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A Significant Association of IL1R2 DraIII T/G Polymorphism with the Risk of Gall Bladder Cancer in Ethnic Kashmiri Population

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Abstract

Background: Chronic inflammation is considered as an emerging area of research interest because of its cognize association with different organ cancers. Recent advances in cancer research have substantiated that targeting cytokines have a strong therapeutic potential in reducing the mortality of inflammation-related cancers. Gallbladder cancer (GBC) has been consistently associated with inflammation mostly due to presence of gallstones which prelude inflammatory response. The Interleukin-1 (*IL1*) gene cluster serves an important function of immunomodulation, thereby regulating interplay between inflammation and cancer. Studies on the association of *IL1* polymorphisms with GS and GBC have shown drastic variations in different populations. Since no such study has been carried out in ethnic Kashmiri population which is known for high incidence of GS disease, we aimed to evaluate the possible role of pro-inflammatory *IL1* family in the pathogenesis of GBC and GS disease.

Methods: A total of 370 individuals (120 GBC, 120 GS and 130 healthy controls) were prospectively recruited. The study analyzed various polymorphisms of *IL1* gene family to predict their association with GBC and gallstone disease. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) was used for genotyping and SPSS 23.0 software was used to calculate odds ratios (ORs) and confidence intervals (CIs). Tissue-specific expression of IL-1αwas done by Quantitative Real-time PCR (qRT-PCR) and the data was analyzed by Graph Pad Prism version 5.

Results: *IL1R2* T/G DraIII 'GG' genotype (OR: 2.65, 95% CI: 1.27-5.53, P=0.011) and 'G' allele (OR: 1.57, 95% CI: 1.10-2.24, P=0.014) indicated a positive association with GBC. Two polymorphisms in the *IL1* gene family (IL-1α+4845G/T and *IL1R1*Pst1C/T) were observed to be insignificant towards GBC in our study cohort. IL-1αmRNA expression did not differ between tumor and adjacent normal GB tissues. Above all none of the studied polymorphisms was significant towards gallstone disease.

Conclusion: We conclude that *IL1R2* DraIII T/G SNP bears a significant association with GBC and could be an important etiological factor for GBC in our population.

Keywords: Gall bladder cancer • Inflammation • Interleukins • Polymorphisms • mRNA expression

Abbreviations: GBC: Gallblader Cancer; GS: Gall Stone disease; IL: Interleukin; PCR: Polymerase Chain Reaction; OR: Odds Ratio; CI: Confidence Interval

Introduction

Gallbladder cancer (GBC) is a multi-factorial disease with diverse risk factors including gallstones, obesity, reproductive factors, chronic infection, and environmental exposure to specific chemicals [1-4]. It is the fifth most common malignant neoplasm of the digestive tract and despite recent advances in the diagnosis and management of gastrointestinal cancers, this cancer presents with a dismal prognosis [5]. The frequency of this cancer increases with age and reaches peak value after fifth decade of life [6]. Worldwide incidence shows striking gender bias and affects females 2–3 times more frequently as compared to males [7].

GBC has been consistently associated with inflammation due to presence of gallstones, blockade of pancreato-biliary duct and chronic infection and intriguingly 1~3% patients of gallstone disease develop gallbladder cancer at any stage of life [8-12]. Accountable of evidences point that genetic susceptibility may contribute to gallbladder carcinogenesis [13,14], however the precise mechanism leading to GBC transformation is yet to be elucidated [15]. It is increasingly recognized that inflammatory pathway genes show association with GBC [16,17]. Whereas the unparalleled role of inflammation in different organ cancers like lung and colorectal cancer is well established, there is limited replicated data that can strongly establish its putative role in GBC [18-21].

Inflammation is a cascade of reactions involving a vast array of genes. Of utmost importance with particular inception towards GBC are the genes of *IL1* family and prostaglandin synthases (cyclooxygenases). The IL-1

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gene located on chromosome 2q14 encodes proteins that are involved in promoting fever, activation of prostaglandin endoperoxide synthase-2 (PTGS2) [13] and differentiation of autoimmune disorders notably monogenic conditions referred as cryopyrin associated periodic syndromes (CAPS) [14]. In addition *IL1* is known to activate vascular endothelial growth factor (VEGF), can trigger the process of angiogenesis [22] and can directly affect gallbladder epithelial cell absorptive function [23].

It has been observed that at least 40% of malignancies worldwide are infectious and inflammation based which accounts for a total of more than 3 million cases per year [24,25]. With respect to GBC, data shows that approximately 50% patients have a history suggestive of chronic inflammation [26-28]. In a population-based study conducted in Shanghai, China, usage of non-steroidal anti-inflammatory drugs (NSAIDs) was associated with a significant 63% reduction in the risk of GBC and it has been already suggested that it can be in-part due to blockade of inflammatory pathway genes or by limiting the activity of prostaglandin synthases [29].

While most of the proteins are controlled by regulated expression, some of their functions are controlled by key polymorphic variants (SNPs) which can potentially alter the splicing process, modify the binding of transcription factors, affect the stability of mRNA or remould the structure of the enzyme. Single nucleotide polymorphisms in Interleukin-1 have been associated regularly with inflammation based cancers including GB cancer, lung cancer and gastric cancer [30-32]. Owing to their role in induction [33] and over expression of associated molecules in neoplastic tissues, including the biliary-tree [34-37], the present study was designed to investigate the role of key polymorphic variants of IL-1 gene family and IL-1α expression in affecting susceptibility to GB cancer and gallstone disease in Kashmiri population. To date, no studies have examined the role of respective *IL1* polymorphisms with the risk of GBC or gallstones in this region.

Subject selection and recruitment

A total of 120 GBC patients, 120 Gall stone Patients and 130 age and sex matched controls were prospectively included. The GBC and gall stone patients were recruited from the Department of Surgical Gastroenterology, Department of General Surgery and Department of Surgical Oncology, Sheri-Kashmir Institute of Medical Sciences (SKIMS) after proper evaluation and diagnosis.

Before sample collection, the participants were informed about the nature of the study and its possible outcome and a written consent was taken. The study was approved by the Institutional Ethics Committee, Skims Vide approval no-138/2013.

Materials and Methods

Genotyping

Genomic DNA was isolated from blood samples by phenol chloroform. Prior to genotyping DNA quality and content was checked by Agarose gel electrophoresis and spectrophotometry respectively (Nanodrop Spectrophotometer, Eppendorf AG, Hamburg, Germany).

Polymerase chain reaction (PCR) was performed using an iCycler Thermal Cycler (Agilent technologies, Malaysia). Respective genotypes of IL-1α +4845G/T, *IL1R1* Pst1 and *IL1R2*DraIII polymorphisms were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. Specific primers were designed and selected using Primer-3, version 0.4.0 software. The list of primers and restriction enzymes utilized are presented in Table 1.

The PCR reaction mixture consisted of Taq DNA polymerase 1.0 U (Ferments), forward and reverse primers (0.5 μmol/l), $MgCl_2$ (50 mmol/l), dNTPs (0.2 mmol/l), and DNA template (250 ng-500 ng) and was subjected to an initial denaturing step of 5 min at 95ºC, then 35 cycles of denaturing for 35s at 95ºC, annealing for 35sfollowed by extension for 35 s at 72ºC, and a final extension step of 10 min at 72ºC. Digestion of the amplified products was carried out by restriction endonucleases (Table 1) by incubation at 37ºC for 16 h. The digested products were resolved on 3% agaroses gel. Furthermore to clarify results, genotyping was randomly repeated on 5% of samples.

mRNA expression analysis

Total RNA was extracted from gallbladder tumor and adjacent normal tissue specimens by using TRIZOL (Sigma Aldrich, USA). Integrity of the mRNA was checked on 1% agarose gel and quantified at 260/280 ratio. Prior to cDNA synthesis, DNAse treatment was given to extracted RNA to remove any traces of genomic DNA. cDNA was synthesized according to manufactures protocol (Fermentas, USA). qRT-PCR (Rotor-Gene Q, Qiagen Hilden, Germany) was performed for the detection of IL-1α mRNA containing Maxima® SYBR Green qPCR Master Mix (2X) and all the samples were run in triplicates accompanied by non-template control (NTC). The assay was validated by normalization against reference gene GAPDH and a melt curve was run to ensure standardization. All amplified products of real time PCR were checked on 2% agarose gel for ensuring the correct amplification products. The data was analyzed by "Delta Delta CT" method and the results were furnished as "fold-change (2–(ΔΔCt))".

Results

Population characteristics

Baseline characteristics of GBC patients and their age and gender matched controls are presented in Table 2. Of the 120 GBC cases, GS patients and 130 healthy controls with complete clinical information and successful genotyping for all the polymorphisms, the mean age was 55.23 ± 9.33 years (range, 32-75 years), 49.12 ± 16.40 years (range, 18-65 years) and 48.11 ± 18.08 (range, 16-75 years) respectively. The mean age and gender distributions were not significantly different among study groups, suggesting an adequate frequency matching. The possibility of population stratification was ruled out by genomic control method. Gallstones were present in 30% of GBC patients and 44.0% of the GBC patients were associated with tobacco usage in some form. All study patients were incident cases and none of the controls had family history of any inflammation related disorder and cancer.

Table 1. Primers and restriction endonucleases used for genotyping respective SNPs.

Genes	Primer Sequence	Ann. T (ºC)	Rest. endo.	Restriction products
IL-1α +4845G/T	F 5'-ATGGTTTTAGAAATCATCAAGCCTAGGGCA-3'	70ºC	SatI	GG: 237bp
	R 5'-ATTGAAAGGAGGGGAGGATGACAGAAATGT-3'			TT: 154bp+83bp
IL1R1 PstI C/T	F 5′-TTGGAGGATGGCCCATGAAGACC-3′	61ºC	PstI	CC: 350 bp
	R 5′-CTGTTACGCGCCCGGATGAAAAA-3′			TT: 253bp+97 bp
IL1R2 DraIII T/G	F 5'- CTTACATGGCTGGTGCCTTT-3'	59ºC	DraIII	TT: 357 bp
	R 5'-TATCTCCCATCCCACATGGT-3'			GG: 194bp+163 bp
	R 5'-GCCCTTCATAGGAGATACTGG-3'			

Association of *IL1* gene polymorphisms with GBC

The distribution and statistical analysis of IL-1α +4845G/T, *IL1R1* Pst1C/T and *IL1R2* Dra111T/G genotypes are shown in Table 3. The observed genotypes for controls were in complete accordance with the Hardy Weinberg equilibrium (p> 0.05). For IL-1α +4845G/T (Figure 1), the genotypic distribution frequency and their alleles revealed no significant association with GBC (OR: 1.08; 95% CI: 0.52-2.27, p=0.85) or GS disease (OR: 0.75, 95% CI: 0.35-1.60, p=0.57). Furthermore, this polymorphism showed no significant association with any of the demographic and clinical parameters of the patients (Supplementary Table 1).

For *IL1R1* Pst1C/T polymorphism (Figure 2), no significant association was observed with either GBC (OR: 1.34; 95% CI: 0.65-2.79, p= 0.54) or GS disease (OR: 0.80, 95% CI: 0.35-1.82, p =0.68), however the 'CT' genotype was observed to be protective and was significantly associated with patient characteristics like rural dwelling (OR: 0.49; 95% CI: 0.26-0.95, p=0.04) and smoking(OR: 0.39; 95% CI: 0.17-0.88, p= 0.02) (Supplementary Table 2).

IL1R2 DraIIIT/G SNP (Figure 3) indicated 'GG' genotype and 'G' allele to be significantly associated with increased risk of GBC with an odds ratio and p value of 2.65 and 0.011 and 1.57 and 0.014 respectively. However this polymorphism presented no association with GS disease (OR: 1.66, 95% CI: 0.76-3.63, p=0.23). In correlation with various demographic and clinical features of GBC patients, a significant association was observed with risk factors of GBC including elderly age (OR: 3.05; 95% CI: 1.40-6.62, p= 0.006) and urban dwelling (OR: 4.8; 95% CI: 1.84-12.50, p= 0.001) (Supplementary Table 3).

In the stratification of GBC patients according to the presence or absence of gallstones, none of the polymorphisms was statistically significant (p>0.05) (Table 4).

Haplotype analysis

Sixteen haplotypes of studied *IL1* cluster polymorphisms were observed in our population as shown in Table 5. GG/CC/TG haplotype was observed to be highly protective towards GBC (OR=0.07, p=0.028) while towards gallstone disease no significance was observed. Haplotype TT/CT/GG was observed to confer nearly 3-fold and 5-fold risk towards GBC and GS respectively, but the results were statistically insignificant. The frequency of other haplotypes did not differ between the patients and controls.

IL-1α mRNA expression

Relative mRNA expression was analyzed to decipher the role of pro-inflammatory IL-1α in the pathogenesis of GBC. Fold change was calculated for each tumor tissue and finally presented as an average fold change in 30

Table 2. Selected characteristics of study subjects.

Variables	GBC Cases (G0) N=120 (%)	Gall stone patients (G1), N=120 (%)	Healthy Controls (G2) N=130 (%)	P-value (G0,G1 vs. G2)
Sex				
Male	42 (35)	36 (30)	45 (4.61)	>0.05
Females	78 (65)	84 (70)	85 (65.38)	>0.05
Age				
Mean age (Males)	62 ± 9.33	49 ± 16.4	48 ± 18.08	>0.05
Mean Age (Females)	56 ± 8.44	45 ± 14.8	46 ± 20.21	>0.05
Dwelling				
Rural	79 (65.83)	85 (70.83)	90 (69.23)	>0.05
Urban	41 (34.17)	35 (29.17)	40 (30.77)	>0.05
Smoking status				
Smoker	44 (36.67%)	-	-	-
Non-smoker	76 (63.33%)			
Family History				
Significant	22 (18.33%)	-	-	-
Non-significant	98 (81.67%)			
GB stone status				
Present	30 (25%)	-	-	-
Absent	90 (75%)			
Histopathology				
WDA	26 (21.66%)	-	-	-
PDA	10 (9.1%)			
MDA	12 (10.9%)			
PA	5 (4.5%)			
SCC	1 (0.9%)			
SRA	3 (2.7)			
MA	2 (1.8%)			
USG/FNAC	61 (55.5%)			

WDA: Well differentiated adenocarcinoma, PDA: Poorly differentiated adenocarcinoma, PA: Papillary adenocarcinoma, SCC: Squamous cell carcinoma, SRA; Signet ring adenocarcinoma, MA: Mucinous adenocarcinoma, USG/FNAC: Ultra sonography guided Fine needle aspiration cytology.

Table 3. Analysis of IL1 cluster gene polymorphisms between study groups.

Gene/SNP	Controls N=130 (%)	GBC N=120 (%)		P-value	Gallstones N=120 (%)		P-value
		Cases (%)	OR (95% CI)		Cases (%)	OR (95% CI)	
			IL-1α +4847 G/T				
GG	39 (30)	33 (27.5)	1 (Reference)	-	39 (32.5)	1 (Reference)	-
GT	67 (51.5)	65 (54.2)	1.15 (0.64-2.03)	0.66	63 (52.5)	0.94 (0.54-1.65)	0.89
TT	24 (18.5)	22 (18.3)	1.08 (0.52-2.27)	0.85	18 (15)	0.75 (0.35-1.60)	0.57
T allele	115 (44.2)	109 (45.4)	1.05 (0.73-1.49)	0.86	99 (41.25)	0.88 (0.62-1.26)	0.53
			IL1R1 Pst1 C/T				
CC	34 (26.2)	38 (31.7)	1 (Reference)	-	32 (26.7)	1 (Reference)	-
CT	76 (58.5)	52 (43.3)	0.61 (0.34-1.09)	0.1	73 (60.8)	1.02 (0.57-1.82)	1
TT	20 (15.4)	30 (25)	1.34 (0.65-2.79)	0.54	15 (12.5)	0.80 (0.35-1.82)	0.68
T allele	116 (44.6)	112 (46.7)	1.09 (0.76-1.54)	0.65	103 (42.9)	0.93 (0.65-1.33)	0.72
			IL1R2 DraIII T/G				
TT	48 (37)	34 (28.3)	1 (Reference)	-	34 (28.3)	1 (Reference)	-
TG	65 (50)	54 (45)	1.17 (0.66-2.07)	0.66	66 (55)	1.43 (0.82-2.50)	0.26
GG	17 (13.1)	32 (26.6)	2.65 (1.27-5.53)	0.011	20 (16.7)	1.66 (0.76-3.63)	0.23
G allele	99 (38)	118 (49.2)	1.57 (1.10-2.24)	0.014	106 (44.2)	1.32 (0.93-1.89)	0.12

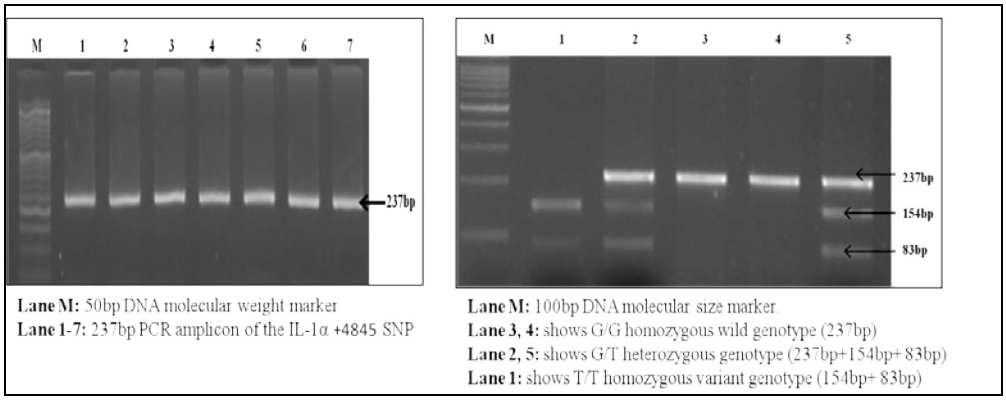

Figure 1. PCR and RFLP picture of IL-1α +4847 G/T polymorphism.

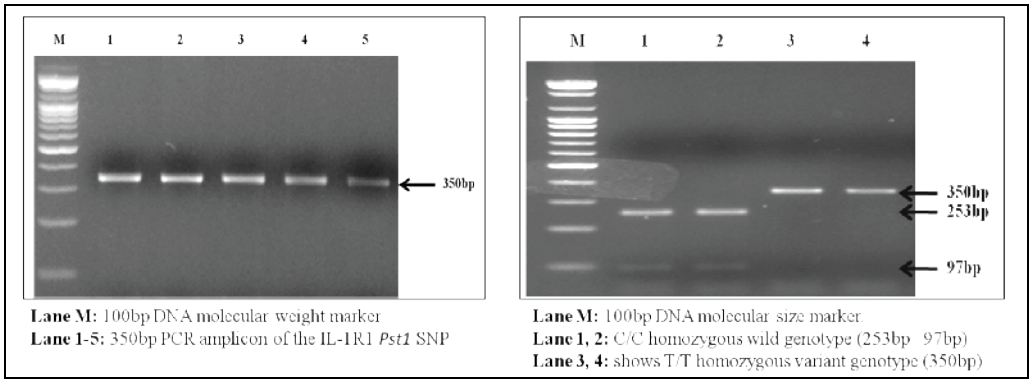

Figure 2. PCR and RFLP picture of IL1R1 Pst1 C/T polymorphism.

GB tumor tissues. An average fold change of 0.98 was observed for IL-1α which showed no variation in its mRNA expression in gallbladder tumor tissues as compared to adjacent normal (Figure 4). IL-1α mRNA expression did not differ between tumor and adjacent normal GB tissues.

Statistical analysis

The distribution of the genotypes in controls was compared with that expected from Hardy-Weinberg equilibrium (HWE) by the chi square (χ^2) test. Odds ratios (ORs) and their 95% confidence intervals (CIs), with adjustments for age, sex and dwelling were calculated by Fisher's exact test/Chi square test as appropriate. Students unpaired't' test was used to compare the mean and standard deviation. All reported p-values were based on two-sided tests. Significance level was taken at $p < 0.05$. Statistical analysis was performed using the software SPSS 23.0 (SPSS Inc., Chicago, Illinois) and Graph Pad Prism version 5.

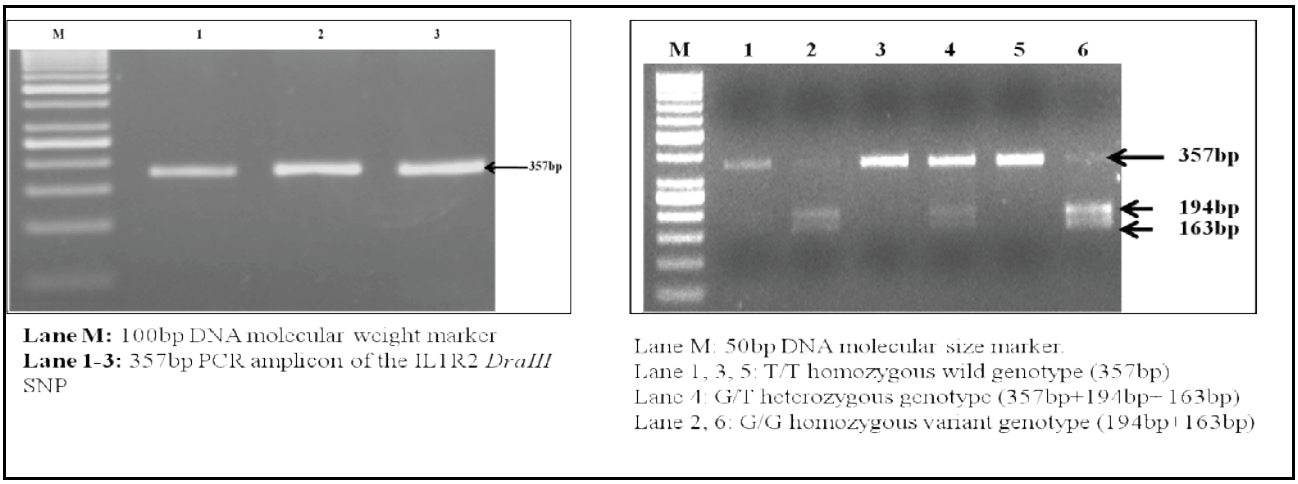

Figure 3. PCR and RFLP picture of IL1R2 DraIII C/T polymorphism.

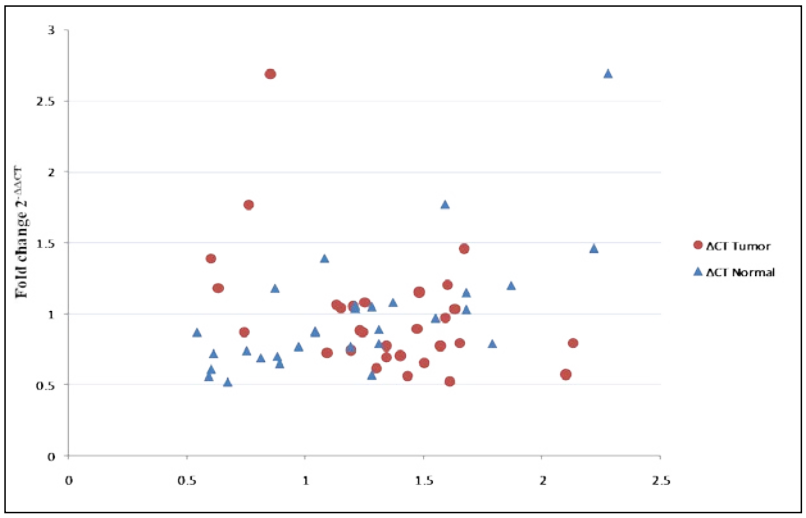

Figure 4. Scatter plot showing relative mRNA expression of IL-1α. Relative mRNA expression of IL-1α has been expressed as ΔCT values (x-axis) and fold change (y-axis) in gallbladder Tumor and adjacent normal tissues.

Table 4. Frequency distribution of IL1 cluster gene in GBC patients stratified according to the presence and absence of gallstones.

SNP Genotype/Allele	Healthy Subjects N=130 (%)	GBC with GS		P-value	GBC without GS		P-value
		N=30 (%)	OR (95% CI)		N=90 (%)	OR (95% CI)	
			IL-1α +4847 G/T				
GG	39 (30)	08 (26.66)	-	-	25 (27.77)	-	-
GT	67 (51.54)	17 (56.66)	1.22 (0.64-2.31)	0.62	48 (53.33)	1.09 (0.57-2.07)	0.87
TT	24 (18.46)	05 (16.66)	0.99 (0.43-2.29)	1.00	17 (18.89)	1.07 (0.47-2.43)	1.00
GT+TT	91 (70)	22 (73.33)	1.06 (0.58-1.94)	0.88	65 (72.22)	1.05 (0.70-1.59)	0.83
			IL1R1 Pst1 C/T				
CC	34 (26.15)	05 (33.33)	-	-	34 (31.11)	-	-
CT	76 (58.46)	17 (53.33)	1.90 (0.59-6.07)	0.31	35 (42.2)	0.46 (0.25-0.86)	0.017
TT	20 (15.38)	08 (13.33)	3.82 (1.04-14.05)	0.06	21 (26.67)	1.05 (0.48-2.28)	1.00
T allele	96 (73.84)	25 (83.33)	1.56 (0.85-2.86)	0.16	56 (62.22)	0.81 (0.54-1.23)	0.34
			IL1R2 DraIII T/G				
TT	48 (36.92)	08 (26.66)	-	-	26 (28.88)	-	-
TG	65 (50)	12 (40)	1.11 (0.42-2.91)	1.00	42 (46.66)	1.19 (0.64-2.21)	0.64
GG	17 (13.07)	10 (33.33)	3.53 (1.19-10.41)	0.02	22 (24.44)	2.39 (1.08-5.28)	0.04
G allele	82 (63.07)	22 (73.33)	1.54 (0.83-2.86)	0.19	64 (71.11)	1.44 (0.80-2.56)	0.24

Table 5. Association of haplotypes of IL1 gene cluster polymorphisms in GBC patients and gallstone patients.

Haplotypes IL-1α			HC N=130 (%)	GBC N=120 (%)	OR, P-value	GS N=120 (%)	OR, P-value
+4845G/T	IL1R1C/T	IL1R2T/G					
GG	CC	TT	5	8	Ref-	7	Ref-
GT	CT	GG	4	10	1.56, 0.69	6	1.07, 1.00
GT	TT	TT	5	4	0.50, 0.66	2	0.28, 0.35
GG	CC	TG	9	1	0.07, 0.028	4	0.38, 0.20
GT	CT	TT	12	4	0.21, 0.06	7	0.32, 0.24
GG	TT	TG	4	5	0.78, 1.00	1	0.18, 0.29
GG	CT	TG	5	5	0.62, 0.68	14	2.00, 0.45
GT	CC	TG	5	5	0.62, 0.68	13	1.86, 0.46
GG	CC	GG	4	4	0.62, 0.67	2	0.29, 0.45
TT	CT	TG	1	5	3.12, 0.60	7	5.00, 0.32
TT	CC	TG	6	2	0.21, 0.18	5	0.59, 0.68
TT	CT	TT	2	4	1.25, 1.00	3	1.07, 1.00
GT	CC	GG	4	2	0.31, 0.35	1	0.18, 0.29
GT	TT	TG	6	9	0.94, 1.00	3	0.36, 0.39
GG	TT	TT	4	1	0.15, 0.29	4	0.71, 1.00
GT	CT	TG	13	11	0.53, 0.49	15	0.82, 1.00

Sixteen haplotypes were identified and p<0.05 was considered significant.

Discussion

IL1 gene family plays an important role in mediating immune responses. IL-1α is a proinflammatory molecule and its downstream effects are mediated by *IL1R1* which is a common receptor of this family. The *IL1R2* is a glycoprotein expressed as a membrane bound and soluble receptor in immune cells particularly monocytes, neutrophills, T and B lymphocytes and acts as a decoy receptor to inhibit proinflammatory activity of *IL1R1* receptor. The synergetic role of cytokines in modulating inflammatory responses is well established and emerging evidence suggests that IL-1α which is produced by activated macrophages can induce PTGS2 synthesis, PGE2 release and accumulation [38-40] in premalignant lesions that culminates in malignancy by moderating pleiotropic effects like proangiogenesis [41,42], antiapoptosis [43,44] and local immune suppression [45,46].

GBC is a multifactorial disease and keeping in view the inflammatory inception of GBC, multiple genetic variants of inflammatory genes in combination might be involved during its transformation. Thus to understand the complex etiology of GBC and its associated risk due to gall stones, this study was attempted to achieve a more comprehensive evaluation of GBC risk considering several genetic variants in *IL1* gene family simultaneously.

We selected three genes located in the *IL1* cluster as candidates for screening. IL-1α which is a proinflammatory cytokine is thought to up-regulate pro-metastatic genes in breast cancer cells and stromal cells [47]. Two SNPs one in the 5'UTR regulatory region (-889C>T) and one in exon 5 of this gene (+4845G>T) have been extensively studied and have not shown association with most of the inflammation based cancers [48-50]. However in correlation with GBC and GS disease these polymorphisms have not been studied yet. We genotyped IL-1α +4845 G/T SNP, where presence of T allele results in an Ala to Ser amino acid substitution at residue 114 of the pro-IL-1α molecule which is cleaved between 112 and 113 amino acid residues. This substitution is believed to affect the proteolytic process [23], influence C reactive protein levels in coronary angiography [24] and has been associated with the development of aggressive periodontitis in Chinese males [25]. In our study cohort, we did not observe any risk associated with minor allele towards either GBC (OR=1.05, p=0.86) or GS disease (OR=.0.88, p=0.53). Subgroup stratification also revealed IL-1α +4845 SNP to be insignificant towards any clinico-pathological characteristic of patients. In addition, when we analyzed the tissue specific relative mRNA expression of IL-1α, we did not observe its modulated expression in tumor tissues. The expression did not vary with tumor grade or tumor type. IL-1α being a pro inflammatory cytokine has been observed to increase the expression of other potent inflammatory molecules like IL-1β and prostaglandin synthases which have been reported to accumulate in tumor and necrotic tissues [51]. It is possible that IL-1α may trigger the early events in inflammation related cancers by modulating expression of other cytokines while keeping its own concentration at check being an autocrine growth factor [52]. Also IL-1α is believed to be less potent inflammatory cytokine as compared to IL-1β which lies in the same gene cluster. Various studies have substantiated that it is IL-1β but not IL-1α that is involved in long term inflammatory responses and in cancers that are believed to have roots in chronic inflammation [53,54].

Two key polymorphisms in *IL1* receptors were genotyped where we observed *IL1R1* Pst1 C/T SNP to be insignificant towards both GBC (p=0.54; OR=1.34; CI=0.65-2.79) and GS disease (p=0.68; OR=0.80; CI=0.35-1.82). However, on subgroup stratification, the heterozygous 'CT' genotype showed protective association with rural dwelling (OR: 0.49, p=0.04), smokers (OR: 0.39, p=0.02) and gall stone absence (OR: 0.46, p=0.017). The TT genotype of this polymorphism has been demonstrated to be associated with decreased percentage of cells expressing *IL1R1* on the intact CD14+ monocyte population [55] indicating that this genotype can have a protective function because of lower expression of membrane-bound *IL1R1*s. In contrast 'C' allele of this polymorphism has been demonstrated to be associated with inflammation [56]. Although this SNP was not significant in our population, our study suggests that 'T' allele of this SNP might have protective function at least in stratified GBC patient subgroups in our population.

On the other hand, *IL1R2* DraIIIT/G polymorphism was observed to be significantly associated with GBC risk. The risk genotype 'GG' nearly conferred 3-fold risk towards GBC however no significant risk was observed towards gallstone disease. Among patients with GBC and GS, the frequencies of *IL1R2* DraIIIT/G polymorphism TT, GT and GG were 28.3%, 45%, 26.6% and 28.3%, 55%, 16.7% respectively. In case of healthy controls, the frequencies of respective genotypes (TT, GT and GG) were 37%, 50% and 13.1% (Table 3). The 'GG' genotype was higher in both GBC and GS patients as compared to healthy controls; however, it reached statistical significance only in case of GBC cases (OR: 2.65, p=0.011). Data stratification on the basis of clinicopathological characteristics of GBC patients revealed a significant association with elderly age group (OR: 3.05, P=0.006), male gender (OR: 1.90, p=0.049) and urban population (OR: 4.8, P=0.001). *IL1R2* DraIIIT/G polymorphism has been observed to have a putative role in the regulation of cell cycle and apoptosis. The

presence of 'G' allele has been observed to be a possible binding site for transcription factor TATA box binding protein-associated factor (TAF-1) [55]. TAF-1 is required for progression of the cell cycle and has been shown to repress apoptosis in mammalian cells [57]. The TAF-1 protein has intrinsic histone acetyltransferase activity [58] and also binds to and modulates the transcriptional activity of cell cycle proteins [59-61]. Because of the activation of regulatory elements of cell cycle and concurrent repression of apoptosis, GBC patients with underlying GG genotype of this SNP can be more predisposed to tissue necrosis as corroborated by subgroup analysis and a more pronounced inflammatory response which may be an important element in triggering the transformation process of GB tissue.

Furthermore, the combined effect of three *IL1* SNPs on the risk of GBC and GS was investigated by haplotype analysis. The GG/CC/TG combination was observed to be protective towards GBC (OR=0.07, p= 0.028) however none of the haplotypes showed any association with gallstone disease. Our data suggests that these polymorphisms might be in low linkage disequilibrium but there is a possibility that *IL1R2* DraIIIT/G also affects GBC susceptibility due to its linkage with any other polymorphism in *IL1* gene that reduces its expression which triggers the inflammation process by less inhibition of *IL1R1* receptor.

Conclusion

In conclusion, this case-control study showed that a common variant located in the promoter region of the *IL1R2* gene is associated with an increased risk of gall bladder cancer, consistent with the view that chronic inflammation is a key impetus to the carcinogenic transformation in the GB. This study also suggests that the screened variants IL-1α +4845G/T and *IL1R1* Pst1 are no prelude to gallstone disease and GB cancer in our population. Further studies are needed that can provide a more comprehensive coverage of genes involved in inflammation-related pathways.

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