Hereditary, familial, and idiopathic chronic pancreatitis are not associated with polymorphisms in the tumor necrosis factor α (*TNF-α*) promoter region or the TNF receptor 1 (*TNFR1*) gene

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Purpose: Genetic alterations that are associated with acute and chronic pancreatitis remain to be identified. **Methods:** The authors investigated two functionally active tumor necrosis factor (*TNF*) promoter region polymorphisms at positions −238 and −308 and the entire coding region of the corresponding TNF receptor 1 (*TNFR1*) gene in 54 patients with hereditary, familial, and idiopathic chronic pancreatitis who were previously tested negative for cationic trypsinogen mutations by direct DNA sequencing. **Results:** In three patients, we detected novel DNA variants in the *TNFR1* gene that did not segregate with the disease. The genotype frequencies of the *TNF* promoter polymorphisms were similar between patients and controls. **Conclusion:** These polymorphisms are not associated with hereditary, familial, or idiopathic chronic pancreatitis. ***Genet Med* 2003:5(2):120–125.**

Key Words: *chronic pancreatitis, gene polymorphisms, hereditary pancreatitis, soluble tumor necrosis factor receptors, tumor necrosis factor α*

Acute and chronic pancreatitis remain among the most difficult diseases to understand and treat because of the retroperitoneal location of the organ and the self-destructive nature of these diseases. Recent advances in understanding acute and chronic pancreatitis emerged from studies in hereditary pancreatitis, a genetic disorder with an autosomal-dominant inheritance pattern and a disease penetrance of approximately 80%. In 1996, a single point mutation in the cationic trypsinogen gene was identified as a primary cause of this disease,[1] and additional disease-causing trypsinogen mutations have been identified (reviewed by Chen et al.[2]). Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene[3,4] and in the pancreatic secretory trypsin inhibitor (*SPINK1*) gene have been linked to familial, idiopathic, and tropical pancreatitis.[5–11] However, none of the previously described mutations has been observed in about 40% of kindreds with hereditary pancreatitis.[12] Thus, in these families with hereditary pancreatitis, as well as in the majority of cases with familial and idiopathic acute and chronic pancreatitis, the underlying genetic mutations are unknown.

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In 1998, a genome-wide genetic linkage study was performed in five families with familial pancreatitis that had no cationic trypsinogen mutations. This analysis suggested that a locus on the short arm of chromosome 12 contains a new gene that is associated with pancreatitis.[13] The tumor necrosis factor receptor 1 (*TNFR1*) gene is located in this broad genomic region. Tumor necrosis factor (TNF), which is the ligand for TNFR1, is of importance in exacerbating acute pancreatitis,[14–16] and elevated levels of soluble TNFR1 have also been observed in severe acute pancreatitis.[15,17,18] In humans, several polymorphisms are known to alter TNF expression. The promoter region of the *TNF-α* gene contains two functionally active polymorphisms. Although not in accordance with recent nomenclature recommendations for human gene mutations,[19] these polymorphisms are frequently cited in the literature as polymorphisms at positions −308 (TNF2 allele, wild-type TNF1 allele) and −238 (TNFA allele, wild-type TNFG allele) relative to the transcription start site.[20] Investigations with the *TNF-α* promoter revealed that, in vitro, the TNF2 allele leads to increased TNF-α expression.[21,22] The −238 promoter polymorphism is located within a regulatory motif and within a *TNF-α* repressor site, suggesting that this variant also affects the transcription of the gene.[23,24] However, conflicting results have been reported regarding the influence of both genetic variants on TNF-α expression (reviewed by Hajeer and Hutchinson[20]). Recently, a preliminary report suggested that the −308 promoter polymorphism is associated with alcoholic chronic pancreatitis.[25]

An investigation of these polymorphisms and of the *TNFR1* gene has not been conducted in patients with hereditary, fa-

milial, and idiopathic chronic pancreatitis. Therefore, we investigated these two polymorphisms within the *TNF-α* promoter region and the entire coding region of the *TNFR1* gene in the context of hereditary, familial, and idiopathic chronic pancreatitis.

MATERIALS AND METHODS

Study population

Patients were recruited from the ongoing University of Pittsburgh hereditary pancreatitis study.[12] The study protocol was approved by the University of Pittsburgh Institutional Review Board. Informed consent was obtained. Hereditary pancreatitis was defined as otherwise unexplained pancreatitis in an individual from a family in which the pancreatitis phenotype appears to be inherited through a disease-causing gene mutation expressed in an autosomal-dominant pattern. Familial pancreatitis was defined as pancreatitis that occurs in a family with an incidence that is greater than would be expected by chance alone and that is not expressed in an autosomal-dominant pattern. Idiopathic pancreatitis was defined as pancreatitis in isolated cases within a family and in which alcohol consumption was excluded.[12]

We investigated 54 patients in which genetic testing of the cationic trypsinogen gene and of the *SPINK1* gene, but not of the *CFTR* gene, was previously performed. Cationic trypsinogen gene mutations have been excluded, but some patients with *SPINK1* mutations were included because *SPINK1* mutations alone are not disease-causing.[6] The patient cohort consisted of 35 patients with hereditary and familial pancreatitis from 32 different families and of 19 patients with idiopathic chronic pancreatitis. In the group of patients with idiopathic chronic pancreatitis, 5 patients with onset of pancreatitis between 3 and 18 years of age did not demonstrate signs of chronic pancreatitis on pancreatic imaging. In these patients, chronic pancreatitis was defined as a condition characterized by at least two episodes of acute pancreatitis requiring hospitalization.

In the group of patients with hereditary and familial pancreatitis, the median age at onset of pancreatic disease was 6 years (range 2–63 years), and in the group of patients with idiopathic chronic pancreatitis, the median age at onset was 8 years (range 3–43 years).

Among the patients with hereditary and familial pancreatitis, 20 patients originated from hereditary pancreatitis families with an autosomal-dominant-appearing inheritance pattern of high penetrance (two or more affected first-degree relatives in successive generations, e.g., parent-child), 9 patients were from hereditary pancreatitis kindreds with an autosomal-dominant-appearing inheritance pattern of low penetrance (two or more affected relatives who are not first-degree relatives of each other, e.g., aunt-nephew, grandparent-grandchild), and 6 patients had a familial-appearing inheritance pattern (two or more affected first-degree relatives in the same generation, e.g., siblings). For analysis of the *TNF-α* promoter polymorphisms, 94 controls were investigated, and for analysis of the *TNFR1* gene, 68 controls were studied. The ethnicity of the patients was Caucasian. The analysis of two exons of the *TNFR1* gene was extended to additional family members, and for an intronic region, 48 other patients with hereditary and familial pancreatitis ($n = 29$ autosomal-dominant-appearing with high penetrance, $n = 8$ autosomal-dominant-appearing with low penetrance, $n = 11$ familial-appearing) were investigated. Previous genetic testing of this patient group was identical with the testing in the original study group with exclusion of cationic trypsinogen gene mutations. The additional patient group consisted of 17 patients from 12 kindreds that were already analyzed in the original cohort, and 31 patients from 25 additional kindreds.

Genetic analysis

Genetic analysis was performed by polymerase chain reaction (PCR) and subsequent direct DNA sequencing.[1] The *TNF* promoter region was investigated with PCR primers and PCR cycle conditions as described previously.[26] For analysis of the entire *TNFR1* coding region and the adjacent intronic regions, PCR primers were designed separately for exons 1, 2–3, 4–5, 6–7, 8–9, 8–10 (Table 1) referring to published GenBank sequences (Accession AC006057). For exons 8–9 and 8–10, Expand Long Template PCR System (Roche Diagnostics Corp., Indianapolis, IN) was used following the manufacturer's instructions. PCR products were purified with GeneChoice PCR purification kits (PGC Scientifics Corp., Frederick, MD) or with the ExoSAP-IT enzymatic purification system (USB Corporation, Cleveland, OH). Additional internal sequencing primers were used for cycle sequencing of the PCR products from exons 1, 2–3, 4–5, 6–7, and for sequencing of the exons 9–10 from the PCR product of exons 8–10. Sequencing reactions were performed using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were purified with Ethanol Precipitation (Applied Biosystems) and were run on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Sequence analysis was performed using Sequencher 3.1 (GeneCodes Corp., Ann Arbor, MI). Novel base changes were verified by sequencing of the opposite strand with corresponding primers. Confirmation of identity was achieved by comparing the resulting sequences with the published *TNF-α* promoter region sequence,[24] and with the published *TNFR1* sequence.[27,28]

Statistical analysis

Differences between patients and controls were assessed with Fisher's exact method. To avoid a biased representation of a genotype due to familial clustering, only one affected individual of each family was considered for statistical analysis. Probability values of <0.05 were considered significant.

RESULTS

TNFR1 gene

The DNA variants in the *TNFR1* gene and the genotype distributions in patients and controls are shown in Table 2.

Table 1
PCR primer pairs and annealing temperatures used for genetic analysis of the *TNFR1* gene

Scanning region	Primer sequences	Temp.	Product size
5′UTR and exon 1	5′-TGTGCTTGTGACTGGACTAC-3′	54°C	~550bp
	5′-CCTACTCCAAAAGGCGGATG-3′		
Exon 2–3	5′-CTGGGCGGAATGTTTCACTG-3′	55°C	~700bp
	5′-CTCACATCCATGCAGTGTCC-3′		
Exon 4–5	5′-ACACTGCATGGATGTGAGTG-3′	61°C	~640bp
	5′-ACTCAGCCAGTAGGACCCTG-3′		
Exon 6–7	5′-GGATGTCCAACAATCTGTGTGG-3′	60°C	~580bp
	5′-GACATGCCTCAGGGCCTATG-3′		
Exon 8–9	5′-CACTGCCAGCTGAGTCCAGG-3′	65°C	~750bp
	5′-GAATTCCTTCCAGCGCAACG-3′		
Exon 8–10 and 3′UTR	5′-TGATGCTTTCTTTCTTTTTCCTC-3′	55°C	~1560bp
	5′-ATCTCACCCCTCAGGATCTG-3′		

Except for the region of exon 8–9, additional internal primers were used for sequencing.

Table 2
Polymorphisms in the *TNFR1* gene and their distribution among patients and controls

Location	Polymorphism	Patients			Controls
Exon 1	Nucleotide 36A>G (P12P)	33/54 (homo and hetero)			*(not applicable)*
Exon 2	*Nucleotide 123T>G (D41E)	1/54 (hetero)			*(not applicable)*
Exon 4	*Nucleotide 362G>A (R121Q)	1/54 (hetero)			1/68 (hetero)
	*Nucleotide 434A>G (N145S)	1/54			0/68
Intron 6	IVS6+10A>G	Hereditary and familial			
		One individual from each family			
		AA 27/57 (47%)	AG 25/57 (44%)	GG 5/57 (9%)	AA 24/68 (35%) Ag 35/68 (52%) GG 9/68 (13%)
		Entire study group			
		AA 39/83 (47%)	AG 37/83 (45%)	GG 7/83 (8%)	(for all patient cohorts $P > 0.05$)
		Idiopathic			
		AA 5/19 (26%)	AG 12/19 (63%)	GG 2/19 (11%)	

Except for intron 6, no differentiation is shown between patients with hereditary or familial pancreatitis and idiopathic chronic pancreatitis. For the IVS6+10A>G polymorphism, 83 patients with hereditary and familial chronic pancreatitis from 57 different families were studied. To avoid a biased representation of a genotype due to familial clustering, only one individual of each family was considered for statistical analysis.
*Novel polymorphism.

Three novel mutations were identified. The first was an exon 2 T>G mutation at position 123 in codon 41 (D41E). In exon 4, a G>A base change at position 362 in codon 121 (R121Q) and an A>G mutation at position 434 in codon 145 (N145S) was identified. The patient with the D41E polymorphism in exon 2 had idiopathic chronic pancreatitis, but was also positive for a N34S/P55S compound heterozygous mutation in the *SPINK1* gene. The R121Q and the N145S polymorphisms were found in patients with hereditary pancreatitis. Sequencing of exon 4 in eight affected and four unaffected family members of the patient with the R121Q mutation revealed that this polymorphism was present only in the initial individual and not in the

other family members. Thus this genetic variant did not segregate with the disease. Moreover, the R121Q mutation was also found in 1 out of 68 controls. The affected father and the unaffected mother of the proband with the N145S polymorphism were both without this variant, suggesting that this variant represents a new mutation. We also observed two previously described genetic variants: a conservative A to G base change in codon 12[27] and a polymorphism in intron 6 (IVS6 + 10A>G).[29] Since we found a trend toward a statistically significant difference between patients with hereditary and familial chronic pancreatitis and controls, we investigated this region in 48 other patients with hereditary and familial pancreatitis. With these additional patients, there was no statistically significant difference between the different patient groups and the controls ($P > 0.05$). We sequenced the *TNFR1* promoter region up to 100 bp from the start of the coding region, but no genetic variants were identified.

TNF-α promoter polymorphisms

The two previously described polymorphisms were identified in the *TNF-α* promoter region: a G>A substitution at position −308 (termed TNF2 allele, wild-type TNF1 allele) and a G>A substitution at position −238 (termed TNFA allele, wild-type TNFG allele) relative to the transcription start site. The genotype distributions of the −308 and −238 *TNF-α* promoter polymorphisms are shown in Table 3. There was no statistically significant difference between the two patient cohorts and the controls ($P > 0.05$). Furthermore, there was no association between the different genotypes and the presence of *SPINK1* mutations or the clinical course of the disease (data not shown).

In the controls, the genotype frequencies for the *TNF-α* promoter region polymorphisms and for the polymorphism in the intron 6 of the *TNFR1* gene were similar to the genotype frequencies of these polymorphisms that are reported in the literature for Caucasians.[29–31]

DISCUSSION

During the past decade, the proinflammatory cytokine TNF and its receptor have been implicated in the mediation of local and systemic manifestations of acute pancreatitis in several clinical and experimental studies.[14–18] Pancreatic acinar cells produce, release, and respond to TNF-α, and TNF has been reported to regulate apoptosis in isolated pancreatic acini and in cerulein-induced experimental pancreatitis.[32] Elevated TNF serum concentrations have been observed in patients with acute pancreatitis,[15,16] and elevated plasma levels of soluble TNFR1 have also been suggested to reflect severity of acute pancreatitis.[15,17] Necrotizing pancreatitis was induced in *TNFR1* knockout mice, and pancreatic injury was attenuated in these genetically determined animals.[14]

TNF and its TNFR1 may also be important in chronic pancreatitis. Elevated levels of soluble TNFRs were found in patients with chronic alcoholic pancreatitis.[33] Recently, TNF-α and other proinflammatory cytokines were reported to activate pancreatic stellate cells, thus suggesting that TNF might participate in the progression from acute pancreatitis to chronic pancreatitis and fibrosis.[34] Finally, in preliminary studies, the *TNF-α* promoter region polymorphism at position −308 and *TNF* microsatellite haplotypes have been associated with alcoholic chronic pancreatitis.[25,35]

The cytokine TNF exists in two homologous forms, TNF-α and TNF-β, which both bind to TNF receptors, TNFR1 and TNFR2, that are present on virtually all cells throughout the body.[36] Several experimental studies further support the assumption that the TNFR1 is important in inflammatory diseases. First, *TNFR1* knockout mice are much more resistant to challenge with bacterial pathogens and endotoxin than wild-type animals or *TNFR2* knockout mice, suggesting that cellular inflammatory responses to soluble TNF are mainly mediated by TNFR1,[37–39] and the TNFR1 receptor has a higher binding affinity for soluble TNF.[37] Second, a ~70 residue "death domain" is found in the TNFR1 that mediates cytotoxic signals and programmed cell death.[40] Finally, the extracellular do-

Table 3
Genotype distribution of the −308 and −238 *TNF* promoter region polymorphisms among patients and controls

	−308 polymorphism			−238 polymorphism		
Patient and control group	*TNF1/TNF1* normal	*TNF1/TNF2* heterozygote	*TNF2/TNF2* homozygote	TNFG/TNFG (normal)	*TNFG/TNFA* (heterozygote)	*TNFA/TNFA* (homozygote)
Patients with hereditary and familial pancreatitis ($n = 32$ from 32 families)	$n = 25/32$	$n = 7/32$	$n = 0/32$	$n = 28/32$	$n = 4/32$	$n = 0/32$
	78%	22%	0%	87.5%	12.5%	0%
Patients with idiopathic chronic pancreatitis ($n = 19$)	$n = 15/19$	$n = 4/19$	$n = 0/19$	$n = 18/19$	$n = 1/19$	$n = 0/19$
	79%	21%	0%	94.7%	5.3%	0%
Controls ($n = 94$)	$n = 64/94$	$n = 27/94$	$n = 3/94$	$n = 87/94$	$n = 7/94$	$n = 0/94$
	68%	29%	3%	93%	7%	0%
	($P > 0.05$)	($P > 0.05$)	($P > 0.05$)	($P > 0.05$)	($P > 0.05$)	($P > 0.05$)

Only one individual of each family was considered for statistical analysis.

mains of the TNF receptors may be released into the systemic circulation. Although the physiological role of this release is not clarified yet, this process is stimulated by inflammation and TNF-α itself.[41] Thus the interactions between TNF and TNFR1 are complex, and genetic variants within the receptor-ligand system may be relevant in acute and chronic pancreatitis.

The TNFR1 protein shows a typical transmembrane structure with a cysteine-rich extracellular domain encoded by exons 1 to 7, with a transmembrane domain constituted by 23 residues of exon 7, and with a cytoplasmic domain composed of the remaining amino acids encoded by exons 7 to 10.[42] Mutations in the extracellular domain of the *TNFR1* gene have been reported to cause a newly recognized subgroup of hereditary fever syndromes (TNF-receptor associated periodic syndrome, TRAPS) by disrupting disulfide bonds between the extracellular receptor domain and the corresponding TNF ligand.[43] Thus it has been hypothesized that other mutations in the *TNF* and *TNF* receptor system might contribute to other inflammatory diseases as well.[43] However, the three novel mutations we observed in the present investigation did not affect sites that were previously reported to participate in the receptor-ligand contact, and the examination of the genetic sequence encoding the transmembrane region and the intracellular region did not reveal further mutations in our patients. We observed a previously described IVS6 + 10A>G polymorphism in intron 6.[29] This polymorphism demonstrated a trend toward a significant association with hereditary and familial pancreatitis, but the evaluation of an additional 48 hereditary and familial pancreatitis patients failed to identify an association. There were no polymorphisms within the promoter region up to 100 bp from the start of the coding region.[28]

In the promoter region of the *TNF-α* gene, two functionally active polymorphisms have been identified at positions −308 and −238.[21–24] In our present investigation, the genotype frequencies were similar between patients and controls, and a correlation between the clinical presentation of the disease, the presence of *SPINK1* mutations, and the different genotypes was not detected. However, additional polymorphisms exist within the *TNF-α* promoter region,[20] and future studies may identify an association with pancreatic disease.

In conclusion, our study represents the first detailed mutational analysis of the entire coding region of the human *TNFR1* and of two functional *TNF-α* promoter polymorphisms in patients with hereditary, familial, and idiopathic chronic pancreatitis. We described three novel polymorphisms in the *TNFR1* gene that do not appear to be disease-associated. Thus these results suggest that mutations in the *TNFR1* gene do not predispose to the development of hereditary, familial, or idiopathic chronic pancreatitis in the USA. Furthermore, it appears that the *TNF-α* promoter region polymorphisms at positions −308 and −238 are not associated with hereditary, familial, or idiopathic chronic pancreatitis.

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