Original Article

Lymphotoxin-alpha +252A/G Single-Nucleotide Polymorphism in Colorectal Cancer in the Ethnic Kashmiri Population: A Case-Control Study

Abstract

Background: Chronic inflammation is an important pathological factor in colorectal tumorigenesis. Lymphotoxin-alpha (LT-α), a pleiotropic pro-inflammatory cytokine has been shown to possess both cancer promoting and cancer inhibiting activities. Several studies have analyzed the association of intronic LT-α+252A/G single-nucleotide polymorphism (SNP) in human LT-α gene with various cancers including colorectal cancer (CRC), but the outcome have been mixed and inconclusive. **Materials and Methods:** The present case-control study analyzed the association of LT-α+252A/G SNP with CRC risk in the ethnic Kashmiri population. The genotype frequencies of LT-α+252A/G intronic SNP were compared between 142 CRC patients and 184 individually matched healthy controls by polymerase chain reaction-restriction fragment length polymorphism method. The association between the LT-α+252A/G SNP and CRC risk was investigated through conditional logistic regression models adjusted for multiple possible confounding (third) variables. Further, the association between CRC risk and various clinico-pathological parameters, demographic variables, and environmental factors within the case group subjects and the SNP under study was also analyzed. **Results:** The overall association between the LT-α+252A/G SNP and the modulation of CRC risk was found to be significant ($P = 0.013$). Further, we found a significant effect modification of the association between the LT-α+252A/G SNP genotypes and CRC risk by gender ($P = 0.046$). We also found that the LT-α+252A/G SNP within the case group was significantly associated with gender ($P = 0.0014$) and lymph node status ($P < 0.0001$). **Conclusion:** This study has demonstrated that LT-α+252A/G SNP is significantly associated with risk of CRC in the ethnic Kashmiri population although the nature of this association could not be deciphered further in a statistically significant manner. However, this study needs to be replicated with larger sample size and if possible in other ethnically defined populations that exhibit comparable incidence of CRC to substantiate and elaborate our findings in a more comprehensive manner.

Keywords: *Case-control study, Colorectal cancer, Kashmir, lymphotoxin-alpha, polymorphism, single-nucleotide polymorphism*

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Introduction

Colorectal cancer (CRC) also known as colon cancer or large bowel cancer includes the neoplasia of the colon, rectum, and appendix and is one of the most common cancers and leading cause of cancer-related mortality and morbidity worldwide. It ranks third among the cancers in men and second in women globally, is the third-most frequent cause of cancer-related mortality with nearly 1.2 million new cases diagnosed each year and approximately 600,000 deaths reported annually.[1,2] The Valley of Kashmir, part of Jammu and Kashmir State, located in Northern India has a unique ethnically defined population with highly distinctive food habits compared with the rest of the country and the world. In Kashmir Valley, CRC represents the third-most common gastrointestinal tract cancer after esophageal cancer and gastric cancer[3] and is the fourth-common cancer among the males and the third among the females.[3,4]

The pathogenesis of CRC is strongly associated with innate immune processes and intestinal inflammation. The chronic inflammation plays a vital role in the promotion of colorectal tumorigenic processes. The chronic inflammation can also stimulate and complement the noninflammatory pro-tumorigenic processes through supply of various mediators

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including cytokines, which promote tumor growth, survival, progression, angiogenesis, invasion, and metastasis.[5,6] Cytokines, both pro-inflammatory and anti-inflammatory, constitute the vital component in the mediation and regulation of inflammatory immune responses which play a key role in the pathogenesis of colorectal tumorigenesis.[7]

Human lymphotoxin-alpha (LT-α) is a pleiotropic pro-inflammatory cytokine, which plays an essential role in the regulation of various immune responses including inflammation, immuno-stimulation, antiviral responses, and cytotoxic activities on infected cells and tumor cells.[8,9] LT-α is a vital mediator of lymphoid neogenesis and plays a key role in the development, organization, and maintenance of secondary and tertiary lymphoid organs including Peyer's patches, lymph nodes, the lymphoid follicles in tonsils, adenoids, and the spleen,[10,11] which are crucial for the initiation and maintenance of adaptive immune responses.

The role of LT-α in colorectal and other tumors is controversial, as it has been shown to possess both cancer promoting and cancer inhibiting activities. LT-α is involved in anti-tumor surveillance through its cytotoxic effect on tumor cells. LT-α is involved in the activation of natural killer (NK) cells, which represent an important component of nonspecific host defense mechanism involved in tumor rejection and inhibition of metastasis. LT-α promotes the differentiation, maturation and recruitment of NK cells to tumor lesions.[12] However, various studies point towards the involvement of LT-α in the development of various cancers including CRC.[13]

The LT-α gene is located within the class III region of the major histocompatibility complex on the short arm of chromosome 6 at position 21.3. The gene encoding LT-α is highly polymorphic and has been reported to contain several different polymorphisms mostly in the form of single nucleotide polymorphisms (SNPs) which are found within the gene promoter besides the intronic and exonic regions. Of all the polymorphisms, the LT-α SNPs designated as rs1041981, rs2239704, rs2229094, rs746868, and rs909253 have been reported to be functionally relevant with regard to the regulation of gene expression and therefore may influence LT-α expression and contribute to the inter-individual variability in the expression and tissue/serum levels of this cytokine.[14,15] This may further result in differences in relevant biological functions including the modulation of inflammatory response and subsequently the differences in susceptibility among different individuals to several diseases including various cancers.[16-20]

The LT-α+252A/G SNP (rs909253) also known as IVS1 +90 A/G SNP representing an adenine (A) to guanine (G) substitution at +252 nucleotide (nt) position is located within the first intron of LT-α gene and has been reported to be significantly associated with differential LT-α expression and activity. The AG and more potently GG genotypes and G allele of LT-α+252A/G SNP have been reported to be associated with increased expression, tissue/serum levels and higher activity of LT-α in comparison to AA genotype and A allele of this SNP.[21-25] The more common +252A allele is sometimes referred to as LT10.5 or LT-α (10.5 kb) whereas the less common +252G allele is referred to as LT5.5 or LT-α (5.5 kb).[26] It has been reported that the less common or variant genotype LT-α+252GG and less common or variant allele LT-α+252G resulted in a 1.5-fold increase in LT-α gene expression in comparison to the more common LT-α+252AA genotype and more common LT-α+252A allele and same has been reflected through the considerably higher tissue and serum levels of LT-α observed in subjects with GG genotype in comparison to the AA genotype.[23,27-29] The LT-α+252A/G SNP also regulates the expression and activity of tumor necrosis factor-alpha (TNF-α) and LT-α+252G allele has also been reported to be associated with increased expression, enhanced tissue/serum levels and higher activity of TNF-α in comparison to LT-α+252A allele.[28,29] Although the exact functional significance of LT-α+252A/G SNP is not known yet fully, it has been proposed that the differential LT-α expression and activity stems from the transcriptional regulatory activity of this SNP. The A to G substitution at +252 bp position has been reported to influence the binding of an unknown nuclear factor (NF) such that the protein binds with higher affinity to the +252G allele, resulting in 1.5-fold higher expression of LT-α protein in comparison to the expression associated with +252A allele.[23] The haplotype specific increased expression, tissue/serum levels, and higher activity of LT-α associated with LT-α+252A/G SNP may result in the constitutive activation of NF-κB and enhanced NF-κB signaling may modulate the inflammatory response and further stimulate or promote the activation of pathogenic pathways involved in several diseases including various types of cancers.

Several studies have analyzed the possible association of LT-α+252A/G SNP with risk of various cancer types including gastric cancer,[17,30] breast cancer,[20] lung cancer,[31] non-Hodgkin's lymphoma,[32,33] oral cancer,[34] endometrial cancer,[35] myeloma,[36] leukemia,[37] bladder cancer,[38] and cervical cancer.[39] However, we found only few studies that have analyzed the possible association of LT-α+252A/G SNP with CRC risk.[18,40]

In this study, we systematically analyzed the possible association between LT-α+252A/G SNP and susceptibility to or risk of CRC in the Kashmiri population through a case-control study design. We also evaluated the possible effect modification of CRC risk by age, gender, and smoking status. Further, we investigated the possible association of LT-α+252A/G SNP with various clinico-pathological parameters, demographic variables and environmental factors including smoking habit and studied their role in modulating the risk of CRC in the population under study.

Materials and Methods

Study subjects.

The present study included two subject groups: Case and control. The case group included 142 individuals recruited consecutively irrespective of their age and gender with primary CRC who underwent surgical resection for primary CRC tumors at the Department of General Surgery, Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, Kashmir. The diagnosis of CRC was confirmed histopathologically. The tumor stage and the tumor grade were classified according to the 8th ed.ition of TNM classification of Union International Control of Cancer. Only those cases who had not received any neoadjuvant chemo or radiotherapy were chosen for this study. All the cases were >18 years old and had no prior history of any malignancy. Blood and tissue samples were obtained from these CRC patients. The control group included 184 healthy individuals with no history or prior diagnosis of any malignant disorder or any other serious disease, which were recruited during the same time period and from the same geographic area and from whom blood was collected and used as control for the present study. The control group included both general population-based subjects and hospital based subjects. The control group subjects were matched to the case group subjects individually for age (±5 years), sex, place of residence (rural/urban), smoking habit, and ethnicity to minimize the confounding effect of these various relevant factors. Both the case and the controls chosen for this study were ethnic Kashmiris.

Data collection

The data relevant to the study concerning all the CRC patients including various clinico-pathological parameters, demographic variables and the environmental factors was obtained and evaluated from the patient medical records, pathology reports and also from the personal interviews with the patients and/or their guardians (for those who were illiterate or unable to communicate). The interviews were conducted in local language for easy and direct communication, which also helped to gather maximum possible relevant information. The data collected included tumor location, Dukes Stage, lymph node status, age, sex, place of residence, ethnicity, smoking habit, and the family history of cancer among several other potential confounding parameters. The relevant data were also obtained for each of the recruited controls mostly through personal interviews and included parameters such as age, sex, place of residence, ethnicity, and smoking habit. The data collection was carried out by research professionals only, to fulfill the requisite quality standards during the course of this study. All the patients and/or their guardians were informed about the study and their willingness to participate in this study was documented using a predesigned questionnaire and same procedure was followed for the controls. All the procedures concerning the study participants including sample procurement and the data collection were carried out in accordance with the ethical standards laid down by the Institutional Ethics Committee, SKIMS and the World Health Organization and the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964 and its seventh amendment, 2013) for experiments in humans.[41]

Sample preparation and DNA extraction

The tumor tissue samples collected after surgical resection were immediately snap frozen in liquid nitrogen and then stored at −80°C until further use for DNA extraction and other experimental purposes. Peripheral blood sample, 3–5 ml from each case and control group individual was collected by venopuncture into ethylene diamine tetra acetic acid coated blood vacutainer collection tubes (purple capped tubes; ADS Hitech Polymers, India) and stored at −80°C until further use. The genomic DNA was extracted from both the tumor tissue and blood specimens using DNeasy™ Blood and Tissue Kit (catalog no. 69504; Qiagen, Germany) and Quick-gDNA™ MiniPrep kit (catalog no. D3024; Zymo Research, USA) according to the manufacturers' instructions. The extracted DNA was stored at −20°C until further use. The qualitative and the quantitative assessments of the extracted genomic DNA samples were carried out by absorbance measurements at 260 nm and 280 nm using ultraviolet-visible Spectrophotometric analysis and also by agarose gel electrophoresis. The DNA extracted from blood samples of case and control group subjects was used for this study.

Single nucleotide polymorphism analysis or genotyping

The LT-α+252A/G SNP was genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.

Lymphotoxin-alpha +252A/G SNP polymerase chain reaction

The PCR for the amplification of LT-α gene region containing the LT-α+252A/G SNP was carried out in a total volume of 25 µL containing 100 ng – 1 µg of genomic DNA, 0.7–1 U Taq DNA polymerase with lX Standard Taq reaction buffer (New England Biolabs, UK), 1.8 mM $MgCl_2$; 0.28 mM deoxynucleotide triphosphate mix (New England Biolabs, UK); 0.56 µM forward and revere oligonucleotide primers (Integrated DNA Technologies, India) and nuclease-protease free water (Qiagen, Germany) added up to a final volume of 25 µL. Alternatively and randomly Phusion DNA Polymerase with Phusion HF Buffer (New England Biolabs, Inc., UK) were used instead of Taq DNA polymerase with Standard Taq reaction buffer to check for any Taq polymerase induced amplification errors.

The PCR conditions used for the amplification of LT-α gene region containing the LT-α+252A/G SNP were as

follows; initial denaturation at 95°C for 6 min followed by 35 cycles of denaturation at 95°C for 45 s; annealing at 64°C for 60 s and extension at 72°C for 45 s followed by a single final extension step at 72°C for 10 min. The oligonucleotide primers used for the amplification of the specific gene region containing the LT-α+252A/G SNP were 5'-CCGTGCTTCGTGCTTTGGACTA-3' (Forward) and 5'-AGAGCTGGTGGGGACATGTCTG-3' (Reverse). The desired PCR product obtained for LT-α+252A/G SNP was 741 bp in size [Figure 1 for PCR gel picture].

Genotyping

The LT-α+252A/G SNP was genotyped using the restriction enzyme *Nco*I. The restriction enzyme was procured from Thermo Fisher Scientific, USA. The digestion was carried out according to the manufacturers' instructions in a 30 μL reaction volume containing 10 μL of PCR product and 10 U of appropriate restriction enzyme and incubated at 37°C overnight. Wild genotype (AA) is not cleaved by *Nco*I enzyme thereby yielding a single 741 bp fragment whereas variant genotype (GG) yields two fragments 545 bp and 196 bp in size. Heterozygous genotype (AG) yields three fragments 741 bp, 545 bp, and 196 bp in size. The digestion products of LT-α+252A/G SNP were separated on a 3% agarose gel stained with ethidium bromide (HiMedia, India) to a final concentration of 0.5 μg/ml [Figure 2 for RFLP digestion gel picture].

Quality control

The quality control included the assessment of genotyping errors including the false estimates of a particular allele or genotype frequency and the evaluation of the reproducibility of the genotyping done. For these assessments, approximately 10% of the patient and control samples selected randomly were re-genotyped. In addition, in each PCR-RFLP setup, previously amplified and genotyped samples representing different genotypic scenarios were included as a positive control. The genotyping reproducibility of the samples was very high for the LT-α SNP with a weighted kappa coefficient of 0.99, which meant a high concordance rate of 99%.[42,43]

Statistical analyses

The frequencies of genotypes and alleles for the SNP under study were obtained through direct counting. The numbers and percentages were calculated and presented for each of the categorical variables along with means, standard deviations, median, and inter-quartile range for continuous variables. Conditional logistic regression analysis was carried out to calculate unadjusted and adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs) to assess the possible association of the relevant SNP genotypes with CRC risk and to assess the possible gene-environment interactions if applicable. In order to eliminate the possible confounding (third) variables, the conditional logistic regression models were adjusted for the known risk factors such as gender, age and smoking habit and with the place of residence. The possible effect measure modification of the association between various genotypes relevant to the SNP under study and CRC risk by various CRC risk factors including age, gender, and smoking status was also included in the conditional logistic regression models and analyzed. The correlation between the genotypes and the clinico-pathological parameters, demographic variables, and environmental factors including smoking habit within the case group was analyzed using Fisher's exact test. The

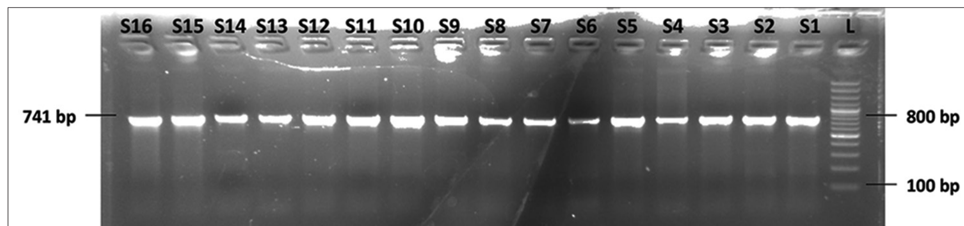

Figure 1: Electrophoresis of LT-α+252A/G SNP PCR products on a 2.5% Agarose Gel. Lanes S1-S16: Amplified PCR products with prominent/desired band 741 bp in size. Lane L: 100 bp Molecular size marker/Ladder

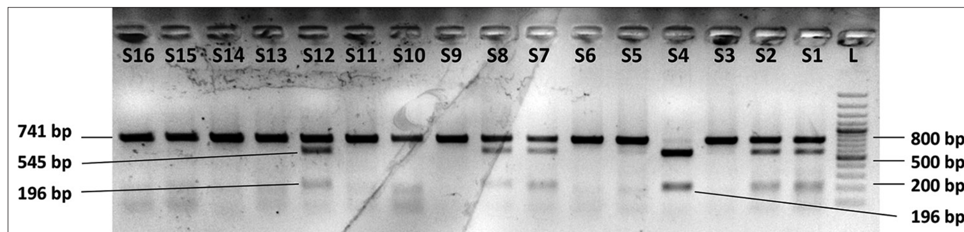

Figure 2: Electrophoresis of LT-α+252A/G SNP genotyping by PCR-restriction fragment length polymorphism on a 3% Agarose Gel. Lanes S1–S16: Restriction digestion products; Wild genotype (AA) is not cleaved by NcoI enzyme yielding a single 741 bp fragment whereas variant genotype (GG) yields two fragments 545 bp and 196 bp. Heterozygous genotype (AG) yields three fragments 741 bp, 545 bp and 196 bp in size. Lanes S1, S2, S7, S8 and S12 show the heterozygous genotype (AG); Lane S4 shows the variant genotype (GG) whereas the Lanes S3, S5, S6, S9, S10, S11. S13, S14, S15 and S16 show the wild genotype (AA) of LT-α+252A/G SNP. Lane L: 100 bp Molecular size marker/Ladder

fitness of the genotype distributions to Hardy-Weinberg equilibrium (HWE) for the allele and the genotype frequencies in the population under study was tested using the Chi-square test. A two sided probability value of or <5% ($P \leq 0.05$) was considered statistically significant for all types of analyses. All statistical analyses were performed using IBM, Armonk, New York, United States.

The effective sample size and the statistical power were computed using the "Genetic Power Calculator" developed by Purcell *et al*. (http://zzz.bwh.harvard.edu/gpc/). We obtained a healthy power score of about 77% for the SNP under study in the present case-control study involving 142 case subjects and 184 controls.

Results

The frequencies of various clinico-pathological parameters, demographic variables, and environmental factors in CRC case subjects and relevant parameters in controls representing the general characteristics of study participants are given in Table 1. The frequencies of the genotypes of LT-α+252A/G SNP for both the case and the control groups are listed in Table 2. The more common AA genotype of LT-α+252A/G SNP was almost equal in frequency among the case group (59.86% [85/142]) and the control group (60.87% [112/184]). The frequency of the heterozygous genotype (AG) in the case group (40.14% [57/142]) was higher than that of the control group (33.7% [62/184]). The variant genotype (GG) was altogether absent in the case group but was present in the control group (5.43% [10/184]). Further, the frequency of the more common LT-α+252A allele was found to be 79.93% (227/284) among the case group subjects and 77.72% (286/368) among the control group subjects. The frequency of the less common LT-α+252G allele was found to be 20.07% (57/284) among the case group subjects and 22.28% (82/368) among the control group subjects. The frequency of the combined variant genotype (AG + GG) in the case group (40.14% [57/142]) was not much different from that of the control group (39.13% [72/184]) [Table 2]. The overall association between the LT-α+252A/G SNP and the modulation of CRC risk was found to be significant (P = 0.013) [Table 2]. However, the nature of this association could not be deciphered further in a statistically significant manner. Further, the genotype frequencies for LT-α+252A/G SNP were found to be in agreement with HWE among the control group subjects (χ^2 = 0.135; P = 0.713) but not among the case group subjects (χ^2 = 8.953; P = 0.002).

The possible effect measure modification or effect modification of the association between LT-α+252A/G

Table 1: General characteristics of study subjects

Characteristics	Colorectal cancer cases (*n*=142)*	Controls (*n*=184)*	Pearson χ^2; *P*
Age (years)			
Mean age (SD) (SEM)	52.68 (15.34) (1.29)	52.22 (14.57) (1.07)	
Age range (median)	21-82 (55)	21-80 (51.5)	
≤50, *n* (%)	66 (46.48)	91 (49.46)	0.29; 0.59
>50, *n* (%)	76 (53.52)	93 (50.54)	
Gender, *n* (%)			
Male	85 (59.86)	102 (55.43)	0.64; 0.42
Female	57 (40.14)	82 (44.57)	
Place of residence, *n* (%)			
Rural	87 (61.27)	101 (54.89)	1.33; 0.25
Urban	55 (38.73)	83 (45.11)	
Smoking status			
Ever	80 (56.34)	94 (51.09)	0.89; 0.35
Never	62 (43.66)	90 (48.91)	
Tumor location			
Colon	58 (40.85)		
Rectum	84 (59.15)		
Tumor grade			
WD	95 (66.90)		
MD and PD	47 (33.10)		
Lymph node status			
Involved	78 (54.93)		
Not involved	64 (45.07)		

Table shows various clinico-pathological parameters, demographic variables and environmental factors in colorectal cancer case subjects and relevant parameters in control subjects from Kashmir. Pearson Chi-square test (χ^2) was used to calculate the *P* values for categorical variables. **n*=Number of subjects or individuals, SD=Standard deviation, SEM=Standard error of mean, WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

Table 2: Lymphotoxin-alpha +252 A/G single nucleotide polymorphism genotype frequency distributions among colorectal cancer cases and matched controls and risk of colorectal cancer*

	CRC cases (*n*=142), *n* (%)*	Controls (*n*=184), *n* (%)*	OR (95%CI); *P*[#]	Adjusted OR[§] (95% CI); *P*[#]	χ^2; Pearson *P* (overall)[#,†]
Genotype					
AA	85 (59.86)	112 (60.87)	**1.0 (Reference)**	**1.0 (Reference)**	8.64; **0.013**
AG	57 (40.14)	62 (33.7)	0.73 (0.44-1.20); 0.210	0.75 (0.45-1.25); 0.264	
GG	0 (0)	10 (5.43)	Not calculable		
AG + GG	57 (40.14)	72 (39.13)	0.92 (0.57-1.46); 0.712	0.94 (0.58-1.51); 0.796	0.03; 0.853
Allele					
A	227 (79.93)	286 (77.72)	**1.0 (Reference)**		
G	57 (20.07)	82 (22.28)	1.14 (0.78-1.67); 0.50		0.467; 0.494

**n*=Number of subjects or individuals, [#]The values in bold indicate significant results. [§]Adjusted ORs (95% CIs) were obtained in conditional logistic regression models when adjusted for age, gender, place of residence and smoking status. ORs (95% CIs) were obtained from conditional logistic regression models, [†]*P*-values calculated using Chi-square tests. CRC: Colorectal cancer, OR: Odds ratio, CIs: Confidence intervals

genotypes and CRC risk by various CRC risk factors including age, gender, and smoking status are summarized in Table 3. On analyzing the effect modification of LT-α+252A/G genotypes by age, gender, and smoking status, it was found that the effect of the combined variant genotype (AG + GG) on CRC risk was significantly influenced by gender ($P = 0.046$). A decreased CRC risk was observed in females (OR, 0.42 [95% CI, 0.19–0.92]; $P = 0.030$).

The numbers and the frequencies of the subsets of various characteristics of the case group subjects under study, i.e., age, gender, dwelling, smoking status, tumor location, tumor grade, and lymph node status for LT-α+252A/G SNP are listed in Table 4. We analyzed the correlation of the LT-α+252A/G promoter SNP with the subsets of these various characteristics of the case group participants. The LT-α+252A/G SNP was significantly associated with gender ($P = 0.0014$). The male participants who carried the heterozygous genotype (AG) were at an increased risk of developing CRC in comparison to females (OR, 3.07 [95% CI, 1.52–6.19]; $P = 0.0017$). The LT-α+252A/G SNP was also significantly associated with smoking status ($P = 0.0141$). The participants who carried the heterozygous genotype (AG) and were smokers were at an increased risk of developing CRC (OR, 2.35 [95% CI, 1.18–4.66]; $P = 0.0163$). The LT-α+252A/G SNP also showed an overall strongly significant association with lymph node status ($P < 0.0001$). Further, the participants who carried the heterozygous genotype (AG) of LT-α+252A/G SNP were at an increased risk of lymph node infiltration (OR, 4.54 [95% CI, 2.21–9.31]); $P < 0.0001$. Some statistical parameters mentioned here are not shown in Table 4.

Discussion

In the present study, we evaluated the role of functional LT-α+252A/G SNP located within the first intron of LT-α gene as a potential modulator of risk of CRC in ethnic Kashmiri population in a case-control study design with 142 case subects and 184 controls.

In the present study, we evaluated the distribution of the LT-α+252A/G SNP genotypes in CRC patients and controls, and found that this LT-α promoter SNP showed an overall significant association with the modulation of the CRC risk in our population. However, the nature of this association could not be deciphered further in a statistically significant manner. The factors that led to this inconclusive result could be both multiple and complex. The more expressive variant genotype LT-α+252GG was totally absent from the case group subjects which possibly led to the haploinsufficiency effect whereby the observed overall association of CRC risk with LT-α+252G allele was not strong enough to express itself clearly. In other words, the association was there but due to lack of LT-α+252GG genotype, the effect of LT-α+252G allele was less pronounced which possibly led to this inconclusively. At the protein level, this possibly means that the increased LT-α expression associated with the heterozygous genotype (AG) in case group subjects will be high compared to the low expressive wild genotype (AA) and this increased expression resulting from a single LT-α+252G allele though was high enough to exhibit that somehow the differential LT-α expression could modulate the CRC risk but not enough to depict this modulation clearly enough. Further studies involving a large number of CRC patients and healthy controls may possibly help to decipher this association in a statistically significant manner and may explain its influence on the CRC risk in a more conclusive manner and substantiate our hypothesis. Our results though inconclusive regarding the nature of risk modulation are still in agreement with one study that reported the association of the LT-α+252A/G SNP with modulation of CRC risk.[40]

In the present study, we also evaluated the possible effect modification of the association between LT-α+252A/G SNP genotypes and CRC risk by age, gender, and smoking status. We found a significant effect modification of association between the combined variant genotype (AG + GG) of

Table 3: Effect modification of lymphotoxin-alpha +252 A/G single nucleotide polymorphism genotypes in presence of various risk factors of colorectal cancer in ethnic Kashmiri Population

Genotype^ and characteristic	CRC cases, *n* (%)	Controls, *n* (%)	OR (95%CI); *P*#	Adjusted OR§ (95% CI); *P*#	χ^2; Pearson *P* (overall)#,†
Age					
Wild and ≤50	39 (27.46)	53 (28.80)	**1.0 (Reference)**	**1.0 (Reference)**	
Variant and ≤50	27 (19.01)	38 (20.65)	0.94 (0.47-1.86); 0.847	0.97 (0.48-1.95); 0.923	0.45; 0.930
Wild and >50	46 (32.39)	59 (32.07)	1.17 (0.17-8.03); 0.876	1.21 (0.17-8.54); 0.847	
Variant and >50	30 (21.13)	34 (18.48)	1.05 (0.14-7.87); 0.959	1.10 (0.14-8.39); 0.929	
Gender					
Wild and male	60 (70.59)	62 (60.78)	**1.0 (Reference)**	**1.0 (Reference)**	
Variant and male	25 (29.41)	40 (39.22)	1.45 (0.77-2.73); 0.247	1.83 (0.93-3.61); 0.081	1.97; 0.161
Wild and female	25 (43.86)	50 (60.98)	**1.0 (Reference)**	**1.0 (Reference)**	
Variant and female	32 (56.14)	32 (39.02)	0.50 (0.24-1.04); 0.064	0.42 (0.19-0.92); **0.030**	3.97; **0.046**
Smoking status					
Wild and nonsmoker	30 (21.13)	55 (29.89)	**1.0 (Reference)**	**1.0 (Reference)**	
Variant and nonsmoker	32 (22.54)	35 (19.02)	0.59 (0.31-1.16); 0.127	0.61 (0.31-1.21); 0.155	4.51; 0.211
Wild and smoker	55 (38.73)	57 (30.98)	0.59 (0.16-2.19); 0.425	0.62 (0.13-2.9); 0.548	
Variant and smoker	25 (17.61)	37 (20.11)	0.83 (0.21-3.34); 0.797	0.90 (0.18-4.41); 0.894	

^Wild refers to AA genotype and variant refers to AG + GG genotype, *n*=Number of subjects or individuals, #The *P* values in bold indicate significant results. §Adjusted ORs (95% CIs) were obtained from conditional logistic regression models when adjusted for age, gender, place of residence and smoking status. The variable under consideration was excluded in the time of analysis, †*P*-values calculated using Chi-square tests. ORs (95% CIs) were obtained from conditional logistic regression models. CRC: Colorectal cancer, CIs: Confidence intervals, OR: Odds ratio

Table 4: Association of lymphotoxin-alpha +252A/G single nucleotide polymorphism with various clinico-pathological parameters, demographic variables and environmental factors in colorectal cancer cases*

Characteristics	*n*=142, *n* (%)	AA (*n*=85; 59.86%), *n* %)	AG (*n*=57; 40.14%), *n* %)	GG (*n*=0; 0%), *n* %)	χ^2; *P* (overall)*
Age (years)					
≤50	66 (46.48)	39 (45.88)	27 (47.37)	0 (0)	0.030; 0.862
>50	76 (53.52)	46 (54.12)	30 (52.63)	0 (0)	
Gender					
Male	85 (59.86)	60 (70.59)	25 (43.86)	0 (0)	**10.14; 0.0014**
Female	57 (40.14)	25 (29.41)	32 (56.14)	0 (0)	
Dwelling					
Rural	87 (61.27)	48 (56.47)	39 (68.42)	0 (0)	2.053; 0.152
Urban	55 (38.73)	37 (43.53)	18 (31.58)	0 (0)	
Smoking status					
Ever	80 (56.34)	55 (64.71)	25 (43.86)	0 (0)	**6.028; 0.0141**
Never	62 (43.66)	30 (35.29)	32 (56.14)	0 (0)	
Tumor location					
Colon	58 (40.85)	37 (43.53)	21 (36.84)	0 (0)	0.632; 0.427
Rectum	84 (59.15)	48 (56.47)	36 (63.16)	0 (0)	
Tumor grade					
WD	95 (66.90)	52 (61.18)	43 (75.44)	0 (0)	3.134; 0.077
MD and PD	47 (33.10)	33 (38.82)	14 (24.56)	0 (0)	
Lymph node status					
Involved	78 (54.93)	59 (69.41)	19 (33.33)	0 (0)	**17.94; <0.0001**
Not involved	64 (45.07)	26 (30.59)	38 (66.67)	0 (0)	

*The values in bold indicate significant results. WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated, OR: Odds ratio

LT-α+252A/G SNP and CRC risk by gender. The female gender decreased the CRC risk. The females carrying the variant allele, LT-α+252A/G in heterozygous form (AG) or variant homozygous form (GG) were at a decreased risk of developing CRC in comparison to males. A plausible explanation of this finding is that the regulation of immune response including the innate and adaptive immune responses and cytokine expression and activity

in humans has been naturally programmed to exhibit a gender-based dimorphism.[44-47] It is now well established that the immune response is regulated in a gender specific manner in which sex hormones, the androgens and the estrogens play an essential role but affect the immune system in opposite ways.[48,49] The androgens have been reported to favor the T Helper 1 (Th1)-type response,[50] whereas as estrogens promote T Helper 2 (Th2)-type immune response.[48] It is also known now through various studies that the cytokines, interferon gamma, a T Helper 1 (Th1)-type cytokine, in males and interleukin-6 (IL-6), a T Helper 2 (Th2)-type cytokine, in females are the main players and regulators of immune system in a gender specific manner.[51-53] In other words, the T Helper 1 (Th1)-type immune response is predominant in males whereas T Helper 2 (Th2)-type immune response is predominant in females. LT-α is a T Helper 1 (Th1)-type cytokine and it is possible that LT-α may possibly be more active and may possibly have a more profound immunomodulatory role in males in comparison to females. This enhanced immunomodulatory role may in part be achieved through increased expression of LT-α in males in comparison to females. Further, the increased expression and circulating levels of LT-α have been associated with the development of various cancers including CRC.[13,54-56] Therefore, with respect to our findings, it is reasonable to argue that the affect of LT-α+252G allele resulting in the increased expression, serum levels and activity of LT-α will be more profound in males in comparison to the females considering the predominance of T Helper 1 (Th1)-type response in males. This may explain our finding that the females carrying LT-α+252G allele were at a decreased risk of developing CRC in comparison to males. However, this hypothesis needs to be mechanistically validated to obtain conclusive evidence. Further studies involving comprehensive mechanistic evaluation of the possible gender specific differences in the expression and circulating levels of LT-α protein are warranted to arrive at a conclusive explanation, which may also possibly substantiate our hypothesis. Further, it is important to emphasize here that the immune response may be regulated differently in a gender-specific manner but the outcome, that is, the preservation of immune homeostasis is similar in both males and females.

In this study, we also evaluated the association of LT-α+252A/G SNP with the numbers and the frequencies of the subsets of various characteristics of the case group subjects under study, i.e., age, gender, dwelling, smoking status, tumor location, tumor grade, and lymph node status.

When stratifying by gender, we found that the male participants who carried the heterozygous genotype (AG) of LT-α+252A/G SNP were at an increased risk of developing CRC in comparison to females. This finding is similar to the one observed in case of effect modification analysis and as such has already been explained.

When stratifying by smoking status, we found that the subjects, who carried the heterozygous genotype (AG) of LT-α+252A/G SNP and were smokers, had an increased risk of developing CRC in comparison to non-smokers. Smoking is an established risk factor of CRC.[57-59] Smoking has been reported to induce inflammatory response[60] by stimulating and increasing the production of various pro-inflammatory cytokines such as TNF-α, LT-α IL-1, IL-6, IL-8 and GM-CSF and by inhibiting and/or decreasing the production of anti-inflammatory cytokines such as IL-10.[60-63] Smoking has also been reported to decrease NK cell function and impair regulatory T cell (Treg) activity.[63] In a broader sense, the smokers in comparison to nonsmokers are exposed to an increased burden of the carcinogenic agents from cigarette/tobacco smoking which increases the risk of developing CRC in comparison to nonsmokers. Further, the heterozygous genotype (AG) is associated with moderate increase in LT-α production in comparison to low producer genotype (AA). Also increased expression and circulating levels of LT-α have been associated with the development of various cancers including CRC.[13,54-56] Thus, in response to our finding, it is reasonable to argue that for the same genotype LT-α+252AG and the level of other environmental exposures, smokers compared to nonsmokers were at an increased risk of developing CRC.

When stratifying by lymph node status, we found that the subjects who carried the heterozygous genotype (AG) of LT-α+252A/G SNP were at an increased risk of lymph node infiltration. As discussed already, the heterozygous genotype (AG) is associated with a moderate increase in LT-α production in comparison to low producer genotype (AA).Further, the increased expression and circulating levels of LT-α have been associated with the development of various cancers including CRC.[13,54-56] and more severe outcome in some cancers,[20,64] which manifests itself through an increased tumor invasion and metastasis and is partly reflected through increased lymph node infiltration. Therefore, with reference to our finding that the carriers of the heterozygous genotype (AG) of LT-α+252A/G SNP were at an increased risk of lymph node infiltration, it is reasonable to argue that the increased LT-α expression, tissue/serum levels and eventually the increased LT-α activity associated with the LT-α+252AG genotype may be responsible for the increased colorectal tumor invasion and metastasis which encompasses increased lymph node infiltration. However, further studies involving comprehensive mechanistic evaluation of the expression and the circulating levels of LT-α protein in various phases of colorectal tumorigenesis and their possible role in tumor progression are warranted to prove our hypothesis.

In this study, we found that the genotype frequencies of LT-α+252A/G SNP among the case group subjects deviated significantly from the HWE. The main reasons responsible for the deviations from the HWE include inbreeding,

consanguinity, population stratification, small population size, migration, mutations, and genotyping errors[65-67] and some of these are possibly responsible for the deviations from HWE observed in our study. The Kashmiri population consisting mostly Muslims represents an almost pure ethnic population[68] among whom consanguineous marriages are quite common and often traditional.[69] Further, due to overall genetic isolation from the rest of the world, the study population also exhibits a considerable degree of stratification and the population is also relatively small in size.[68] The genotyping errors resulting from technological and human reasons or both also represent an important reason behind the deviation from HWE in association studies but the genotyping errors are unlikely to be responsible for the deviations from HWE observed in the present study because we consistently obtained a high degree of the genotyping reproducibility for the samples studied. We obtained a weighted kappa coefficient of 0.99, which corresponded to a high concordance rate of 99% for the SNP studied.

To the best of our knowledge, the present study is the first of its kind with regard to examining the association of LT-α+252A/G SNP in the Kashmiri population. The use of clinically diagnosed and histopathologically confirmed CRC samples, involvement of population-based controls in addition to the hospital-based controls which were recruited from the same geographical area during the same time period and were matched to the case group subjects individually for age, sex, place of residence (rural/urban), smoking habit and ethnicity to minimize the confounding effect of these relevant factors and the adjustment of results for multiple potential confounding (third) variables can be described as the major strengths of this study. The major limitation of this study is the modest sample size to detect comprehensively the gene-gene and gene-environment interactions which usually require much larger sample size. Therefore, more of the similar studies but with a larger sample size and if possible incorporating other ethnic populations are needed to substantiate our findings or elaborate our findings in a more comprehensive manner regarding the association of the SNP under study with risk of CRC. However, these limitations are unlikely to affect the outcome of this study.

Conclusion

We have demonstrated through this study that the LT-α+252A/G intronic SNP is significantly associated with risk of CRC in the ethnic Kashmiri population. However, the nature of this association could not be deciphered further in a statistically significant manner. Further studies involving a large number of CRC patients and healthy controls may possibly help to decipher this association in a statistically significant manner and may explain its influence on the CRC risk in a more conclusive manner. We have also demonstrated that there is a significant effect modification of the association between LT-α+252A/G SNP genotypes and CRC risk by gender. Further, we have also demonstrated that there is a significant association between LT-α+252A/G SNP and some characteristics of the case group subjects including gender, smoking status and lymph node status.

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Conflicts of interest

There are no conflicts of interest.

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