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Exploring the Role of Lymphotoxin-alpha +252A/G SNP in Colorectal Cancer among Kashmiri Ethnic Groups: A Case-Control Analysis

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Abstract

Chronic inflammation is a key factor in the development of colorectal cancer (CRC). Lymphotoxin-alpha (LT-α), a multifunctional pro-inflammatory cytokine, has shown both tumor-promoting and tumor-suppressing properties. The relationship between the intronic LT-α +252A/G single-nucleotide polymorphism (SNP) and various cancers, including CRC, has been widely studied with inconsistent findings. This study investigated the association between the LT-α +252A/G SNP and CRC susceptibility in the Kashmiri population. The frequencies of LT-α +252A/G SNP genotypes were compared between 142 CRC patients and 184 healthy controls using PCR-RFLP. Logistic regression models, adjusted for potential confounders, were used to assess the association between the SNP and CRC risk. The study also evaluated the relationship between clinicopathological characteristics, environmental factors, and the SNP in the case group. A significant association was found between the LT-α +252A/G SNP and CRC risk ($P = 0.013$). The effect of this association was influenced by gender ($P = 0.046$). In addition, significant correlations were observed between the SNP and gender ($P = 0.0014$) and lymph node status ($P < 0.0001$) in the case group. Our findings suggest that the LT-α +252A/G SNP is associated with CRC risk in the Kashmiri population, although further research is needed to clarify the specifics of this association. Replication of the study with larger sample sizes and among different populations is necessary to confirm and enhance the results.

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

Introduction

Colorectal cancer (CRC), encompassing neoplasms of the colon, rectum, and appendix, is one of the most prevalent cancers globally and a major contributor to cancer-related morbidity and mortality. It is the third most common cancer in men and the second in women worldwide, accounting for over 1.2 million new cases annually and approximately 600,000 deaths each year [1, 2]. In the Kashmir Valley, located in the northern part of India, CRC is the third most common gastrointestinal cancer, following esophageal and gastric cancers [3], and ranks as the fourth most common cancer in men and the third in women [3, 4]. The region is home to a unique ethnic population with distinct dietary habits, setting it apart from other regions both in India and globally.

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The development of CRC is closely linked to the body's immune response and intestinal inflammation. Chronic inflammation plays a pivotal role in the initiation and progression of colorectal tumors, not only fueling inflammatory responses but also driving pro-tumorigenic processes by releasing various mediators, such as cytokines. These cytokines promote tumor growth, survival, angiogenesis, invasion, and metastasis, underscoring the complex interaction between inflammation and cancer development [5, 6]. Both pro-inflammatory and anti-inflammatory cytokines are key players in modulating immune responses and are central to the pathogenesis of colorectal cancer [7].

Lymphotoxin-alpha (LT-α) is a versatile pro-inflammatory cytokine that significantly influences immune regulation, including inflammatory responses, immune stimulation, antiviral defenses, and cytotoxic activity against tumor and infected cells [8, 9]. LT-α also plays a crucial role in lymphoid tissue development, particularly in secondary and tertiary lymphoid organs such as Peyer's patches, lymph nodes, and the spleen [10, 11]. These organs are essential for initiating and maintaining adaptive immune responses, further emphasizing the importance of LT-α in immune-mediated processes related to tumorigenesis.

Lymphotoxin-alpha (LT-α) is a multifunctional pro-inflammatory cytokine that regulates immune responses such as inflammation, immune stimulation, antiviral activity, and cytotoxic effects on infected or tumor cells. LT-α is also a key player in lymphoid tissue formation, being essential for the development and maintenance of lymphoid structures such as Peyer's patches, lymph nodes, and the spleen, which are crucial for initiating and sustaining adaptive immune responses.

The role of lymphotoxin-alpha (LT-α) in cancer development, including colorectal cancer (CRC), is complex and remains controversial, as it shows both cancer-promoting and cancer-inhibiting properties. LT-α plays a key role in immune defense, particularly in tumor surveillance, through its cytotoxic effects on cancer cells. It also activates natural killer (NK) cells, which are critical in the immune system's defense against tumors and in preventing metastasis. LT-α is involved in enhancing the differentiation and recruitment of NK cells to tumors. However, despite these protective actions, research has also indicated that LT-α may contribute to the development of several cancers, including CRC [12].

The LT-α gene resides in the class III region of the major histocompatibility complex (MHC) on chromosome 6 at position 21.3. This gene is known for its high polymorphism, with numerous variations, particularly single nucleotide polymorphisms (SNPs), present in both its promoter and other regions. Some of the SNPs, such as rs1041981, rs2239704, rs2229094, rs746868, and rs909253, have been shown to influence gene expression. These genetic variations can affect LT-α production, leading to differences in immune responses, which may impact an individual's vulnerability to diseases, including cancer [13-20].

The LT-α +252A/G SNP (rs909253), also known as IVS1 +90 A/G, refers to a genetic variation where adenine (A) is replaced by guanine (G) at the +252 nucleotide position within the first intron of the LT-α gene. This polymorphism has been linked to changes in LT-α expression and activity. Specifically, the AG and GG genotypes, as well as the G allele, have been associated with higher LT-α expression, increased tissue and serum levels, as well as greater activity, compared to the AA genotype and A allele [21-25]. The more common +252A allele is referred to as LT10.5 or LT-α (10.5 kb), while the less frequent +252G allele is known as LT5.5 or LT-α (5.5 kb) [26]. Studies show that individuals with the LT-α +252GG genotype and G allele exhibit a 1.5-fold higher LT-α gene expression compared to those with the LT-α +252AA genotype, with corresponding increases in LT-α levels in both tissue and serum [23, 27-29].

Additionally, the LT-α +252A/G SNP is involved in regulating tumor necrosis factor-alpha (TNF-α), with the G allele linked to elevated TNF-α expression, and higher serum and tissue levels compared to the A allele [28, 29]. The exact functional role of the LT-α +252A/G SNP remains unclear, it is suggested that the variation affects transcriptional regulation. The A to G substitution at the +252 position is believed to enhance the binding of a yet-to-be-identified nuclear factor (NF), which binds with greater affinity to the +252G allele, leading to a 1.5-fold increase in LT-α protein expression compared to the +252A allele [23]. The SNP's influence on LT-α expression and activity may trigger the activation of NF-κB, enhancing NF-κB signaling. This, in turn, could modulate inflammatory responses and potentially drive the activation of pathways linked to various diseases, including several types of cancer.

Numerous studies have explored the potential link between the LT-α +252A/G SNP and the risk of various cancers, such as 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, only a limited number of studies have examined the connection between the LT-α +252A/G SNP and the risk of colorectal cancer (CRC) [18, 40].

In this research, we conducted a comprehensive case-control study to investigate the potential association between the LT-α +252A/G SNP and CRC susceptibility in the Kashmiri population. Additionally, we evaluated how factors such as age, gender, and smoking status might influence the relationship between the SNP and CRC risk. We further explored the possible connections between the LT-α +252A/G SNP and various clinicopathological features, demographic characteristics, and environmental factors like smoking, to understand their role in modulating CRC risk within the studied population.

Materials and Methods

Study groups

This study involved two groups: the case group and the control group. The case group consisted of 142 patients with primary colorectal cancer (CRC) who were selected consecutively based on their diagnosis at the Department of General Surgery, Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, Kashmir. All patients underwent surgery for CRC, and the diagnosis was confirmed histologically. Tumor staging and classification followed the TNM system (8th edition) from the Union for International Cancer Control. Only patients who had not undergone neoadjuvant therapy, including chemotherapy or radiotherapy, were included. All participants were over 18 years old and had no previous cancer history. Blood and tissue samples were collected from these patients. The control group included 184 healthy individuals with no personal or family history of cancer or significant illness, matched for age, sex, place of residence (rural/urban), smoking habits, and ethnicity to the case group. Both groups consisted of ethnic Kashmiris.

Data gathering

Data for CRC patients, including clinical details, demographic information, and environmental factors, were collected from medical records, pathology reports, and personal interviews. If patients were unable to communicate, interviews were conducted with their guardians. Interviews were carried out in the local language to ensure clarity and obtain accurate information. The collected data included tumor characteristics such as location, stage (Dukes), lymph node involvement, and factors like age, sex, residential area, ethnicity, smoking habits, and family cancer history. For the control group, data were gathered via interviews, focusing on similar demographic details. Data collection was performed by trained research staff to maintain high-quality standards. All participants, including patients and controls, provided informed consent using a pre-designed questionnaire. This study adhered to ethical guidelines set by the Institutional Ethics Committee of SKIMS and the World Health Organization's Declaration of Helsinki (1964, with its seventh amendment in 2013) [41].

Sample collection and DNA extraction

After surgical resection, tumor tissue samples were promptly frozen in liquid nitrogen and stored at −80 °C for later DNA extraction and other analyses. Blood samples (3–5 ml) from both case and control individuals were collected using venipuncture into EDTA-coated vacutainer tubes (purple caps; ADS Hitech Polymers, India) and stored at −80 °C until DNA extraction. Genomic DNA was isolated from both tumor tissues and blood samples using the DNeasy™ Blood and Tissue Kit (catalog no. 69504; Qiagen, Germany) and the Quick-gDNA™ MiniPrep Kit (catalog no. D3024; Zymo Research, USA) by following the manufacturer's protocols. The extracted DNA was stored at −20 °C for subsequent use. DNA quality and concentration were assessed by spectrophotometry at 260 nm and 280 nm, along with agarose gel electrophoresis to verify integrity. The DNA derived from blood samples of both groups was used for the SNP genotyping analysis.

SNP Genotyping Methodology

To analyze the LT-α+252A/G SNP, the PCR-RFLP technique was employed for genotyping.

PCR Amplification of LT-α+252A/G SNP Region

PCR amplification of the region containing the LT-α+252A/G SNP was conducted in a total reaction volume of 25 µL, which included 100 ng to 1 µg of genomic DNA, 0.7–1 U Taq DNA polymerase with 1X standard reaction buffer (New England Biolabs, UK), 1.8 mM

MgCl2, 0.28 mM dNTP mix (New England Biolabs, UK), and 0.56 µM of both forward and reverse primers (Integrated DNA Technologies, India), along with nuclease-free water (Qiagen, Germany) to bring the final volume to 25 µL. In some cases, Phusion DNA Polymerase (New England Biolabs, UK) and its associated buffer were used instead of Taq polymerase to minimize errors due to amplification.

The PCR cycling conditions were as follows: initial denaturation at 95 °C for 6 minutes, followed by 35 cycles consisting of denaturation at 95 °C for 45 seconds, annealing at 64 °C for 60 seconds, and extension at 72 °C for 45 seconds, with a final extension at 72°C for 10 minutes. The primers used for amplification were 5'-CCGTGCTTCGTGCTTTGGACTA-3' (forward) and 5'-AGAGCTGGTGGGGACATGTCTG-3' (reverse). The resulting PCR product, corresponding to the LT-α+252A/G SNP, was 741 base pairs in length, see **Figure 1** for gel image of PCR results.

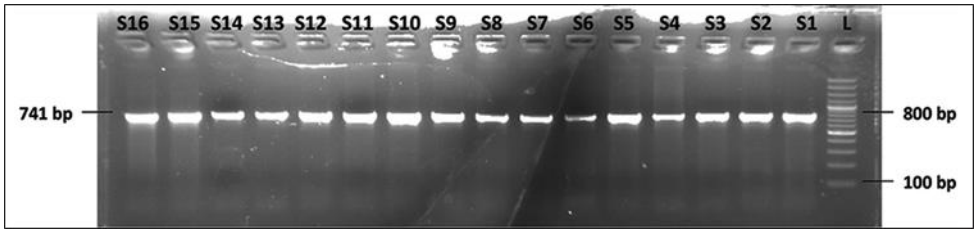

Figure 1. Electrophoresis of LT-α+252A/G SNP PCR products on a 2.5% Agarose Gel. Lanes S1-S16: Amplified PCR products with prominent/desired band 741 bp in size. Lane L: 100 bp Molecular size marker/Ladder

Genotyping of LT-α+252A/G SNP

The LT-α+252A/G SNP was genotyped using the NcoI restriction enzyme (Thermo Fisher Scientific, USA). In a 30 µL reaction mixture, 10 µL of PCR product was combined with 10 U of enzyme and incubated overnight at 37 °C. The AA genotype remains intact (741 bp), while the GG variant is cut into two fragments (545 bp and 196 bp). The AG genotype results in three fragments: 741 bp, 545 bp, and 196 bp. The digestion products were analyzed by electrophoresis on a 3% agarose gel stained with ethidium bromide (0.5 µg/ml), as shown in **Figure 2**.

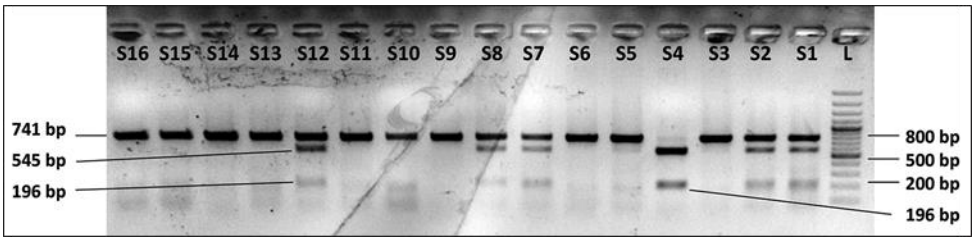

Figure 2. Gel image of LT-α+252A/G SNP genotyping using PCR-RFLP. The AA genotype shows one 741 bp band, the GG variant displays two fragments (545 bp and 196 bp), and the AG genotype produces three bands (741, 545, and 196 bp). Lane L shows the molecular marker

Quality Control

To ensure accurate genotyping, we performed quality control measures to assess potential errors, including misclassification of alleles and genotype frequencies and checked the reproducibility of results. For this, about 10% of both were randomly selected and re-genotyped. Additionally, each PCR-RFLP run included previously genotyped samples as positive controls representing different genotypic patterns. The reproducibility of genotyping was very high, with a weighted kappa coefficient of 0.99, indicating 99% agreement in results [42, 43].

Statistical Analysis

Genotype and allele frequencies for the SNP were calculated via direct counting. Categorical variables were presented as counts and percentages, while continuous variables were summarized by mean, standard deviation, median, and interquartile range. Conditional logistic regression was employed to compute unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) to assess the SNP-genotype's association with CRC risk, including possible gene-environment interactions. To adjust for confounders, models accounted for age, gender, smoking status, and residence. The analysis also considered the modification of CRC risk by these factors. Fisher's exact test was used to examine correlations between genotypes and clinicopathological factors, demographics, and environmental variables within the case group. Hardy-Weinberg equilibrium was assessed using the Chi-square test. A P-value ≤ 0.05 was considered statistically significant. All analyses were conducted using IBM SPSS, Armonk, New York.

The study's statistical power, based on the effective sample size, was calculated using the "Genetic Power Calculator" by Purcell *et al.* (http://zzz.bwh.harvard.edu/gpc/), yielding a power score of approximately 77% for the SNP in this case-control study with 142 cases and 184 controls.

Results and Discussion

Table 1 presents the distribution of various clinicopathological, demographic, and environmental factors for both CRC patients and control subjects. The distribution of LT-α+252A/G SNP genotypes is shown in **Table 2**. Among the cases, the AA genotype was seen in 59.86% (85/142) of individuals, closely mirroring the frequency found in the control group at 60.87% (112/184). The AG heterozygous genotype was more prevalent in the case group (40.14% [57/142]) than in the control group (33.7% [62/184]). Interestingly, the GG variant genotype was not found in the case group but accounted for 5.43% (10/184) of the control group. When looking at allele frequencies, the LT-α+252A allele was more common in the cases (79.93% [227/284]) than the controls (77.72% [286/368]), while the LT-α+252G allele was found in 20.07% (57/284) of cases and 22.28% (82/368) of controls. The combination of the variant genotypes (AG + GG) was 40.14% (57/142) in the case group, which was similar to the control group's frequency of 39.13% (72/184) **(Table 2)**. Statistically significant results were obtained between the LT-α+252A/G SNP and CRC risk (P = 0.013) **(Table 2)**, though further analyses did not yield consistent findings. Hardy-Weinberg equilibrium was observed in the control group ($\chi 2 = 0.135$; P = 0.713), but the case group did not follow this pattern ($\chi 2 = 8.953$; P = 0.002).

Table 1. Demographic and clinical characteristics of study participants

Characteristics	Colorectal cancer cases (n=142)	Controls (n = 184)	Pearson $\chi 2$; P-value
Age (years)			
Mean age (SD) (SEM)	52.68 (15.34) (1.29)	52.22 (14.57) (1.07)	0.29; 0.59
Age range (median)	21-82 years (55 years)	21-80 years (51.5 years)	
≤ 50 years (n (%))	66 (46.48%)	91 (49.46%)	
> 50 years (n (%))	76 (53.52%)	93 (50.54%)	
Gender			0.64; 0.42
Male	85 (59.86%)	102 (55.43%)	
Female	57 (40.14%)	82 (44.57%)	
Place of Residence			1.33; 0.25
Rural	87 (61.27%)	101 (54.89%)	
Urban	55 (38.73%)	83 (45.11%)	
Smoking Status			0.89; 0.35
Ever	80 (56.34%)	94 (51.09%)	
Never	62 (43.66%)	90 (48.91%)	
Tumor Location			
Colon	58 (40.85%)		
Rectum	84 (59.15%)		
Tumor Grade			
Well-differentiated (WD)	95 (66.90%)		
Moderately/Poorly differentiated (MD/PD)	47 (33.10%)		
Lymph Node Status			

Involved	78 (54.93%)
Not involved	64 (45.07%)

Table 1 summarizes various clinical, demographic, and environmental factors for colorectal cancer patients and control participants from Kashmir. The analysis was done using Pearson's Chi-square test to assess categorical variables. *n refers to the number of participants in each group, SD indicates standard deviation, and SEM denotes the standard error of the mean. WD represents well-differentiated, MD refers to moderately differentiated, and PD stands for poorly differentiated tumor grades.

Table 2. Frequency distribution of LT-α+252 A/G SNP genotypes and their association with colorectal cancer risk

Genotype/Allele	CRC cases (n = 142)	Controls (n = 184)	OR (95% CI); P-value	Adjusted OR (95% CI); P	χ2; Pearson P-value (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)		0.467; 0.494
G	57 (20.07%)	82 (22.28%)	1.14 (0.78-1.67); 0.50		

n refers to the number of individuals in each group. Bolded values represent statistically significant results. Adjusted odds ratios (ORs) and their confidence intervals (CIs) were calculated using conditional logistic regression, adjusted for age, gender, smoking status, and place of residence. Overall statistical significance was assessed using Pearson's Chi-square test. CRC: Colorectal cancer, OR: Odds ratio, CI: Confidence interval.

The relationship between the LT-α+252A/G genotypes and colorectal cancer (CRC) risk, influenced by factors like age, gender, and smoking, is summarized in **Table 3**. When evaluating the interaction of these factors, a notable influence of gender on the association of the combined variant genotype (AG + GG) with CRC risk was observed (P = 0.046). Specifically, females carrying this genotype had a lower likelihood of developing CRC (OR = 0.42 [95% CI = 0.19–0.92]; P = 0.030).

Table 4 outlines the distribution of various characteristics among the case group subjects, including age, gender, smoking habits, tumor location, tumor grade, and lymph node involvement, all about the LT-α+252A/G SNP. A significant relationship was identified between this SNP and gender (P = 0.0014), with male participants showing a higher risk for CRC if they carried the heterozygous AG genotype compared to females (OR = 3.07 [95% CI = 1.52–6.19]; P = 0.0017).

Furthermore, smoking status was found to have a significant association with the SNP (P = 0.0141), with smokers who had the AG genotype at an elevated CRC risk (OR = 2.35 [95% CI = 1.18–4.66]; P = 0.0163). The LT-α+252A/G SNP also exhibited a very strong correlation with lymph node status (P < 0.0001), with individuals carrying the AG genotype being much more likely to experience lymph node involvement (OR = 4.54 [95% CI = 2.21–9.31]; P < 0.0001). Some statistical details are not presented in **Table 4**.

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

Genotype^ and characteristic	CRC cases (n (%))	Controls (n (%))	OR (95% CI); P-value[#]	Adjusted OR[§] (95% CI); P-value[#]	χ^2; Pearson P-value (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 at 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 clinicopathological 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	48 (56.47%)	36 (63.16%)	0 (0%)	

	(59.15%)				
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

In this research, we investigated the role of the LT-α+252A/G SNP, situated in the first intron of the LT-α gene, as a factor influencing the risk of colorectal cancer (CRC) in the Kashmiri ethnic population, using a case-study design with 142 CRC patients and 184 control subjects.

We examined the distribution of LT-α+252A/G genotypes among CRC cases and controls, finding a significant association between the SNP and CRC risk in the study population. However, this relationship could not be definitively clarified due to the lack of statistical significance in further analysis. The reason for these findings could be attributed to various complex factors. Notably, the more strongly expressed LT-α+252GG genotype was completely absent in the case group, possibly leading to a haploinsufficiency effect. This absence might have resulted in a less pronounced effect of the LT-α+252G allele on CRC risk, despite its presence. In simpler terms, while an association was evident, the absence of the GG genotype likely diminished the clarity of this effect. This phenomenon might reflect higher LT-α expression linked to the heterozygous AG genotype in the case group compared to the AA genotype, but not at a level sufficient to provide conclusive evidence. Larger-scale studies involving a higher number of patients with CRC and controls could potentially yield more definitive results, helping to further elucidate the role of this SNP in CRC risk and reinforcing our hypothesis. Although our results did not conclusively demonstrate the mechanism of risk modulation, they are in line with previous studies suggesting a link between LT-α+252A/G and CRC risk.

Our analysis also explored whether age, gender, and smoking status modified the association between the LT-α+252A/G genotypes and CRC risk. We observed a significant gender-based modification of the relations between the combined various genotypes (AG + GG) and CRC risk, with females carrying these genotypes showing a lower risk of CRC compared to males. This observation might be explained by the established differences in immune regulation between genders, influenced by sex hormones like androgens and estrogens. Androgens tend to favor T Helper 1 (Th1) responses, whereas estrogens promote Th2 responses. In males, LT-α, a Th1-type cytokine, may be more active, potentially having a stronger immunomodulatory role. The higher LT-α expression in males might explain why the effect of the LT-α+252G allele is more pronounced in men. In contrast, females, with their predominant Th2-type immune response, may exhibit a lesser immunological impact from the LT-α+252G allele. While this hypothesis offers a potential explanation, it requires further validation through mechanistic studies to confirm the gender-specific effects of LT-α in CRC.

In this study, researchers also examined the relationship between LT-α+252A/G SNP genotypes and different characteristics of the CRC patient group, including age, gender, tumor location, smoking status, tumor grade, and lymph node involvement. Stratifying by gender revealed that male participants carrying the heterozygous AG genotype had a significantly higher risk of developing CRC compared to females, aligning with our earlier findings of gender-based effect modification.

We observed that smokers carrying the AG genotype had a higher risk of CRC compared to non-smokers. Smoking is a well-established risk factor for CRC and is known to stimulate the production of pro-inflammatory cytokines, including TNF-α and LT-α. It also inhibits anti-inflammatory cytokine production and impairs immune cell function, which can contribute to the development of CRC. As smokers are exposed to more carcinogenic compounds, the combined effect of smoking and the LT-

α+252AG genotype likely contributes to an elevated CRC risk.

In terms of lymph node status, our researchers understood that participants with the AG genotype were more likely to experience lymph node infiltration, a sign of more severe cancer progression. Increased LT-α expression, which is linked to the AG genotype, had relations with the development and metastasis of different cancers, including CRC. This elevated LT-α expression could be responsible for promoting metastasis and tumor invasion, as reflected in increased lymph node infiltration. Also, additional studies are needed to explore the mechanistic role of LT-α in tumor progression.

The genotype frequencies of the LT-α+252A/G SNP in the case group did not adhere to Hardy-Weinberg equilibrium (HWE). Possible causes for this deviation include inbreeding, population stratification, small sample size, or errors in genotyping. The Kashmiri population, predominantly Muslim, has a high rate of consanguineous marriages and is genetically isolated, which could contribute to these deviations. However, we believe genotyping errors were unlikely to have influenced our findings, as we achieved a very high degree of reproducibility in genotyping with a weighted kappa coefficient of 0.99. Our study confidentially is, the first to examine the LT-α+252A/G SNP in the Kashmiri population. Strengths of the study include the usage of clinically diagnosed CRC samples, the involvement of both population-based and hospital-based controls matched by age, sex, residence, smoking status, and ethnicity, and the adjustment for multiple confounding factors. However, the study's relatively modest sample size is a limitation, particularly when it comes to detecting gene-environment and gene-gene interactions, which often require larger samples. Future studies with larger populations, possibly including other ethnic groups, are necessary to substantiate these findings and provide a more comprehensive understanding of the LT-α+252A/G SNP's role in CRC risk.

Conclusion

In this study, we have shown that the LT-α+252A/G SNP in the intronic region of the LT-α gene is significantly linked to CRC risk in the Kashmiri population. However, the exact nature of this association remains unclear due to the lack of statistical importance in the analysis. Larger-scale studies, including more healthy controls and CRC patients, are necessary to better clarify this relationship and provide more conclusive insights into how this SNP influences CRC risk. Also, we have observed a notable gender-based edit of the relationship between LT-α+252A/G genotypes and CRC risk. Our findings also highlight significant correlations between the LT-α+252A/G SNP and various characteristics of CRC patients, including lymph node involvement gender, and smoking status.

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