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Research Article

Association of Chemokine and Chemokine Receptor Gene Polymorphis (MCP1 A-2518G and CCR2-V64I) with Urinary Bladder Cancer: A Study in Kashmiri Population

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Abstract

Epidemiological evidence points to a connection between inflammation and a predisposition for the development of cancer. Several pro-inflammatory gene products have been identified that lead to variations in the production and concentration of inflammatory proteins. A case-control study was conducted in chemokine and chemokine receptor genes (*MCP-1 A-2518*G and CCR2-V64I) to elucidate the possible role of these SNPs as risk factors in urinary bladder cancer (UBC) development. Using the polymerase chain reaction-restriction fragment length polymorphism approach, we investigated the genotype distribution of 120 bladder cancer patients in comparison with 155 cancer-free controls from the same geographical region. Significant differences were observed in the distribution of genotypes in MCP-1 A-2518G between the control and bladder cancer patients (0.36 vs 0.47; p=0.000). Homozygous variant GG frequency of *MCP-1 A-2518G* in cases was found to be 20% as against 5.1% in controls ($p<0.05$). No significant association was found in *CCR2-V64I* genotypes ($p = 0.09$) between the controls and patients with the frequency of homozygous variant (64I) being 15% and 20% (0.15 vs. 0.20) respectively. Interestingly, *CCR2-V64I* heterozygote (wt/64I+64I) frequencies were significantly increased in the higher grades/stages of bladder cancer patients ($p< 0.05$). Based on above mentioned results, we conclude that the *MCP-1 -2518A/G* polymorphism is associated with genetic susceptibility to the risk for bladder cancer.

Keywords: Chemokine; Heterozygote; CCR2-V64I; Polymorphism

1. Introduction

Urinary bladder cancer ranks ninth in worldwide cancer incidence. It is the seventh most common malignancy in men and the 17th most common in women (Ploeg et al., 2009). Bladder cancer ranks

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as the seventh leading cancer and accounts for 5.90% of all prevalent cancers in a Kashmiri population (Arshad et al., 2012). Bladder cancer has been associated classically with exogenous and environmental risk factors, which primarily include smoking and occupational exposure (Clavel et al., 2007; Clayson et al., 2008).

An association between the development of cancer and inflammation has long been appreciated (Balkwill et al., 2001). The inflammatory response orchestrates host defenses to microbial infection and mediates tissue repair and regeneration, which may occur due to infectious or non-infectious tissue damage. Epidemiological evidence points to a connection between inflammation and a predisposition for the development of cancer, i.e. long-term inflammation leads to the development of dysplasia. Epidemiological studies estimate that nearly 15 percent of worldwide cancer incidence is associated with microbial infection. Although smoking is a well-recognized risk factor for bladder cancer, direct and indirect evidence points to inflammation as another predisposing factor. The use of chronic indwelling catheters and chronic bladder inflammation are two factors associated with an increased bladder cancer risk (Groah et al., 2002). Furthermore, inflammatory infiltrates are common in the stroma of patients with bladder cancer but not in healthy control subjects (Cresswell et al., 2001). History of urinary tract infection significantly increases the risk of bladder cancer, especially in individuals who have three or more infections (Kantor et al., 1984). Chemokines are small, potent and inducible proinflammatory cytokines, which are implicated in many biological processes, such as migration of leukocytes, embryogenesis, angiogenesis, hematopoiesis, atherosclerosis, tumor growth and metastasis, and HIV-infection (Mackay et al., 1997; Luster et al., 1998; Yoshie et al., 2001; Struyf et al., 2003; Vandercappellen et al., 2008).Variations in individual inflammatory responses could explain the considerable variability in the clinical course of disease and therapy response among patients with tumors of similar grades and stages.

Chemokines are important in many biological events such as embryogenesis, wound healing, tumor growth, and angiogenesis. MCP-1(monocyte chemoattractant protein-1) CCL2 is a member of the CC chemokine subfamily characterized by the absence of an amino acid between conserved cysteines at the amino-terminal end of the molecule. Several MCP-1 polymorphisms have been studied in relation to disease susceptibility or severity. It is well known that genetic variants of genes have an impact on the products of genes, which are associated with different kinds of diseases. It has been shown that the polymorphism A2518G in the regulatory region of the MCP-1 gene influences MCP-1 expression in response to inflammatory stimuli (Rovin et al., 1999). MCP-1(CCL 2) is currently believed to mediate its actions through one receptor, CC chemokine receptor 2 (CCR2). CCR2 is a CC chemokine receptor that shows affinity for CCL2, CCL7, CCL8 and CCL13 ligands. CCR2 is mainly expressed by memory T lymphocytes, monocytes, dendritic cells, B cells and basophils. The CCR2-64I mutation (G→A substitution at position 190 in the CCR2 chemokine receptor gene) results in a valine→isoleucine substitution at position 64 in the CCR2 protein (Smith et al., 1997; Kostrikis et al., 1998).

In this study, a population-based case-control study was conducted to examine the distribution and/or association between the polymorphic variants of *MCP-1 A-2518G* and *CCR2-V64I* and bladder cancer in the Kashmiri population.

2. Materials and Methods

2.1 Sample Collection/ Storage

Peripheral blood was obtained from each subject and stored at −20°C till use. A histopathological report of the collected bladder tumor tissues corresponding to the blood samples of the patients was obtained from the Department of Pathology of the Sher-I-Kashmir Institute of Medical Sciences (SKIMS). After histopathological confirmation of bladder cancer tissues, their corresponding blood samples were then only used for genotype analysis by restriction fragment length polymorphism (RFLP).

2.2 Study & Subjects

120 peripheral blood samples from urinary bladder cancer patients were collected in the urology ward and related clinics in SKIMS between March 2008 and August 2011. A pool of 155 control subjects was recruited from the same hospital,each belonging to the same geographical area,the same ethnic background and an approximately similar age group. The mean age was 58.5 ± 12 years for the cases and 56.6 ± 11.6 years for the controls (p =0.52). The controls were healthy and , free from any cancer and were frequency-matched to the cases in terms of sex and smoking status.Written informed consent was obtained from each recruited subject and the study was approved by the local institutional ethical committee. Cases and controls went through a face-to-face interview during hospital admission using standard questionnaires.

2.3 DNA Extraction and Polymerase chain Reaction- Restriction Fragment Length Polymorphism

DNA was extracted from blood samples (n = 275, controls and cases) using standard proteinase-K digestion, phenol/chloroform extraction and ethanol precipitation methods. Previously reported primers (Szalai et al., 2001; Abdi et al., 2002) for the *CCR2-V64I* polymorphism, sense primer 5′-TTG GTT TTG TGG GCA ACA TGA TGG-3′ and antisense primer 5′-CAT TGC ATT CCC AAA GAC CCA CTC-3′ were used for the ampilification of the target regions of the polymorphism; for the MCP-1 A-2518G polymorphism, sense primer 5′-TCT CTC ACG CCA GCA CTG ACC-3′ and antisense primer 5′-GAG TGT TCA CAT AGG CTT CTG-3′ (Biotools, B & M Labs, Madrid, Spain) were used. PCR was carried out in a final volume of 25 ųL containing 50 ng genomic DNA template, 1×PCR buffer (Biotools, B & M Labs, Madrid, Spain) with 2 mmol/L $MgCl_2$, 0.4 mmol/L of each primer (Genscript, Piscataway, NJ), 50 mmol/L dNTPs (Biotools, B & M Labs), and high-fidelity 1U *Taq polymerase* (Biotools, B &M Labs). For PCR amplification, the standard protocol was used as follows: one initial denaturation step at 95°C for 7 min, followed by 35 denaturation cycles of 30 s at 94°C, 30 s of annealing (56°C for *CCR2-V64I* and 52 °C for *MCP-1 A-2518G*) and 30 s of extension at 72°C, followed by a final elongation cycle at 72°C for 5 min.

For RFLP, 173-bp PCR product *CCR2-V64I* was digested with 5 U *BsaBI* (Fermentas Inc., MD) (5 U at 37°C for 16 h) restriction enzyme and the digested products were separated by electrophoresis on a 3% agarose gel and visualized using ethidium bromide. The AA variant type genotype contains a unique restriction site that results in 149 bp and 24 bp products, and the GG wild type genotype is not cut and displays a band of 173-bp, while the heterozygous form AG displays all the three bands(173 bp, 149 bp and 24 bp). In *MCP-1 A2518G* polymorphism, the PCR product of 234 bp was digested with *PvuII* (Fermentas Inc., MD) (5 U at 37°C for 16 h) and the digested products were separated in 2% agarose gel and later visualized with ethidium bromide. Wild type allele (GG) did

not contain the *PvuII* site and thus displayed the same PCR product of 237 bp whereas variant allele (AA) contained the *PvuII* site, giving rise to 159 and 75bp products. The heterozygote displayed all the three bands (234 bp, 159 bp and 75 bp). For quality control, each PCR reaction used distilled water instead of DNA as a negative control, and more than 20% of the samples were analyzed twice.

2.4 Statistical Analysis

The cases and controls were compared using the χ^2 test for categorical variables like sex and smoking status of the demographic variables. A goodness-of-fit χ^2 test was used to determine whether the polymorphisms were in Hardy-Weinberg equilibrium between cases and controls. Odds ratios (ORs) were used as estimates of the relative risk, and 95% confidence intervals (CIs) were calculated to estimate the association between certain genotypes or other related risk factors of bladder cancer.

3. Results

In this study, we included a total of 120 confirmed transitional cell carcinoma of the urinary bladder cases and 155 healthy controls. Cases included more males than females (88% vs. 12%) compared to controls, while smoking status was frequency matched among cases and controls (p= 0.1). Table 1 shows demographic information and other parameter histories for the study subjects.

Table 1 Clinico-epidemiological variables of UBC patients used for mutational analysis of chemokine and chemokine receptor gene polymorphism

Parameter	Variable	Cases(n=120)	Controls(n=155)	p value
Sex	Males: Females:	106 (88.33) 14(11.67)	105 (67.8) 50 (32.2)	0.00
Age	≤50 >50	22 (18.33) 98 (81.67)	63 (40.8) 92 (59.2)	0.01
Dwelling	Rural: Urban:	88 (73.33) 32 (26.67)	110 (70.9) 45 (29.1)	0.06
Smoking status	Smokers: Nonsmokers:	84 (70.0) 36 (30.0)	105 (67.8) 50 (32.2)	0.1
Differentiation grade	I: II: III: IV:	10 (8.3) 52(43.3) 50 (41.7) 06 (5.0)		
Histological type *	S: MI:	74 (61.67) 46 (38.33)		
Stage	PTa: PT1: PT2: PT3:	36 (30.0) 52 (43.3) 25 (19.1) 07 (05.8)		

Histological Types *Histopathological Type: MI = Muscle Invasive, S = Superficial

MCP-1 A 2518G and *CCR2-V64I* genotype distributions of wild- and variant-type alleles among different clinicopathologic characteristics are shown among the Kashmiri ethnic population for the cases (Table 2).

Table 2 The distribution of MCP-1 A-2518G and CCR2-V64I genotype frequencies in patients with bladder cancer and control groups and their related clinicopathological characters

Variables	**Cases (n= 120)** *CCR2V61I*		**Controls (n=155)** *CCR2V61I*		**OR (95%C.I)**	**p value**
	wt	wt/64I+64I	wt	wt/64I+64I		
Overall genotypes	82 (68.3%)	38 (31.6%)	110 (70.9%)	45 (29.0%)	1.1 (1.2-3.3)	0.6
Age						
≤ 50 Years	12	10	42	21		0.3
> 50 Years	70	28	70	22	1.6 (0.4-6.5) 1.2 (0.52-3.8)	0.4
Gender						
Female	08	06	40	10	3.0 (0.9-9.0)	0.08
Male	74	32	65	40	0.7 (0.4-1.4)	0.7
Dwelling						
Rural	58	30	75	35	1.1(0.6-1.6)	0.7
Urban	24	08	35	10	1.1 (0.7-1.7)	0.7
Smoking						
Yes	52	32	75	30	1.5 (0.8-2.5)	0.1
No	30	06	32	18	0.3 (0.1-0.7)	0.05
Variables	**Cases (n= 120)** *MCP-1 A-2518G*		**Controls (n=155)** *MCP-1 A-2518G*		**OR (95%C.I)**	**p value**
	AA	AG+GG	AA	AG+GG		
Overall genotypes	32 (26.6%)	88 (73.3%)	60 (38.7%)	95 (61.2%)	1.7 (1.0-2.7)	**0.03**
Age						
≤ 50 Years	05	19	20	40	2.0 (1.0-3.6)	0.2
> 50 Years	27	69	32	55	1.1 (0.7-1.9)	0.6
Gender						
Female	05	09	15	35	0.7 (0.3-1.2)	0.6
Male	27	79	45	60	2.2 (1.6-2.5)	0.006
Dwelling						
Rural	20	50	45	65	1.7 (0.8-3.0)	0.09
Urban	12	38	15	30	1.5 (0.6-3.0)	0.3
Smoking						
Yes	20	64	37	68	1.7 (0.8-3.0)	0.09
No	12	24	23	27	1.7 (0.5-3.5)	0.2

The genotype frequencies observed in cases and controls in both the SNPs were in Hardy-Weinberg equilibrium. In *CCR2 V64I* SNP, frequencies of wt, wt/64I and 64I genotypes among controls were 70.9%, 26.4%, and 2.5%, while in cases allele frequencies were 68.3%, 23.3%, and 8.3%, respectively and this difference was statistically insignificant (p = 0.09; Table 3).
The frequency of the wild wt allele in controls was 0.84, compared to 0.80 in cases, while the variant 64I allele frequency in cases was slightly higher at 0.20 as against 0.15 in controls, but the difference was not statistically significant (p = 0.3; Table 3). Interestingly, when further stratified into different groups of pathological classification of bladder cancer, an important finding of our study was that we observed a very high frequency of variant alleles (wt/64I+64I: Val/Iso) in higher grades/stages (III+IV/pT2 or higher) of cases as against the low grades/stages (I+II/pTa+pT1) with an OR 4.0 (CI = 2.5–7.5). Variant alleles (AG+AA: Val/Iso) accounted for 43% and 41% while the wild-type allele (GG) accounted for 57% and 59% in higher grades/stages of bladder cancer respectively (Table 4).

Table 3 The distribution of MCP-1 A-2518G and CCR2-V64I genotype frequencies in patients with bladder cancer and control groups

Genotype/Allele	Controls (%)	Patients (%)	p value
Genotype MCP-1 A-2518G			
AA	60 (38.7)	32 (26.6)	0.000
AG+GG	87 (56.1)	64 (53.3)	
GG	08 (05.1)	24 (20.0)	
Allele MCP-1 A-2518G`			
A	199 (64.1)	128 (53.3)	0.01
G	111 (35.8)	112 (46.7)	
Genotypes CCR2-V64I			
Wt	110 (70.9)	82 (68.3)	0.09
Wt/64 I	41 (26.4)	28 (23.3)	
64 I	04 (2.5)	10 (8.3)	
Allele CCR2-V64I			
wt	261 (84.1)	192 (80.0)	0.3
64 I	49 (15.8)	48 (20.0)	

On the other hand, variant alleles (wt/64I+64I: Val/Iso) accounted for 16% and 21% while the wild type allele accounted for 84% and 59% in lower-grades/stages of bladder cancer respectively (Table 4).These differences between the higher-grade/stage and low-grade/stage bladder cancer in the distribution of *CCR2* V64I were statistically significant (p<0.05; Table 4). In *MCP-1 A-2518G*, frequencies of the AA, AG and variant GG genotypes among controls were 38.7%, 56.1% and 5.1% respectively, while in cases allele frequencies were 26.3%, 53.3% and 20.0% and this difference of genotypes among cases and controls was statistically significant (p=0.000; Table 3). The frequency of the wild type A allele in controls was 0.64, compared to 0.53 in cases, while the variant G allele frequency in cases was moderately higher at 0.47 than in controls at 0.36, in controls and the difference was observed to be statistically significant (p=0.01;Table 3). In contrast to *CCR2 V64I*, no association was observed between *MCP-1 A-2518G* and pathological characteristics of bladder cancer patients.

Table 4 Distributions of genotypes in the patient group according to the pathological type, stage and grade of the tumors

	CCR2 V64I		**OR (95% C.I)**	**P value**	**MCP-1**		**OR (95% C.I)**	**p value**
	wt	wt/ 64I+64 I			AA	AG+ GG		
Differentiation Grade								
I+II	52	10	4.0 (1.6-8.8)	0.002	22	40	2.0 (0.8-4.8)	0.1
III+IV	32	24			10	48		
Tumor Stage								
PTa+PT1	70	18	2.6 (1.2-5.2)	0.03	22	66	1.3 (0.5-2.7)	0.5
PT2+higher	19	13			10	22		
Histological Type								
S	62	12	3.9 (1.9-7.4)	0.001	15	39	1.1 (0.4-2.3)	0.8
MI	26	20			17	49		

In *CCR2* V64I, the frequency of rare allele (wt/64I+64I) in cases was 31.6% and 29% in controls and this difference was observed to be insignificant (p=0.6: OR=1.1: CI=1.2-3.0; Fig. 1)

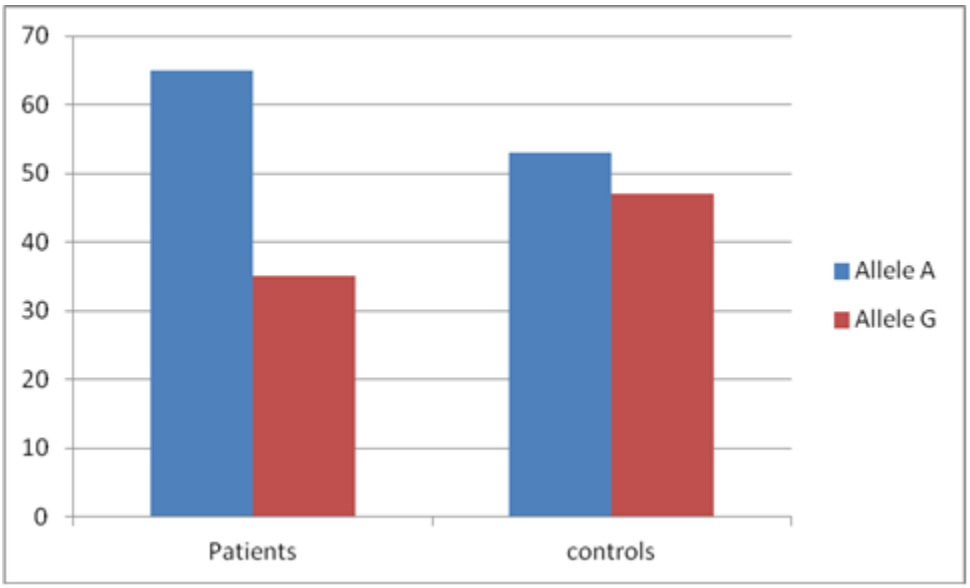

Fig 1. Distribution of *MCP-1 A-2518G* genotype frequencies in patients with bladder cancer and control groups

On the other hand rare alleles (AG+AA) in *MCP-1 A-2518G* were abundantly found in cases with 1.7 fold risk with a frequency of 731.3% and frequency of rare alleles was 61% in controls and this difference was observed to be significant (p=0.03:OR=1.1 : CI=1.2-3.0;Table 2;Fig. 2).

No association with gender, age and smoking status was observed between genotypes of *CCR2 V61I* or *MCP-1 A-2518G* among cases and controls.

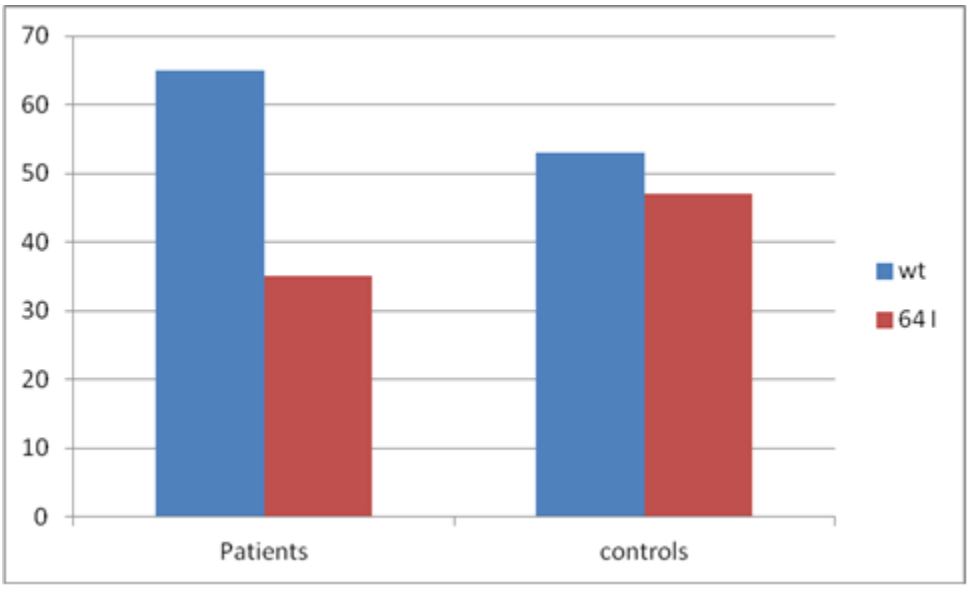

Fig 2. Distribution of CCR2-V64I genotype frequencies in patients with bladder cancer and control groups

4. Discussion

Cytokines, expressed by various cancer and immune cells, bind to specific receptors and activate distinct signal pathways to transcriptionally activate a plethora of downstream factors. It is postulated that chemokines and their receptors play diverse roles in malignant tumor progression, particularly as key mediators of tumor-stroma interactions (Balkwill et al., 2004; Karnoub et al., 2006). Polymorphisms affect expression and functional characteristics (Faure et al., 2000; Nickel et al., 2000; Karpus et al., 1997). Since this polymorphism has been reported only twice in bladder cancer, further evaluation was imperative, to elucidate the conformity of the results in the backdrop of different ethnic backgrounds. Thus, we conducted a case-control polymorphic study of *MCP-1 A-2518G* and *CCR2 V64I* to assess the role of these SNPs in bladder cancer patients from the Kashmir region (North India).

In the case of *CCR2 V64I*, the spectrum of genotypic distribution of wt, wt/64I and 64I observed in controls was 70.9%, 26.4% and 2.5% compared to 68.3%, 23.3% and 8.3% in cases respectively. The allele frequency of the wild- type allele was found to be 84% in controls compared with 80% in cases, while as variant allele frequency was 15.8% in controls and 20% in cases. The allelic as well as genotypic distribution in cases and controls clearly shows a non-significant association with predisposition to bladder cancer ($p>0.05$). This finding is in stark contrast to the previous report (Narter et al., 2010) in cases of bladder cancer where the distribution of wt, wt/64I and 64I was 54.2%, 31.9% and 13.9% ; however, the frequency of genotypes in controls was reported to be

77.7%, 19.6% and 2.6%, which was in agreement with our results. Further, our results in *CCR2 V64I* differ with a recent study conducted by Chen et al., (2011) on oral cancer. In contrast to this, our results in *CCR2 -V64I* were in accordance with Bektas-kayun et al., (2012) conducted on oral cancer reporting almost similar allelic as well genotypic distributions in both cases and controls. In some previous studies, *CCR2-V64I* 64I / 64I genotypes have been reported as risk factors for endometrial (Attar et al., 2010) and bladder cancers (Narter et al.,2010) but our study shows no association with either of the variant homozygotes or heterozygotes and thus does not conform to these reports. However, an important finding in our study was that *CCR2-64I* homozygotes and heterozygotes presented abundantly in high grade and advanced stage bladder cancer cases compared to *CCR-V64* I alleles (Table 4). This difference in the distribution of variant allele (AG+AA) showed a strong significance in advanced tumors ($P < 0.05$). This finding was not in agreement with the earlier study of Narter et al., (2010). Discrepancies observed in our and many other reports with regard to the polymorphism in *CCR2 V64I* contributes to the differences between individuals or races in susceptibility and severity of disease

The distribution of *MCP-1 A-2518G* genotypes AA, AG and GG observed in controls was 38.7%, 56.1% and 5.1%,respectively, compared to 26.3%, 53.3% and 20.0% in cases. The allele frequency of wild type A allele was found to be 64% in controls as against 53.3% in cases while as variant allele frequency was seen as 35.8% in controls and 46.7% in cases respectively. The allelic/genotypic distribution of *MCP-1 A-2518G* in cases and controls clearly shows a significant association with predisposition to bladder cancer ($p<0.05$). G allele and GG genotype of MCP-1 were significantly increased in patients which appear to be risk factors, as compared to those of controls. This finding is in stark contrast to the previous report (Narter et al., 2010) which showed no association of *MCP-1 A-2518G* with bladder cancer. Our study is in agreement with the studies showing increased risk for *MCP-1 2518* GG genotype and G allele of different cancer types including breast (Ghilardi et al., 2005), bladder (Vazquez-Lavista et al., 2009), nasopharynx (Tse et al., 2007), and endometrial (Attar et al., 2010). In addition to the increased frequency of genotypes, the overexpression of MCP-1 was also associated with different types of tumors such as glioma, ovarian, esophageal, lung, breast, and prostate cancer (Luo et al., 2007; Cai et al., 2009; Melgarejo et al., 2009). Further prognostic analysis revealed that high expression of MCP-1 and increased serum levels were correlated with the advanced tumor stage, lymph node metastasis, and early relapse (Hefler et al., 1999; Lebrecht et al., 2001; Lebrecht et al., 2004).

The A2518G SNP in the regulatory region of MCP-1 might cause increased promoter activity via inflammatory molecules such as interleukin and chemokine, and is also associated with elevated circulating levels of MCP-1 (Tse et al., 2007). Homozygous GG individuals produce significantly higher MCP-1 levels than those with the AA genotype (Luo et al., 2007). In the present study, the abundance of genotypes of GG, which is correlated with circulating levels of MCP-1 in the previous studies, perhaps elevates the risk of bladder cancer by increasing the transcription of gene product (Tse et al., 2007). There have been conflicting results related to the effect of CCR2 -64I genotype on the expression levels. While some authors suggested that *CCR2-64I* genotype increased the protein levels, others did not confirm these results. The *CCR2-64I* variant in our study does not seem to be a risk factor for the development of bladder cancer possibly because this polymorphic variant has been reported to be protective in the development and progression of development of breast cancer and cervical cancer (Zafiropoulos et al., 2004; Coelho et al., 2005). Although the current

study has some interesting observations, there are some limitations as the expression of MCP-1 and CCR2 could not be measured.

5. Conclusion

In conclusion, our findings demonstrate that *MCP-A1 2518G* may be a risk factor for the development of bladder cancer and *CCR2-V64I* bears no association in the predisposition for bladder cancer in Kashmiri patients. Our assumption is based on the fact that *MCP-1 A 2518G* variant alleles (GG and AG) is also associated with elevated circulating levels of MCP-1 and that increased activity may increase the risk of bladder cancer development. Further studies with larger sample groups are necessary to clarify the role of this chemokine receptor in the natural history of bladder cancer.

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