RESEARCH COMMUNICATION

Do TNFA -308 G/A and IL6 -174 G/C Gene Polymorphisms Modulate Risk of Gallbladder Cancer in the North Indian Population?

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Abstract

Objectives: Gallbladder carcinoma (GBC) is highly aggressive neoplasm which arises in the background of gall stones and inflammation. GBC affects women 2-3 times more frequently than men. Pro-inflammatory TNFA and IL6 gene polymorphism has been associated with various inflammatory diseases. The aim of this study was to investigate whether TNFA -308 (G/A) and IL6 -174 G/C polymorphisms within flanking region of the genes are associated with GBC susceptibility. Methods: The promoter polymorphisms were genotyped using PCR-RFLP in 200 healthy subjects and 124 GBC patients. Results: Frequency distribution of TNFA -308 (G/A) and IL6 -174 G/C were not significantly different in GBC patients in comparison to healthy controls. However, frequency of TNFA -308 (G/A) polymorphism in female GBC patients without gallstone were significantly different (p-value= 0.006) when compared to healthy female subjects (OR=3.054; 95% CI=1.39-6.72). Conclusion: These results suggest that TNFA -308 (G/A) polymorphism may influence the susceptibility of female gender gallbladder cancer in absence of gallstones while IL6 -174 G/C polymorphism does not seem to be playing significant role in the susceptibility to gallbladder cancer.

Key Words: TNFA - IL6 - gallbladder carcinoma - genetic susceptibility - gallstones - inflammation - polymorphism

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Introduction

Gallbladder cancer (GBC) is a highly malignant neoplasm with poor prognosis, and most cases are diagnosed at an advanced stage (Chianale et al., 1990). GBC affects women 2–6 times more frequently than men (Lazcano-ponc et al., 2001). Epidemiological studies have revealed worldwide geographical, ethnic and cultural variation in the incidence of gallbladder cancer (Diehl et al., 1983; Bamba et al., 1988). In India, the incidence of gallbladder carcinoma is very high in the north Indian populations as compared with south India (ICMR, 1996). The highest incidence rate of GBC is up to 23 per 100,000 for women from North India.

Gallbladder cancer is multi-factorial disease and associated risk factors identified so for include cholelithiasis, obesity, reproductive factors, chronic infection and environmental exposure to specific chemicals (Lazcano-Ponc et al., 2001). However, there is very limited information about the molecular pathogenesis involved in gallbladder cancer development (Wistuba et al., 2004). Gallstones and subsequent inflammatory changes are believed to be important risk factors for GBC. Chronic inflammation predisposes to the development of various forms of cancer which indicates that local inflammatory mediators are responsible for their development and strong evidences support the genetic basis for the susceptibility to tumor development (Macarthur et al., 2004). Cytokines which are local mediators of inflammatory immune response have been found to affect gallbladder epithelial cell absorptive function, similar to the proinflammatory agents LPS and PGE2 (Robert et al., 2000).

Pro-inflammatory cytokine TNF plays an important role in cell proliferation, differentiation and apoptosis. TNFA gene consists of four exons and three introns mapped on chromosome 6p21.3. The most reported promoter region polymorphism of TNFA -308 G to A substitution has been found to be associated with susceptibility to a number of inflammatory diseases including various cancers such as cervical (Goven et al., 2006), lung (Shih et al., 2006), bladder (Nonomura et al., 2006) and breast (Azmy et al., 2004). Functionally, rare allele of this polymorphism (A) increases the production of TNFA (Wilson et al., 1992).

IL6, a phosphorylated glycoprotein containing 185 amino acids, is a pleiotropic cytokine involved in different physiologic and pathophysiologic processes such as inflammation, bone metabolism, synthesis of C-reactive protein, and carcinogenesis (Asschert et al., 1999; Diel et

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al., 2002). The human IL-6 gene (IL6) is mapped to chromosome 7p21-24 (Bowcock et al., 1988). The common IL6-174 G/C polymorphism has also been shown to influence in vivo protein expression by reducing transcription rates (Jones et al., 2001; Vickers et al., 2002). Belluco et al., (2003) demonstrated that circulating IL-6 levels are significantly higher in IL6–174 G/G homozygotes with colon cancer compared with carriers of a C allele, and correlated significantly with the presence of hepatic metastases.

Long standing gallstones are generally present in 65-80% patients of gallbladder cancer where 1~3% patients of gallstone disease may develop gallbladder cancer. Gallstones might cause direct mechanical irritation to the surrounding mucosal surface leading to delayed or incomplete emptying with subsequent bile stasis and dilation of the gallbladder, which predisposes to chronic inflammation. In addition, chronic infection and subsequent inflammation by *Salmonella typhi*, *Salmonella paratyphi*, *Helicobacter pylori* and *Helicobacter bilis* have also been implicated as aetiological factors for GBC (Dutta et al., 2000; Leong et al., 2002; Randi et al., 2006). Our aim of this study was to investigate whether TNFA -308G/A and IL6 -174 G/C polymorphisms modulate the risk of gallbladder cancer.

Materials and Methods

Subjects

The present case control study comprised 124 consecutive cases of histopathological or cytologically proven GBC from Department of Gastroenterology and Gastro-surgery of Sanjay Gandhi Post Graduate Institute of Medical Sciences Lucknow, UP (India). The clinical profile of patients was based on hospital investigations. Staging of cancer was documented according to American Joint Committee on Cancer (Misra et al., 2003). A total of 124 GBC patients were enrolled which consists of 45 males and 79 females. Out of 124 cases, 65 GBC patients had gallstones. A total of 200 age and sex matched healthy subjects were recruited from staff of our Institute and the unrelated persons visiting the hospital for minor medical or surgical problems The inclusion/exclusion criteria, demographic profile and clinical characteristics of the subjects were same as described earlier (Pandey et al., 2006). After obtaining informed consent, all individuals were personally interviewed for information on their ethnicity, food habits, occupation, drinking and tobacco usage. The study was approved by the Ethical Committee of our Institute.

Genomic DNA Isolation

Five ml blood samples were collected from normal healthy subjects and GBC patients in EDTA vials and kept frozen till DNA extraction. The genomic DNA was isolated from peripheral blood using salting out method (Miller et al., 1988).

TNFA-308 G/A Polymorphism

TNFA-308 G/A polymorphism were analyzed by polymerase chain reaction- restriction fragment length polymorphism (Marsh et al., 2003). Cycling was performed as follows: a pre PCR step of 5 min denaturation at 94˚C followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 59˚C for 30 sec, extension at 72˚C for 10 sec, and final extension at 72˚C for10 min. Resulting PCR products of 117 bp was subjected to restriction digestion with NcoI (New England Biolabs Inc. Beverly, MA, USA) in a 10 µl digestion mixture at 37˚C for overnight. The restriction enzyme digestion resulted in 3 different sizes of polymorphic DNA fragments: GG (117 bp), GC (117+97 +20 bp) and CC (97 +20 bp) which were genotyped by 15% nondenaturing polyacrylamide gel electrophoresis and ethidium bromide staining.

IL6 -174 G/C Polymorphism

Polymorphism analysis was performed according to protocols based on the tetra-primer amplification refractory mutation system (ARMS)-polymerase chain reaction (PCR) (Ye et al., 2001) using two primer pairs to amplify the two alleles of SNP, respectively, in a single PCR reaction. The PCR primers used were forward inner primer (G allele) 5'-GCACTT TTCCCC CTAGTTG TGTCTT CCG-3', reverse inner primer (C allele) 5'-ATTGTGCAATGTGACGTCCTTTAGCTTG-3', forward outer primer 5'-GACTTC AGCTTT ACTC TTTGTCAAGACA-3' and reverse outer primer 5'-GAATGAGCCTCAGACATCTCCAGTCCTA-3'. Each PCR reaction was performed in a total volume of 15 µl, containing 100 ng genomic DNA, 10 pmol of each inner primer, 1 pmol of each outer primer; 200µM deoxynucleoside triphosphate, 2.5 µM MgCl2 and 2.5U Taq DNA polymerase in the buffer supplied by the manufacturer (Bangalore Genei, Bangalore India). PCR cycling conditions for the assay were 95˚C for 2 min, followed by 35 cycles of touchdown reactions at 95oC for 1 min and 72˚C for 1 min for the first cycle, decreasing by 1˚C per cycle until annealing temperature reached to 63˚C and then continuing at 63˚C in the annealing step of the remaining cycles with extension at 72˚C for 1 min and a final extension step at 72˚C for 5 min. After PCR amplification, a 5 µl aliquot of PCR products was resolved by 15% non-denaturing polyacrylamide gel electrop horesis and visualized by ethidium bromide staining.

Quality control

Twenty per cent of samples from both patients and controls were re-genotyped by other laboratory personnel and no discrepancy in genotyping was noticed.

Statistical Analysis

The sample size was calculated by using Quanto version1.1 software with 80% statistical power. Differences in genotype frequencies between GBC and healthy subjects were assessed by chi-square test or Fisher's exact test. To examine Hardy-Weinberg equilibrium, a goodness of fit $\chi 2$ test was used. Age and sex are the significant covariates for the GBC so, age and sex adjusted odds ratio (OR) and 95% confidence interval (CI) associated with the putative at-risk TNFA and IL6 loci were calculated by unconditional logistic regression analysis. The significant association was considered when

Table 1. Frequency Distribution of Genotypes/ Alleles of TNFA -308G/A Polymorphism in GBC Patients and Healthy Subjects

Genotypes/ Alleles	Healthy subjects n=200 (%)	GBC n=124 (%)	*p*- value	Adjusted OR (95% CI)
GG	153 (76.5)	97 (78.2)	-	1 (Reference)
GA	41 (20.5)	22 (17.7)	0.827	0.94 (0.52-1.70)
AA	6 (3.0)	5 (4.0)	0.555	1.47 (0.41-5.29)
*G	346 (86.5)	216 (87.1)	0.979	1 (Reference)
*A	54 (13.5)	32 (12.9)	0.979	1.01 (0.62-1.63)
Males	n=82	n=45		
GG	53 (64.6)	35 (77.8)	-	1 (Reference)
GA	26 (31.7)	9 (20.0)	0.192	0.55 (0.23-1.35)
AA	3 (3.7)	1 (2.2)	0.578	0.52 (0.05-5.23)
*G	130 (79.3)	79 (87.8)	-	1 (Reference)
*A	34 (20.7)	11 (12.2)	0.093	0.53 (0.25-1.13)
Females	n=118	n=79		
GG	100 (84.7)	62 (78.5)	-	1 (Reference)
GA	15 (12.7)	13 (16.5)	0.279	1.58 (0.68-3.64)
AA	3 (2.5)	4 (5.1)	0.268	2.68 (0.47-15.31)
*G	216 (91.5)	137 (86.7)	-	1 (Reference)
*A	20 (8.5)	21 (13.3)	0.067	1.90 (0.96-3.76)

* Number of chromosomes

p-value is < 0.05. All analyses were performed using the SPSS statistical analysis software, version 11.5 (SPSS, IL, USA).

Results and Discussion

The mean age ± SD of GBC patients and healthy subjects were 49.4 ± 9.34 and 50.0 ± 11.4. Gallstones were present in 52.4 % of GBC patients. Tobacco usage either by smoking or chewing was present in 18 GBC patients and alcohol usage was reported by 6 patients. Most of the patients were in advanced stages of cancer (stage 3 and stage 4).

In this preliminary study, we studied two polymorphism namely TNFA -308 (G/A) and IL6 -174G/C to show whether these two polymorphisms modulate the risk of gallbladder cancer or not. The role of pro-inflammatory and immuno-modulatory cytokine tumour necrosis factor (TNF) and interleukin-6 (IL6) is established in the pathogenesis of chronic inflammatory diseases (Lee at al., 2006; Skyora et al., 2006). The frequencies of polymorphic alleles of TNFA -308 (G/A) and IL6 -174G/C were consistent with the Hardy-Weinberg equilibrium in our healthy subject population.

In this case-control study, the frequency distribution of TNFA -308 (G/A) polymorphism was almost similar between GBC patients and healthy subjects at genotype and allele levels. However, after segregation of patients

Table 2. Frequency Distribution of Genotypes and Alleles of TNFA -308G/A Polymorphism Based on Gender in GBC Patients Stratified with Presence and Absence of Gallstones and Healthy Subjects

Genotype/ Allele	Healthy Subjects n=200 (%)	GBC n=59 (%)	*p*- value	Adjusted OR (95% CI)	GBC with gallstone n=65 (%)	*p*- value	Adjusted OR (95% CI)
GG	153 (76.4)	43 (72.9)	-	1 (Reference)	54 (83.1)	-	1 (Reference)
GA	44 (22.1)	12 (20.3)	0.743	0.13 (0.54-2.40)	10 (15.4)	0.489	0.78 (0.35-1.73)
AA	3 (1.5)	4 (6.78)	0.128	2.88 (0.74-1.39)	1 (0.15)	0.553	0.56 (0.06-4.97)
G*	350 (87.4)	98 (83.1)	-	1 (Reference)	118 (90.8)	-	1 (Reference)
A*	50 (12.6)	20 (16.9)	0.251	1.40 (0.79-2.49)	12 (9.2)	0.270	0.68 (0.34-1.35)
Males	n=82	n=26			n=19		
GG	62 (75.0)	20 (76.9)	-	1 (Reference)	15 (78.9)	-	1 (Reference)
GA	18 (21.9)	5 (19.2)	0.355	0.59 (0.19-1.80)	4 (21.1)	0.217	0.46 (0.13-1.59)
AA	2 (3.1)	1 (3.8)	0.989	0.98 (0.10-0.11)	0 (0.0)	0.999	--
G*	142 (85.9)	45 (86.2)	-	1 (Reference)	34 (89.5)	-	1 (Reference)
A*	22 (14.1)	7 (13.5)	0.341	0.65 (0.27-1.58)	4 (10.5)	0.075	0.36 (0.11-1.11)
Females	n=118	n=33			n=46		
GG	91 (77.1)	23 (69.7)	-	1 (Reference)	39 (84.8)	-	1 (Reference)
GA	26 (22.1)	7 (21.2)	0.121	2.24 (0.81-6.24)	6 (13.0)	0.761	1.18 (0.41-3.35)
AA	1 (0.8)	3 (9.1)	0.060	5.97 (0.93-8.56)	1 (2.20)	0.936	1.11 (0.10-2.77)
G*	208 (88.2)	53 (80.3)	-	1 (Reference)	84 (91.3)	-	1 (Reference)
A*	28 (11.8)	13 (19.7)	0.006	3.054 (1.39-.72)	8 (8.70)	0.668	1.22 (0.50-2.97)

* Number of chromosomes

Table 3. Frequency Distribution of Genotypes/ Alleles of IL-6 -174G/C Polymorphism in GBC Patients and Healthy Subjects

Genotypes/ Alleles	Healthy subjects n=200 (%)	GBC n=124 (%)	*p*- value	Adjusted OR (95% CI)
GG	153 (76.4)	97 (78.2)	-	1 (Reference)
GC	44 (22.1)	25 (20.2)	0.693	0.89 (0.49-1.60)
CC	3 (1.50)	2 (1.6)	0.660	0.60 (0.06-5.90)
*G	350 (87.4)	219 (88.3)		1 (Reference)
*C	50 (12.6)	29 (11.7)	0.590	0.87 (0.51-1.47)
Males	n=82	n=45		
GG	62 (75.0)	36 (80.5)	-	1 (Reference)
GC	18 (21.9)	9 (19.5)	0.800	0.88 (0.633-2.38)
CC	2 (3.1)	0 (0)	0.999	0 (0)
*G	142 (85.9)	81 (90.2)	-	1 (Reference)
*C	22 (14.1)	9 (9.8)	0.381	0.67 (0.27-1.64)
Females	n=118	n=79		
GG	91 (77.1)	61 (77.1)	-	1 (Reference)
GC	26 (22.1)	16 (20.5)	0.711	0.87 (0.41-1.83)
CC	1 (0.8)	2 (2.4)	0.767	1.53 (0.09-25.82)
*G	208 (88.2)	138 (87.3)	-	1 (Reference)
*C	28 (11.8)	20 (12.7)	0.839	0.933 (0.48-1.82)

*Number of chromosomes

Table 4. Frequency Distribution of Genotypes and Alleles of IL-6 -174G/C Polymorphism in GBC Patients Stratified on the Basis Presence and Absence of Gallstones and Healthy Subjects

Genotype/ Allele	Healthy Subjects (n=200)	GBC without gallstone (n=59)	*p*- value	Adjusted OR (95% CI)	GBC with gallstone (n=65)	*p*- value	Adjusted OR (95% CI)
GG	153 (76.4)	48 (81.7)	-	1 (Reference)	49 (75.0)	-	1 (Reference)
GC	44 (22.1)	11 (18.3)	0.555	0.79 (0.35-1.76)	14 (21.9)	0.878	0.95 (0.46-1.93)
CC	3 (1.5)	0 (0.0)	0.999	0	2 (3.1)	0.872	1.21 (0.12-12.4)
*G	350 (87.4)	109 (90.8)	-	1 (Reference)	112 (85.9)	-	1 (Reference)
*C	50 (12.6)	11 (9.2)	0.362	0.70 (0.33-1.50)	18 (14.1)	0.955	0.98 (0.52-1.84)
Males	n=82	n=26			n=19		
GG	52 (75.0)	20 (78.3)	-	1 (Reference)	16 (83.3)	-	1 (Reference)
GC	15 (21.9)	6 (21.7)	0.868	0.98 (0.29-3.24)	3 (16.7)	0.670	0.74 (0.19-2.95)
CC	2 (3.1)	0 (0.0)	0.999	0 (0)	0 (0)	0.999	0 (0)
*G	119 (85.9)	46 (89.1)	-	1 (Reference)	35 (91.7)	-	1 (Reference)
*C	19 (14.1)	6 (10.9)	0.54	0.71 (0.24-2.10)	3 (8.3)	0.414	0.59 (0.12-2.12)
Females	n=118	n=33			n=46		
GG	101 (77.1)	28 (83.8)	-		33 (71.7)	-	1 (Reference)
GC	29 (22.1)	5 (16.2)	0.420	0.62 (0.20-1.98)	11 (23.9)	0.866	1.08 (0.46-2.52)
CC	1 (0.8)	0 (0)	1.00	0	2 (4.3)	0.502	2.64 (0.16-45.1)
*G	231 (88.2)	61 (91.9)	-	1 (Reference)	77 (83.7)	-	1 (Reference)
*C	31 (11.8)	5 (8.1)	0.384	0.61 (0.20-1.85)	15 (16.3)	0.656	1.08 (0.56-2.49)

* Number of chromosomes

and controls on the basis of gender, the frequency of variant 'A' allele of TNFA -308 (G/A) polymorphism was higher in female GBC patients (13.3%) in comparison healthy female subjects (8.5%), showing risk up to 1.9 fold (95% CI=0.96-3.76) with borderline significance (p-value= 0.067). No significant difference was observed in male GBC patients when compared with healthy male subjects (Table 1). TNFA acts to induce a number of pro-inflammatory genes, such as cytokines, angiogenic factors which contribute towards tumor formation, growth, invasion and metastasis to other sites. Many of the actions of TNFA may occur by the stimulation of stromal tissue, tumour-associated macrophages and fibroblasts. These cells may then produce inflammatory cytokines including TNF itself, as well as some of the angiogenic factors. Immune responses vary with gender by affecting monocyte, granulocyte and cytokine production (Bouman et al., 2005). Available evidence from animal studies suggests that sex hormones regulate immune responses in vivo (as reviewed by Ansar et al., 1985). Experimental and clinical evidences indicate that immune reactivity is greater in females than in males and suggests that gonadal steroids may play an important role in the regulation of the immune response. Indeed, many cells of the immune system have been found to possess functional sex hormone receptors, such as CD8-positive T cells, B cells and, notably, monocytes/macrophages (Cutolo et al., 2005). Our results hypothesize that female sex hormones and TNF variant allele induces higher expression of TNFA which leads to the progression of gallbladder

carcinogenesis. Baskaran et al., (2005) suggested that the female sex hormones may have a role in the pathogenesis of gallbladder cancer and that progesterone expression has a prognostic significance. Nakamura et al., (1989) studied 21 primary gallbladder carcinoma patients and found that estrogen and progesterone receptors are found only in cytoplasm of cancer cells.

On stratification of GBC patients according to presence or absence of gallstones, no significant difference was observed in frequency of genotype and allele of TNFA -308 (G/A) polymorphism. The frequency of variant 'A' allele of TNFA -308 (G/A) polymorphism showed low risk in GBC male patients having gallstone with border line significance (p-value =0.075; OR=0.36; 95% CI= 0.11-1.11) as compared to healthy male subjects. However, in female GBC patients the frequency of variant 'A' allele was higher in absence of gallstone with highly significant difference (p-value= 0.006; OR=3.054; 95% CI=1.39-6.72) when compared with healthy female subjects. (Table 2) Duell et al., (2006) reported proinflammatory TNFA-308 polymorphisms, in combination with proinflammatory conditions, may influence the development of pancreatic cancer. Long-standing gallstones are well-established risk factor for GBC but up to 40% patients do not have associated gallstones. Although TNFA-308 related risk of GBC should have arisen in the background of inflammation resulting from gallstones but the present results show otherwise. It suggests that chronic inflammation due to factors other than gallstones may be responsible for higher susceptibility to TNFA mediated risk of GBC in these patients. These factors may include chronic infections.

In numerous studies, chronic infections with specific microorganisms have been implicated as aetiological factors for GBC. Infection by *Salmonella typhi* and subsequent chronic inflammation has been associated with increased risk of GBC (Dutta et al., 2000). In addition, cultures grown from bile samples have found *Salmonella typhi* more frequently in patients with GBC that in individuals without biliary neoplasia (Nath et al., 1997). There are also preliminary reports that indicate that *Helicobacter* species might colonize the biliary tract and these species have been implicated as a cause of chronic cholecystitis and GBC (Leong et al., 2002). Therefore, the results of our study suggest that chronic infections in the background of TNF-308 A allele may impart higher susceptibility of GBC in females.

In IL6-174G/C polymorphic alleles there were no significant differences between GBC patients and healthy subjects at genotype and allele levels. Based on the gender analysis, the frequencies of IL6 -174G/C in male and female in GBC patients were also found to be non-significant (p>0.05) when compared with healthy subjects, both at genotype and allele levels (Table 3). Further analysis based on the presence or absence of accompanying gallstone showed that the frequencies of IL6 -174G/C genotypes and alleles were almost similar in GBC patients with and without gallstones compared with healthy subjects. (Table 4) Previous functional *in vivo* studies have shown that IL 6 gene production is increased with an increased inflammatory response, associated with presence of G allele in IL6 -174 G/C SNP of the promoter region. Wehbe et al (2006) studied whether the altered gene expression of IL6 enhances tumor growth in cholangiocarcinoma. They found that epigenetic regulation of gene expression by IL6 can contribute to tumor progression by altering promoter methylation and gene expression of growth-regulatory pathways. Recently, Chen et al (2007) found that levels of IL1, 6 and 8 in gallbladder mucosa homogenates were significantly higher in *Helicobacter pylori* infected cholecystitis group. However, present studies shows no relation between IL 6 -174G/C polymorphism with gallbladder cancer which shows that some other related SNPs of IL6 -174G/C may be more important in determining the inflammatory state and disease progression in gallbladder cancer.

This is the first study to show the association of TNFA -308G/A and IL6 -174G/C polymorphism with GBC. In conclusion, our result shows that variant allele of TNFA -308G/A polymorphism modulated the risk of female gender GBC with absence of gallstones. However, IL6 -174G/C does not influence the susceptibility for GBC. Furthermore, our data highlight the need for further studies to clarify the association of gender variation and cytokine polymorphism as many confounding factors like chronic infections may contribute to the risk of developing cancer of the gallbladder.

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