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A single-nucleotide polymorphism in tumor necrosis factor-α (−308 G/A) as a biomarker in chronic pancreatitis

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ABSTRACT

Objective: Chronic pancreatitis is a gradual, long-term inflammation of the pancreas that results in alteration of its normal structure and function. The study aims to investigate the role of −308 (G/A) polymorphism of TNF-α gene in chronic pancreatitis.
Material and methods: A total of 200 subjects were included in this case–control study. A total of 100 in patients admitted in the Gastroenterology Unit of Gandhi Hospital and Osmania General Hospital, Hyderabad were included in the present study. An equal number of healthy control subjects were randomly selected for the study. The genotyping of TNF-α gene was carried out by tetra-primer ARMS PCR followed by gel electrophoresis. The TNF-α levels were assayed by enzyme-linked immunosorbent assay.
Results: A significant variation with respect to the genotypic and allelic distribution in the disease group when compared to control subjects [OR = 2.001 (1.33–3.005), $p < 0.0001^{**}$] was observed. Subjects homozygous for the A allele had higher TNF-α levels compared to G allele.
Conclusion: The present study revealed a significant association of the TNF-α gene promoter polymorphism with chronic pancreatitis. Thus, TNF-α genotype can be considered as one of the biological markers in the etiology of chronic pancreatitis.

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1. Introduction

Chronic pancreatitis (CP) is an ongoing inflammatory condition in which pancreatic secretory parenchyma is destroyed and replaced by fibrous tissue, ultimately leading to malnutrition and diabetes. The prevalence of chronic pancreatitis in France is 26 per 100,000 people. This prevalence is not unsimilar to the middle of three estimates from Japan, but considerably lower than the figure of 114–200 per 100,000 in South India (Braganza et al., 2011). The main symptom of chronic pancreatitis is usually pain, identical to that of acute pancreatitis which is usually a constant and disabling pain.

Recently, chemokines have been contemplated as essential factors in the development of chronic pancreatitis (Ito, 2007). They are elucidated as important factors that affect inflammation by increasing fibrosis which is an important feature of chronic pancreatitis. Thus, inflammatory chemokines are evidently associated with the chemo-attraction of leukocytes in early-stage chronic pancreatitis, leading to the movement of monocytes into the pancreas thereby resulting in the formation of pancreatic fibrosis (Grady et al., 1997; Inoue et al., 2002; Saurer et al., 2000).

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine with strong immune-stimulatory activity. It is involved in the inflammatory responses by stimulating the cytokine production, increasing the expression of adhesion molecules, and neutrophil activation, and it also acts as a co-stimulator for T-cell activation and antibody production by B-cells (Vassalli, 1992). TNF-α secretion is controlled primarily at the transcription level, and inter-individual differences in TNF-α secretion are associated with TNF-α promoter variant (Beranek et al., 2003).

Single-nucleotide polymorphisms positioned within the gene promoters have been shown to affect transcription factor binding and hence gene expression. One of the common G/A polymorphisms has

Abbreviations: TNF, tumor necrosis factor; ARMS PCR, amplification refractory mutation system polymerase chain reaction; CP, chronic pancreatitis; CT scan, computed tomography scan; ERCP, endoscopic retrograde cholangiopancreatography; EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; OR, Odds ratio; CI, class intervals; ELISA, enzyme-linked immunosorbent assay.

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been described at position −308 (TNF-308 G/A). The −308 polymorphism could probably affect the cell-type and stimulus specific control of TNF synthesis at the transcriptional level. A genetic predisposition to produce elevated TNF levels, due to the presence of the −308A polymorphism, may change the course of an immune response such that an individual is more susceptible to the disease (Abraham and Kroeger, 1999).

Given the local and systemic immune-stimulating effects of TNF-α, we hypothesized that promoter polymorphism of this gene may be a genetic modifying factor for chronic pancreatitis (Beranek et al., 2003). Hence, the aim of the present study was to investigate the qualitative and quantitative variation of TNF-α in the etiology of chronic pancreatitis.

2. Materials and methods

2.1. Study population

The study design was a prospective case control study conducted with a total of 200 unrelated individuals. One hundred clinically evaluated chronic pancreatitis (CP) patients referred and admitted in the Gastroenterology Unit of Gandhi Hospital and Osmania General Hospital, Hyderabad between March 2008 and March 2010 were involved in the present study. The diagnosis of chronic pancreatitis was established on the findings of pancreatic duct dilation or pancreatic calcifications, or on the histological findings of chronic pancreatitis of various diagnostic tests like X-ray, CT scan, ERCP, etc. (Abraham and Kroeger, 1999). An alcoholic origin of chronic pancreatitis was assumed when the alcohol intake was more than 80 g/day in men for at least two years. For smoking status, a person who had smoked at least once a day for > 1 year in his or her lifetime was regarded as a smoker (Özhan et al., 2010). An equal number of healthy control subjects were randomly selected for the study from the individuals visiting our institute for regular health checkup. As male preponderance was observed in the patient group, more number of male controls was included in the study for better comparative analysis. Among these controls, 40% of them were found to be alcoholics at random. A structured proforma was used to seek information on dietary habits, smoking, alcohol consumption, family history, etc. Written informed consent was obtained from all the subjects, included for the study. The study was also approved by the Institutional Ethical Committee.

2.2. DNA isolation

5 ml of venous blood was drawn from each individual in vacutainers with and without EDTA for the separation of plasma and serum respectively and stored at −70 °C until use. Genomic DNA was isolated from whole blood by following the salting out procedure of Lahiri and Nurnberger (1991).

2.3. Genotyping of the TNF-α gene

The A and G alleles at position −308 in the promoter region of the TNF-α gene were determined using the amplification refractory mutation system polymerase chain reaction (ARMS-PCR) methodology (Ye et al., 2001). Each PCR reaction was carried out in a total volume of 10 μl, containing 30 ng of template DNA, 10 pmol of each inner primer, 1 pmol of each outer primer, 200 μM dNTP, 2.5 mM of $MgCl_2$, 20 mM Tris–HCl pH 8.4, 50 mM KCl and 0.5 U Taq polymerase (B. Genei). The cycling conditions were as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 61 °C for 1 min and 72 °C for 1 min. The final extension step was at 72 °C for 2 min. The amplified products were: 323 bp internal control, 224 bp G allele and 154 bp A allele which were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. The gel was visualized under ultraviolet light with a 100-base pair ladder.

2.4. Estimation of TNF-α level

Concentrations of TNF-α (Invitrogen®) in plasma were measured using a commercial ELISA kit according to the manufacturer's protocols. In brief, 50 μl incubation buffer was added to different wells designated as samples and standards. 100 μl of standard or control samples was added to each well. After 2 h of incubation at room temperature on a constant shaker (500 ± 50 rpm), the reaction solution was aspirated and the wells were washed 4 times with wash buffer; 100 μl of biotinylated anti-TNF-α conjugate was then added to each well and incubated for another 1 h on the shaker at room temperature. Then the aspiration/wash steps were repeated as mentioned above, followed by adding of 100 μl of streptavidin–HRP solution to each well. The microplate was allowed to stand for 30 min at room temperature. The aspiration steps were repeated. Then stabilized chromogen (100 μl) was added to each well followed by 30 min of incubation at room temperature in the dark. The optical density of each sample was determined at 450 nm and represented in ng/ml. The TNF-α concentration for each sample was calculated from the standard curve obtained.

2.5. Statistical analysis

Statistical analysis was done by using the computer software Statistical Package for Social Sciences-SPSS for windows (version 19.0) (Chicago, IL). ORs and 95% CIs were estimated by conditional logistic regression analyses based on the comparison of genotypes between patients with the disease and healthy controls, and by adjusting the potential confounders such as age, sex, smoking and alcoholism. Hardy–Weinberg equilibrium was conducted to compare the observed and expected genotype frequencies among cases and controls, respectively. A two-sided P-value < 0.05 was considered to be statistically significant. For analyses of genotype frequencies, the wild-type category was the reference group.

3. Results

The study population (Table 1) consisted of 100 patients with CP with an age range of 13–70 years, as well as 100 control subjects with an age range of 20–70. CP group consisted of 95.0% males and 5.0% females whereas control group consisted of 94.0% males and 6.0% females. Patients and control subjects were derived from the same geographic location and are representative of South Indian population from Andhra Pradesh. A significant difference was observed with respect to mean age (<0.000**) smokers (<0.000**) and alcoholics (<0.000**) in patients compared to control subjects.

Table 2 gives the genotype distribution of TNF-α (−308 G/A) gene in control subjects and patient group. The distribution of the TNF-α

Table 1
Demographic and clinical parameters of the study groups (CP).

Parameter	Controls		CP		OD (CI)	p value
	N	%	N	%		
Gender						
Males	94	94	95	95		
Females	6	6	5	5	0.82 (0.22–2.92)	0.378*
Age						
<40 years	78	78	52	52		
≥40 years	22	22	48	48	3.25 (1.77–6.10)	<0.000**
Addictions						
Smokers	22	22	49	49		
Non-smokers	78	78	51	51	0.29 (0.15–0.56)	<0.000**
Alcoholics	40	40	76	76		
Non-alcoholics	60	60	24	24	0.21 (0.11–0.38)	<0.000**

* p < 0.05.
** p < 0.0001.

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