

TGF- β 1 (C-509T) Gene Promoter Polymorphism and its Influence on Chronic Pancreatitis

Kavutharapu Sri Manjari, Akka Jyothy, Porika Shraavan Kumar, Boddu Prabhakar, Prathibha Nallari, Ananthapur Venkateshwari

Kavutharapu Sri Manjari, Akka Jyothy, Ananthapur Venkateshwari, Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, India

Porika Shraavan Kumar, Department of Gastroenterology, Gandhi General Hospital, Secunderabad, India

Boddu Prabhakar, Department of Gastroenterology, Osmania General Hospital, Hyderabad, India

Prathibha Nallari, Department of Genetics, Osmania University, Hyderabad, India

Correspondence to: Ananthapur Venkateshwari, Asst. Professor, Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, India

Email: venkateshwari@yahoo.com

Telephone: +91-40-23403681

Received: March 7, 2013 Revised: April 5, 2013

Accepted: April 10, 2013

Published online: January 21, 2015

ABSTRACT

AIM: Chronic pancreatitis is a long-standing inflammation of the pancreas that alters its normal structure and function. The aim of the study is to investigate the role of promoter polymorphism in the TGF- β 1-509 (C/T) gene in chronic pancreatitis.

METHODS: A total of 100 chronic pancreatitis patients and 100 control subjects were included in the present study. The genotyping of promoter polymorphism in the TGF- β 1-509 (C/T) gene was carried out by tetra-primer ARMS PCR followed by agarose gel electrophoresis. The serum TGF- β 1 levels were measured by enzyme-linked immunosorbent assay. Statistical analysis was applied for the significance of the results.

RESULTS: The genotype distribution was 57% of C/C, 38% of C/T and 5% of T/T in control subjects, whereas in chronic pancreatitis patients they were 37% of C/C, 39% of C/T, and 24% of T/T genotype respectively. A significant variation in the distribution of T allele was observed in the patient group compared to control subjects [OD = 0.410 (0.261-0.643), $p < 0.000^{**}$]. There was a significantly

higher levels of TGF- β 1 levels in CP patients compared to healthy subjects.

CONCLUSION: The present study found a significant association between the TGF- β 1-509 (C/T) gene promoter polymorphism and chronic pancreatitis. Further, the levels were also found to be elevated in patients compared to control subjects. Thus, TGF- β 1-509 (C/T) genotype can be considered as one of the risk factor in the etiology of the disease.

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Key words: Chronic inflammation; Pancreatic fibrosis; Extra cellular matrix; Cytokines; Polymorphism

Manjari KS, Jyothy A, Kumar PS, Prabhakar B, Nallari P, Venkateshwari A. TGF- β 1 (C-509T) Gene Promoter Polymorphism and its Influence on Chronic Pancreatitis. *Journal of Gastroenterology and Hepatology Research* 2015; 4(1): 1447-1450 Available from: URL: <http://www.ghrnet.org/index./joghr/1023>

INTRODUCTION

Chronic pancreatitis (CP) is characterized by the slow, progressive development of structural abnormalities within the pancreas. These morphological changes lead to progressive pancreatic functional impairment^[1]. One of the key features of chronic pancreatitis is pancreatic fibrosis, which has received wide attention over the past few years, largely due to the identification and characterization of stellate cells in the pancreas. The progressive replacement of pancreas-specific tissue by ECM-rich connective tissue may lead to the development of an exocrine and endocrine insufficiency of the gland.

In human tissues, normal homeostasis requires intricately balanced interaction between cells and the network of secreted proteins known as the extracellular matrix (ECM). These cooperative interactions involve numerous cytokines acting through specific cell-surface receptors. The balance between the cells and the extracellular matrix when perturbed can result in a disease^[2]. This is clearly evident by

the interaction mediated by the cytokine transforming growth factor β 1 (TGF- β 1), a growth factor demonstrating documented and well-known profibrogenic activity^[1].

Transforming growth factor- β 1 is a cytokine produced by both immune and non immune cells. TGF- β 1 controls the differentiation, proliferation, and state of activation of all immune cells, wound healing and angiogenesis and is implicated in immune abnormalities linked to cancer, autoimmunity, opportunistic infections, and fibrotic complications. The present study is aimed at evaluating the qualitative and quantitative role of TGF β 1 in the etiology of chronic pancreatitis.

METHODS

Study population

A total of 200 unrelated individuals were included in the present study. One hundred clinically evaluated chronic pancreatitis (CP) patients referred to the Gastroenterology unit of Gandhi Hospital and Osmania General Hospital, Hyderabad during the two years from 2008 to 2010 were included in the present study. An equal number of healthy control subjects were randomly selected among the individuals visiting our Institute for regular health checkup was also included for comparative studies. 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, before initiation of the study. The study was also approved by our Institutional Ethical committee.

DNA separation

5ml 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 *et al* (1991)^[4].

Genotyping of the TGF- β 1 gene

The polymorphism for TGF- β 1 C-509T (rs1800469) was genotyped based on Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method. The allele-specific primers are as follows: forward primer, 5'CTACGGCGTGGAGTGCTGAG 3', and reverse primer, 5'AAGGGGCAACAGGACACCTGGG 3' for the C allele and 5'AAGGGGCAACAGGACACCTGGA 3' for the T allele. A 349 base pair region (- 488 to -836 from the transcription start site) in the TGF- β 1 gene promoter was targeted for amplification. The optimized reaction conditions consisted of 40 ng of genomic DNA in a reaction volume of 15 μ L containing 1X reaction buffer, 1.5 μ M MgCl₂, 30 μ M of each dNTP, 0.16 μ M of each primer, and 0.3U of Taq DNA polymerase. The cycling conditions were as follows: an initial denaturation at 94°C for 4 minutes, followed by 35 cycles at 94°C for 30 s, 61°C for 20 s and 72°C for 30 s. The final extension step was at 72°C for 6 min.

The amplified products were electrophoresed on an agarose gel (1.5 %) stained with ethidium bromide. The presence of 349bp bands in both the C and T lanes based on 100–base pair ladder indicated heterozygous CT genotype whereas the presence of 349 bp band either in C lane or T lane represents homozygous CC genotype and homozygous TT genotype respectively (Figure 1).

Estimation of TGF- β 1 levels by ELISA

Concentrations of TGF- β 1 (Invitrogen®) in plasma were estimated using a commercial ELISA kit according to the manufacturer's

protocols. In brief, 50 μ L detector antibody was added to different wells designated as samples and standards. 50 μ L of standard or control samples were added to each well. After 2 hours of incubation at room temperature on a constant shaker (500 \pm 50 rpm), the reaction solution was aspirated and the wells were washed 4 times with wash buffer; 100 μ L of TGF- β 1 conjugate was then added to each well and incubated for another 30 min on the shaker at room temperature. Then the aspiration/wash steps were repeated as mentioned above, followed by adding of 100 μ L of substrate solution to each well. The microplate was allowed to stand for 30 min at room temperature in the dark. After adding 100 μ L of stop solution, the optical density at 450 nm of each sample was determined and represented in ng/mL. The TGF- β 1 concentrations for each sample were calculated from the standard curve obtained.



Lane 1 and 2: Heterozygous C/T; Lane 3 and 4: Homozygous C/C; Lane 5 and 6: Homozygous T/T; Lane 7: 100 bp ladder.

Figure 1 Gel picture showing TGF- β 1genotypes.

Statistical analysis

Differences among subject groups in clinical characteristics were analysed with *t*-tests or Fisher's two-tailed exact tests. Genotype frequencies were tabulated by direct counting and allele frequencies estimated from the observed number of genotypes. Chi-square (χ^2) analysis was used to test for deviation of allele frequencies from Hardy–Weinberg equilibrium. The distributions of the TGF- β 1genotype and allele frequencies in healthy and CP group were also analyzed by Chi-square or Fisher's two-tailed exact tests. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were also assessed. The relationship between genotype and disease status was analyzed by multiple logistic regression analysis for potential confounding factors including age, gender, smoking, consanguinity and alcohol status. TGF- β 1 serum levels within CP and healthy groups were compared between subjects carrying different genotypes using the "*t*" test. *P*-values <0.05 were considered to be statistically significant. Clinical parameters were compared with repeated measure ANOVA test. The data analysis was performed using a statistical package (SPSS Inc., version 19.0, Chicago, IL, USA).

RESULTS

The study population consisted of 100 CP patients of which 94 (94.0%) males and 6 (6.0%) females with an age range of 13-70 years, as well as 100 control subjects of an age range 20-70 [(95.0% males and 5 (5.0%) females] were considered (Table 1). 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 smokers (<0.000**) and alcoholics (<0.000**) in patients compared to control subjects.

Table 2 gives the genotype distribution of TGF- β 1 (-509 C/T) gene in control subjects and patients with chronic pancreatitis. Among the controls the genotype distribution was 57% of C/C, 38% of C/T and 5% of T/T, whereas in chronic pancreatitis patients 37% were of C/C, 39% of C/T, and 24% were of T/T genotype. A significant variation was observed in the patient group compared to control subjects [OD=0.410 (0.261-0.643), p <0.000**] with respect to allelic distribution.

Table 3 gives the mean serum TGF- β 1 levels with respect to TGF- β 1 genotype between controls and chronic pancreatitis patients. A significant increase in the serum TGF- β 1 levels in CP patients (219.30 \pm 163.907) was observed when compared to controls (168.55 \pm 100.22) (p <0.0001**).

DISCUSSION

Chronic pancreatitis an inflammatory, usually painful, condition characterized by progressive glandular destruction, fibrosis and loss of endocrine and exocrine function culminating in pancreatic failure, along with associated complications to the surrounding viscera and vasculature^[5].

Fibrosis is a wound-healing response by which the body attempts to repair itself following injury. Acute and more commonly, chronic injury from a wide variety of insults leads to organ fibrosis. Organ systems have different cellular and molecular mechanisms that result in fibrosis^[6]. Fibrous tissue contains extracellular matrix but in different ratios to normal tissue. In particular, there is a significantly

increased amount of type I collagen, with progressive fibrosis eventually leading to distortion of the normal organ architecture. The distorted architecture, along with loss of normal cellularity may, lead to a loss of function of the underlying organ^[7].

Pancreatic fibrosis is associated with major alterations in both the quantity and composition of the extracellular matrix (ECM). Accumulation of ECM, including fibrillar collagens, fibronectin, laminin, hyaluronan, and proteoglycans, results from both increased synthesis and decreased degradation^[8]. An imbalance of the two dynamic processes, fibrogenesis and fibrolysis leads to fibrosis. Genes that govern the production and degradation of fibrous tissue are important with regard to the etiology of chronic pancreatitis. These genes could represent genetic markers of progression rather than susceptibility towards a certain disease.

Cytokines play an important role in regulating the immune response during acute pancreatitis^[9], and cytokines may also be secreted by inflammatory cells that infiltrate the pancreas in chronic pancreatitis^[10]. As TGF- β 1 is one of the most important profibrogenic cytokine and a potent activator of pancreatic stellate cells, its role in fibrogenesis and fibrolysis cannot be ruled out. The expression of TGF- β 1 is influenced by polymorphisms in the TGF- β 1 gene. In particular, there is a C>T promoter polymorphism at nucleotide position -509 (position relative to transcriptional start site defined in GenBank NM_000660 and NT_011109) that alters a Yin Yang 1 (YY1) transcription factor consensus binding site (-CCATCTC/TG-) and is associated with higher circulating concentrations of TGF- β 1 in plasma^[11]. TGF- β 1 has been shown to be of particular importance in biosynthesis and turnover of extracellular matrix. The present study revealed a significant association of TT genotype and T allele with the disease compared to control subjects.

Pancreatic stellate cells may play a central role in pancreatic fibrosis in regulating the fibrotic destruction of the organ^[12,13]. Results from *in vitro* studies revealed that these cells are stimulated by alcohol and other inflammatory cytokines^[13-15]. Earlier studies with animal models have shown that cytokines participate in the immunologic processes during chronic pancreatitis^[16,17]. Awad *et al*^[18] (1998) found an association between leucine at codon 10 and elevated TGF- β 1 concentrations in patients with cystic fibrosis. A preliminary study by Howell *et al*^[19] showed no association between TGF- β 1 +915 polymorphism and CP. Studies by Yamada *et al* (1998) and Yokota *et al* (2000) revealed conflicting results and linked the proline at codon 10 with the highest serum concentrations in Japanese^[20,21]. Elevated levels of TGF- β 1 observed in the present study may stimulate transcription and biosynthesis of extracellular matrix proteins and functions as an activator of pancreatic stellate cell proliferation. These alterations may lead to pancreatic fibrosis and ductular cell proliferation, resulting in chronic pancreatitis.

Table 1 Demographic and clinical variables of controls and chronic pancreatitis.

Parameter	Controls		Chronic pancreatitis		χ^2 value	p value
	N	%	N	%		
Gender						
Males	94	94	95	95		
Females	6	6	5	5	0.096	0.756
Age (mean age)						
< 40 yrs	58	58	52	52		
\geq 40 yrs	42	42	48	48	0.727	0.477
Addictions						
Smokers	22	22	49	49		
Non-smokers	78	78	51	51	15.919	<0.000**
Alcoholics	37	37	76	76		
Non-alcoholics	63	63	24	24	30.945	<0.000**
Consanguinity	9	9	17	17		
Non-consanguinity	91	91	83	83	0.093	<0.000**

* p < 0.05, ** p <0.0001

Table 2 Genotypic and allelic frequencies of TGF- β 1 genotypes in controls and chronic pancreatitis.

Study group	C/C		C/T		T/T		Total	Allelic frequencies	
	N	(%)	n	(%)	N	(%)		C	T
Controls	57	(57)	38	(38)	5	(5)	100	0.35 (152)	0.66 (113)
CP	37	(37)	39	(39)	24	(24)	100	0.21 (48)	0.79 (87)

P <0.000** (OD=0.410, 0.261-0.643)

Table 3 TGF- β 1 genotypes and their corresponding mean serum levels of TGF- β 1 in control subjects and chronic pancreatitis.

TGF- β 1	Controls (N)	Mean \pm SD	Patients (N)	Mean \pm SD	t-value	p -value
Genotype:						
C/C	57	119.21 \pm 54.97	37	148.65 \pm 103.26	1.7985	0.075
C/T	38	192.95 \pm 69.63	39	191.67 \pm 155.50	0.0464	0.963
T/T	05	386.00 \pm 111.66	24	373.13 \pm 158.06	0.1721	0.865
TOTAL	100	219.30 \pm 163.91	100	168.55 \pm 100.22	2.6416	0.009*

It is a well-defined fact that TGF- β 1 strongly stimulates the synthesis of extracellular matrix components, including collagen, fibronectin, and proteoglycan, and a major candidate in the fibrogenesis that occurs in different chronic inflammatory diseases, including chronic pancreatitis. Earlier studies by Mola *et al* 1999, on the pancreatic tissue samples of chronic pancreatitis patients by in situ hybridization and immunohistochemistry have clearly showed overexpression of TGF- β 1 suggesting its role in CP^[22]. Similar studies by Satoh *et al* 1998 have shown a significant increase in TGF- β 1 expression^[23]. A study by Muller *et al* 1999 on transgenic mice showed an enhanced TGF- β 1 expression in the development of fibrosis and inflammation in pancreas during CP^[24].

In conclusion, TGF- β 1-509 (C/T) gene can be considered as one of the biomarker in the etiology of the disease and may contribute to the pathogenesis of chronic pancreatitis by increasing proliferation of pancreatic stellate cells. Thus, further analysis on a larger sample size is required to confirm the possible association of TGF- β 1 in chronic pancreatitis

ACKNOWLEDGMENTS

The authors acknowledge financial support from University Grants Commission (UGC), New Delhi [File No: 36-185/2008 (SR)].

CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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Peer reviewer: Raffaele Pezzilli, Department of Internal medicine and Gastroenterology, Sant'Orsola-Malpighi Hospital, Via Massarenti 9, 40138 Bologna, Italy.