allele frequency, the frequency of D allele, as a risky allele, in the cases was more than the control samples (0.0427 vs 0.0245, respectively) (P=0.0206). Hence, W/D genotype risk effect was calculated as (OR=1.77, CI: 1.09-2.90; P=0.0206) compared with WW genotype (Table 3).

Table 2: Summary of clinic-pathological data and several demographic variables of the carcinoma patients and the control groups

Characteristics	*N*	*Cases*	*N*	*Controls*	*P-value*
Age	359	51.6±11.6	536	42.5±12	P<0.05*
BMI	320	27.4±5	513	25.3±4.4	P<0.05*
Height	321	159.5±6.8	516	161.3±5.9	P<0.05*
Weight	344	69.7±13.2	529	66.2±11.7	P<0.05*
Marital status	897				
Married		331 (92.2%)		461 (85.7%)	P<0.05*
Divorced		3 (0.8%)		1 (0.2%)	
Widowed		6 (1.7%)		5 (0.9%)	
Never-married		19 (5.3%)		71 (13.2%)	
Abortion	642				
Yes		117 (45.3%)		115 (29.9%)	P<0.05*
No		141 (54.7%)		269 (70.1%)	
The family history of cancer	870				
Yes		192 (57.1%)		371 (69.5%)	P<0.05*
No		144 (42.9%)		163 (30.5%)	
History of cancer	847				
Yes		23 (7.2%)		4 (0.8%)	P<0.05*
No		298 (92.8%)		522 (99.2)	

Table 3: Distribution of the *CCR5D32* genotypes and the allelic frequencies

Genotypes/Models/Alleles		*Breast carcinoma*	*Normal individuals*	*OR (CI 95%)*	*P-value*
		Number (%)	Number ()		
Genotypes	$CCR5^{W/W}$	428 (91.45)	542 (95.09)		
	$CCR5^{W/D}$	40 (8.55)	28 (4.91)	1.77 (1.09-2.90)	0.0206
	$CCR5^{D/D}$	0 (0)	0 (0)		
Alleles	W	896 (95.72)	1112 (97.54)		
	D	40 (4.28)	28 (2.46)	1.77 (1.09-2.90)	0.0206

Wild: W; Deletion: D.

Discussion

In our study, there was a significant association between heterozygous genotype (W/D) and breast cancer risk in Iranian breast cancer patients. Moreover, D allele, as a risky allele, in the cases was more than the control samples.

The relation between genetic and environmental factors is widely investigated. In recent years, immunogenetics have an essential role in the pathogenesis of breast cancer. CCR5 is the receptor for chemotactic chemokines macrophage inflammatory protein-1 alpha (MIP1-α), MIP1β, and RANTES (CCL3, CCL4, CCL5). In addition, the *CCR5D32* is located in the *CCR5* promoter, which encodes a non-functional receptor (33, 34). Moreover, *CCR5D32* allele has been shown to have a high frequency, among the different Caucasian populations (35, 36). A meta-analysis of an Indian population indicates that the *CCR5D32* was associated with the risk of breast cancer (37). On the other hand, the *CCR5D32* genotype has been correlated with better prognosis in patients of the Nijmegen discovery cohort with postmenopausal breast tumour (Nijmegen, Netherlands). The individuals, carrying the *CCR5D32* genotype, demonstrate a longer metastasis-free survival (MFS) in this population (38).

In agreement with our investigation, in a meta-analysis study, the *CCR5D32* was associated with the risk of breast cancer in the Indians (37). In the Netherlands population, the individuals carrying the *CCR5D32,* displayed a longer MFS in the postmenopausal subgroup of the initial cohort (38). In the Turkish population, the frequency of heterozygous genotypes was an independent risk factor for the breast cancer progression (39). In addition, another study reported a significant association between I/I and I/D alleles in the breast cancer patients with the *CCR5D32* mutation; carried out with 500 breast cancer patient samples in a Pakistani population (40). Similar to our study, heterozygous genotype (W/D) in the breast patients was more than the control samples in a Pakistani population (41).

Furthermore, there was a strong association between the expressions CXCR5 in the breast cancer tissues from the German population (42). On the other hand, *CCR5D32* was not connected to the risk of cancer (43). Moreover, in some studies, there was no significant correlation between this polymorphism and the risk of breast cancer such as in the North of India and Brazil (44-46).

CCR5 increases T cell responses to cancer cells *via* modulating the activation of helper-dependent $CD8^+$ T cell (47). Besides, CCR5 exerts main regulatory effects on the immunity mediated by $CD4^+$- and $CD8^+$ T cell. CCR5 has a particular, ligand-dependent role in improving anti-tumor responses. Efficient tumor rejection requires the expression of CCR5 *via* the $CD4^+$ and $CD8^+$ T cells (48). Furthermore, CCR5 was found to be mainly expressed in the $CD8^+$ T lymphocytes, and the frequency of $CCR5^+CD8^+$ Cytotoxic T lymphocytes (CTLs) was higher in tumor-infiltrating lymphocytes (TIL), compared with the human peripheral blood mononuclear cells (PBMC) (48).

The activation of CCR5 in the $CD4^+$ cells contributed to increasing the expression of CD40 ligand, resulting in antigen-presenting cells (APCs) maturation and improved $CD8^+$ T-cell

cross-priming as well as tumor infiltration. CCR5 leads to the reduction of chemical (3-methylcholanthrene)-induced fibrosarcoma growth and incidence in mice (47). The *CCR5D32* has been associated with the lower expression of CCR5 and higher CC-chemokine secretion levels of $CD4^+$ cells (49). In this respect and from the results of our study, the reduction in CCR5 leads to a decrease in the immune system defense against a tumour (especially a decrease in the T cell response).

Another explanation of our results may be related to the downstream pathways of the CCR5, as a protective factor for breast cancer. The activity of CCR5 regulates breast cancer development in a p53-dependent manner in MDA-MB-231 and MCF-7 cell lines (20). Mutation inactivates the p53 tumor suppressor gene in approximately half of all human tumors (50). Prevention of CCR5 expression leads to increase the proliferation of tumor cells in wild-type p53 without affecting the tumor cell proliferation in p53 mutation carriers in tumour xenograft models (20). In this line, the data from a breast cancer clinical study (547 patients in a cohort study) displayed that disease-free survival (DFS) was shorter in individuals carrying *CCR5D32* allele and expressed wild-type p53. The activity of CCR5 affects breast cancer development in a p53-dependent manner. All downstream pathways of *CCR5D/D* and *CCR5W/D* are abolished or severely impaired in patients with *CCR5D32*, respectively. Consequently, activation of p53 mediated by CCR5 is diminished. In this line, p53, as a tumor suppressor, may be silenced in the *CCR5D32* patients. Furthermore, two p38-dependent pathways, Gi and JAK2, help CCR5 to regulate p53 transcriptional activity. These p38-dependent pathways seem to be impaired/ silenced in the participants with the *CCR5D32* allele. Collectively, breast cancers with normal p53, grow quicker and relapse faster in the D32 carriers (20).

Conclusion

CCR5D32 may affect the risk of the breast cancer in our population. The potential use of CCL5/CCR5 may be a target for preventing breast cancer metastasis. In addition, it can be used as a marker of DNA damage/repair in the tumor cells. Thus, further functional studies are needed to investigate pathways related to the CCR5, p53 and T cell response influencing the risk of breast cancer.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of interest

The authors report no conflicts of interest.

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