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Newly identified genetic variant rs2294693 in *UNC5CL* gene is associated with decreased risk of esophageal carcinoma in the J&K Population–India

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Abstract: Esophageal cancer is the second most common type of cancer after lung carcinoma in the state of Jammu and Kashmir (J&K). The understanding of genetics in Esophageal cancer development is poor in the state. Genome wide association studies (GWAS) has proved to be unsurpassed tool in identification of new loci associated with different cancers. GWAS in Chinese population has identified SNP rs2294693 present in *UNC5CL* (UNC-5 Family C-Terminal like) to be associated with non-cardia gastric cancer. We performed a case control association study and genotyped the SNP rs2294693 using Taqman allele discrimination assay in 566 individuals (166 esophageal cancer patients and 400 controls) belonging to the J&K population. A statistically significant protective association with allelic odds ratio of 0.73 (0.56–0.94 at 95% CI) and p value = 0.016 was observed. This is the first study in relation to esophageal cancer in the Jammu and Kashmir population, so far it has been studied in association with gastric carcinoma in the Chinese population only. The results indicate that the polymorphism rs2294693 is associated with esophageal cancer susceptibility and the mutant (T) allele might be a protective factor for esophageal cancer among Jammu and Kashmir population. Further the functional characterization of the variation is also warranted.

Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth leading cause of death from cancer throughout the world (Zhang *et al.*, 2012; Yousefi *et al.*, 2018). It has been observed that about 5,72,034 new cases have been reported in 2018 and each year accounts for about 90% of the 456,000 ESCC (esophageal squamous cell carcinoma) cases (Abnet *et al.*, 2018). The mortality rate of esophageal cancer closely follows the geographical patterns for incidence (Kachala, 2010). Esophageal cancer has been divided into two major subtypes: Esophageal Squamous Cell Carcinoma (ESCC) and Esophageal Adeno Carcinoma (EAC) (Blot and McLaughlin, 1999). ESCC is most prevalent in developing countries like India and Iran, while EAC is common in developed countries like the USA (Mir and Dar, 2009; Barbhuiya *et al.*, 2018). About 90% of the esophageal cancers are ESCC, 5% are EAC, and 5% are other rare forms of esophageal cancer in Kashmir valley (Mir and Dar, 2009). A number of risk factors are associated with esophageal carcinoma. Alcohol consumption, hot drinks, and smoking are being primarily associated with ESCC, and obesity, smoking, and acid reflux are associated with EAC (Hazelton *et al.*, 2015).

Esophageal cancer is the second-largest prevailing cancer in Jammu and Kashmir, India, after lung cancer (Dikshit *et al.*, 2012), and it accounts for 60% of all cancers along with gastric cancer in the region (Shah *et al.*, 2020). The population of Jammu and Kashmir (J&K) is highly diverse in terms of ethnicity and culture (Sharma *et al.*, 2018). The population of Jammu and Kashmir is ethnically distinct and have special dietary habits and weather. Due to these climatic conditions of Kashmir valley, the population usually depend on preserved foods like smoked pickle, sundried vegetables, and these food products contain carcinogens like N-nitrosodimethylamine (NDMA), methyl nitrosourea (NMU), N-nitrosopyrrolidine (NPYR), that could lead to adverse effects in various organs leading to favorable condition for cancers (Chikan *et al.*, 2012). Studies have

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shown that both cases, as well as controls in the population of J&K, are exposed to the same type of dietary habits. These studies also suggest that there is a chance of a higher rate of esophageal cancer in the population that is exposed to common dietary risk factors than those without such exposures (Murtaza et al., 2006). There has been a rapid increase in ESCC in Kashmir according to the cancer registry of SKIMS (Sher-E-Kashmir Institute of Medical Sciences) from years 2001–2003. Incidence of ESCC is low in Jammu and Ladakh compared to Kashmir valley (Khuroo et al., 1992; Iqbal et al., 2015). Alterations in the genetic makeup of an individual play an important role in esophageal carcinogenesis (Xing et al., 2003). Genome-wide studies have been widely used to identify the novel common variants associated with different cancers. GWAS (Genomic Wide Association Studies) in the Asian population identified variant rs2294693 of UNC5CL associated with gastric non-cardia cancer risk (Hu et al., 2016). This genetic variant in UNC5CL falls in a genomic region that is in close proximity with two SNPs, rs10484761 and rs2494938, which are a hotspot for upper gastrointestinal cancers and has been associated with esophageal squamous cell carcinoma and non-cardia gastric cancer in Chinese populations (Hu et al., 2016). In the current study, we tried to explore the genetic variant in the population of J&K (Jammu and Kashmir) to find if it is associated with the increased or decreased risk of ESCC. It should be noted that both the frequency of SNPs and the extent of LD varies significantly between different populations (Erichsen and Chanock, 2004). In order to determine the clinical implication of the variant, it is essential to replicate the newly identified variants in other ethnicities (Jing et al., 2014) to ascertain and strengthen the findings of GWAS.

Materials and Methods

Sampling
A total of 566 individuals (166 cancerous and 400 healthy controls) were recruited for the study after approval from the Institutional Ethical Review Board (IERB)–Shri Mata Vaishno Devi University (SMVDU) under notification number (SMVDU/IERB/16/43) dated 12/05/2016. The cancer cases were recruited only after the histopathological confirmation. All the patients recruited were neither on chemotherapy nor radiotherapy. All the control samples recruited had no prior family history of ESCC or related malignancy. A 2 mL-venous blood sample was collected, and written informed consent was obtained from all the participants. Details of both cases and controls have been coded and no information which reveals the identity of the subjects has been divulged. The details of cases and controls are given in Tab. S1.

DNA isolation
The genomic DNA was isolated by phenol–chloroform method given by Ghatak et al. (2013) and Flexi gene® DNA Kit. The DNA was quantified using the Eppendorf's Bio-spectrophotometer^{TM} at wavelengths of 260 nm and 280 nm. The amount of DNA was calculated using the following formula: DNA ng/mL = OD at 260 nm × 50X

dilution factor. The ratio at 260/280 was taken as criteria to check the purity of DNA.

Genotyping
The TaqMan allele discrimination assay was adopted to perform the genotyping using real-time PCR (Agilent Stratagene M × 3005p, Wald Brown, Germany) following the procedure given by Bhat et al. (2019) (21). TaqMan ®SNP Genotyping assay labelled with FAM and VIC (ThermoFisher Scientific). Following is the primer sequence of the probes used in the study: TGGTATATTGGAAATTGAGACAGCA (A/G) CTTCTCTGCATCAAGGAGACTCTTA; and UNG Mastermix II (Applied Biosystems, ThermoFisher Scientific, Foster City, CA, USA) were used. Following thermal cycling conditions were adopted; 10 min at 95°C, 40 cycles of 95°C for 15 s, and 60°C for 1 min. In each 96-well, 3 NTC's (non-template controls) were used for quality control. The genotype data were analyzed by Agilent MX3005p Post PCR detection software. As a quality control measure, 40 cases and 40 controls were randomly selected and validated by genotyping, no mismatch was found.

Statistical analysis
The Chi-square (χ^2) analysis was performed to test the genotypic frequency distribution for the Hardy–Weinberg equilibrium. Logistic regression analysis was used to estimate the odds ratio (OR), at 95% confidence interval (CI) and respective level of significance as a p-value. Covariates for adjustment in this study were age, gender, and BMI (Body mass index). The statistical analyses was performed using the Statistical Package for the Social Sciences (SPSS) version 23.

Results

The case-control association study included 166 cases and 400 controls (N = 566). The genotypic and allelic frequency distribution for the studied population is given in Tab. 1. The frequency distribution of the population was in accordance with the Hardy-Weinberg equilibrium (p = 0.10). The T allele of the SNP (single nucleotide polymorphism) rs2294693 was significantly associated with the reduced risk of esophageal carcinoma in the Jammu and Kashmir population, having an allelic odds ratio (OR) 0.73 (95% CI, 0.56–0.94, p = 0.016) (Tab. 1). XT (TT + TC) genotypes under the dominant model when compared against CC genotype yielded corrected OR 0.5 (0.34–0.84, at 95% CI, p = 0.006). After correction with confounding factors like age, gender, and BMI, the observed OR observed was 0.5 (0.2–0.9, at 95% CI, p = 0.039).

Discussion

UNC5CL is also called ZU5 or ZUD (Stelzer et al., 2018). It has pro-inflammatory activity and specific expression in the epithelium, which demonstrates its association with mucosal diseases like Inflammatory Bowel Disease (IBD), and it needs to be explored in future genetic association studies (Heinz et al., 2012). In the current study, the interaction of the UNC5CL gene was also predicted using the Genemania

TABLE 1

Allele frequency distribution and risk associated with variant rs2294693 of *UNC5CL* in the studied population group from the Jammu and Kashmir, India

Gene/SNP		Total samples (566)				
		Allelic frequency distribution		Genotypic frequency distribution		
		T	C	TT	CT	CC
UNC5CL/rs2294693	Cases (N = 166)	0.497	0.503	0.241	0.512	0.247
	Controls (N = 400)	0.575	0.425	0.30	0.55	0.15
Odds ratio (95% CI)		0.73 (0.56–0.94)		0.5 (0.2–0.9)		
p-value *p*-value*		0.016		0.039		

Note: *Corrected with age, gender, and BMI.

tool of BioGRID version 3.4 (Stark *et al.*, 2006). *UNC5CL* has been found to be physically interacting with *NF-k Band REL* gene (Fig. S1). *NF-κB* is a protein-coding gene and transcription regulator (Stelzer et al., 2018). *NF-κB* has an important role in the initiation, development, and metastasis of human cancers (Xia *et al.*, 2014). *REL* is a proto-oncogene and plays a vital role in cell differentiation and lymphopoiesis. *REL/NFkB* pathway regulates immunity, apoptosis, and cell proliferation. Many studies have shown a correlation between *REL/NF-κB* factors, gene expression, and their role in malignancy (Kojima *et al.*, 2004). It has been indicated that functional inactivation of *NF-κB* in many tumor cell types induce apoptosis and alienate oncogenicity (Rayet and Gelinas, 1999). The interactive functional role of *UNC5CL*, *NF-κB*, and *REL* in the pathogenesis of cancer needs further elucidation.

In a combined Asian population (Beijing, Henan, and Korea), a GWAS study found that the variant rs2294693 of *UNC5CL* showed risk with gastric non-cardia cancer ($p = 2.50 \times 10^{-8}$) and nominal risk for cardia cancer ($p = 1.47 \times 10^{-2}$) (Hu *et al.*, 2016). In the above study, T is the reference allele, and C is the risk allele. The result showed that EAF (Effect Allele Frequency) in cases is 0.277, and EAF in controls is 0.226 with OR = 1.14 (1.09–1.20, $p < 0.05$). The present study shows that the variant rs2294693 of *UNC5CL* gene is showing protection against esophageal carcinoma in the population of J&K population. The frequency of allele T in controls (0.575) is higher than cases (0.497), which may be a protective factor for esophageal cancer in the studied population. These results may be due to differences in ethnicity, lifestyle, and disease prevalence, as well as possible limitations due to the relatively small sample size.

The above-investigated pattern of association can occur when the investigated variant is correlated, through interactive effects or linkage disequilibrium (LD), with a causal variant (Lin *et al.*, 2007). Single nucleotide polymorphism (SNPs) varies in frequencies within ethnic groups (Moorjani *et al.*, 2013; Sharma *et al.*, 2017). It is pertinent to evaluate the variants, which are in strong LD with the studied SNP, in order to find the contributory variant associated with disease. We also evaluated the LD of the variant rs2294693 of the *UNC5CL* gene using the

Ensemble database (www.ensemble.org). The variant rs2294693 is an intronic SNP in *UNC5CL*, but it is in close proximity with 5'UTR variant rs9369260 of *TSPO* gene (Translocator protein gene) (Fig. S2). *TSPO* has been associated with cell proliferation, apoptosis, and mitochondrial function. It has been investigated that the expression of *TSPO* protein is associated with tumor malignancy (Bhoola *et al.*, 2018). *TSPO* encodes PBR (peripheral type benzodiazepine receptor) protein, which has been linked to regulation of apoptosis and immune response; because of these functions, it has been associated with a number of diseases and cancers. It has been investigated that expression level of PBR is increased in a number of malignancies, which also include esophageal cancer. PBR is considered an independent prognostic factor in ESCC (Chen *et al.*, 2017). We can hypothesize that as the rs2294693 in *UNC5CL* and rs9369260 in *TSPO* gene are in close proximity with each other, there must be a mechanism of interaction between the two and a pathway that links these two genes with ESCC.

This variant has not been studied so far in any Indian population. Our study is the first one to explore this variant in association with ESCC in J&K. Evaluation of this SNP in different Indian ethnic groups will present a better insight towards its significance, and future studies should address whether these differences in association may be related to the local incidence of cancer or not. Though the power of the study is above 90% on the basis of the incidence of ESCC in the J&K region, it is very important to replicate the study in the large cohort for a conclusive statement.

Drug response is just as complex as disease genetics, resulting not only from underlying genotypic variation at many loci, but also from variation at the level of gene expression, post-translational modification of proteins, drug dose, drug interactions, diet, and other non-genetic factors. Therefore, individual genes will be associated with effects on drug response. To have no data from the population severely undermines the importance of research that needs to be undertaken in these specific domains. This present study investigated the association of *UNC5CL* gene polymorphisms with esophageal cancer among an Indian subpopulation. The mutant allele T of the genetic variant

rs2294693 of *UNC5CL* gene is a protective factor for the development of esophageal cancer in the studied population. Evaluation of this SNP in different Indian ethnic groups will present a better insight towards the significance, and future studies should address whether these differences in association direction may be related to the local incidence of cancer or not. These results are the first step towards the genetic elucidations of cancers in the region. This Region in the past has suffered heavily due to the turmoil related to political conditions which led to the shifting of focus from diseases and looking at the recent data, these diseases have now taken alarming proportions. It is worth to mention that the region studied is the lung cancer capital of India, where smoking is at the highest. The esophagus being the doorway for lung cancer is the primary site and hotspot for the malignancy. The investigated information can be used to create diagnostic and prognostic markers. Cancer controls programs can be successful across the globe only if the cancer menace is uprooted at the regional level also. SNP study helps the medical practitioners to provide the treatment on the basis of the assessment of risk. As already informed in a previous revision, the focus on these SNPs will help in establishing the biomarkers for the esophageal cancers in the region.

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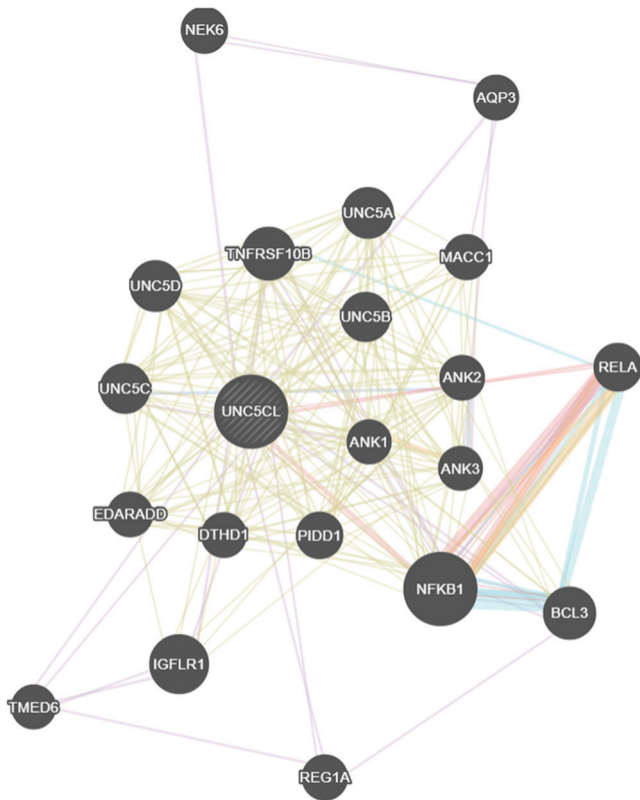
Supplementary Files

SUPPLEMENTARY TABLE 1

**Details and clinical features of the cases and controls of J&K Population, India**

Characteristics	Cases	Controls	*p*-value
Age (±SD) in years	59.1 ± 12.09	56.1 ± 13.18	0.01
BMI (±SD) in kg/m^2	21.3 ± 4.2	27.7 ± 5.26	<0.01
Metastasis	Yes = 68 No = 98		
Family History	Yes = 22 No = 46 Unknown = 98		

Note: SD: Standard deviation

SUPPLEMENTARY FIGURE S1. The Genemania network interaction of *UNC5CL* with *NF-k Band REL*.
We found the moderate protein interaction of *UNC5CL* gene with *NF-κB* (Nuclear factor-κB) and *REL* (proto-oncogene).
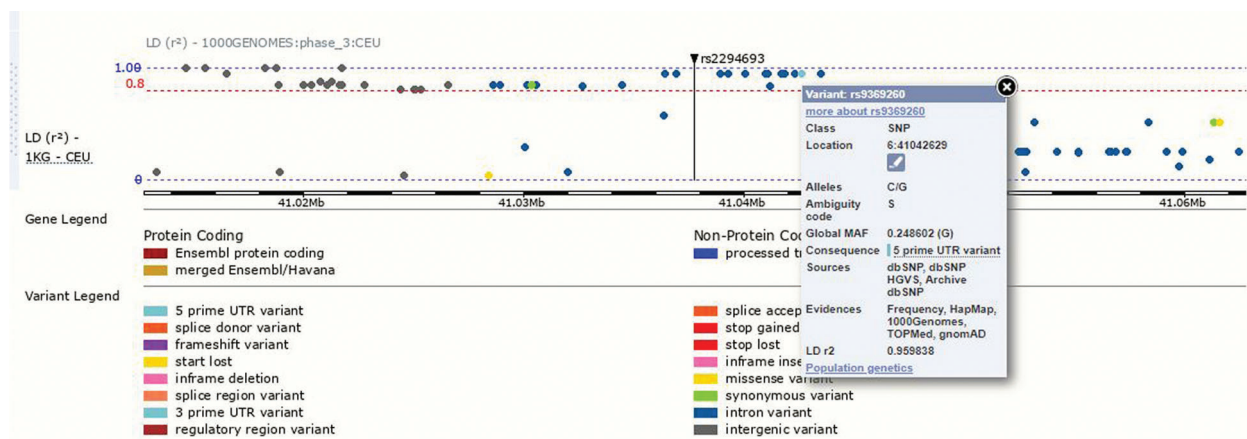
SUPPLEMENTARY FIGURE S2. The variant rs2294693 of *UNC5CL* shows close proximity with 5'UTR variant rs9369260 of the *TSPO2* gene.