ORIGINAL ARTICLE

Influence of cytokine and ICAM-1 gene polymorphisms on susceptibility to chronic pancreatitis

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Aims: To test the hypothesis that single nucleotide polymorphisms (SNPs) within genes (or their promoter regions) encoding cytokines, growth factors, and intercellular adhesion molecules modulate the risk of development of chronic pancreatitis (CP).

Methods: DNA was extracted from peripheral blood leucocytes or formalin fixed, paraffin wax embedded tissue from 53 patients with CP and 266 healthy controls. SNPs within the interleukin 1β (IL-1β), IL-6, IL-8, tumour necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF) gene promoter regions and the transforming growth factor β1 (TGFβ1) and intercellular cell adhesion molecule 1 (ICAM-1) genes were genotyped by the amplification refractory mutation system polymerase chain reaction or 5′ nuclease (Taqman®) techniques. Patient–control comparisons were made using 2 × 2 contingency tables and χ^2 analyses.

Results: A non-significant decrease in the frequency of the IL-8 −251 AA genotype and a non-significant increase in the frequency of the ICAM-1 +469 GA genotype was seen in patients compared with controls. No associations were identified between SNPs in the promoter regions of the IL-1β, IL-6, or TNFα proinflammatory cytokines genes or the TGFβ1 and VEGF genes and susceptibility to CP.

Conclusions: This preliminary study suggests that genetic polymorphism within several cytokine genes is unlikely to influence susceptibility to CP, but the possible role of IL-8 and ICAM-1 polymorphisms in the development of this disease requires further investigation.

Chronic pancreatitis is characterised clinically by severe abdominal pain together with malabsorption and, in advanced disease, diabetes mellitus. The triggering event cannot be determined in every case. However, chronic excess alcohol consumption is the most common aetiological factor, with other cases occurring as a result of chronic pancreatic duct occlusion, an inherited genetic predisposition (hereditary pancreatitis), or autoimmune pancreatitis.

The presence of T cells[1] and human leucocyte antigen (HLA) class II upregulation[2] within chronic pancreatitis strongly suggests that inflammatory processes are important in the development and progression of this disease, representing an additional mechanism for disease pathogenesis that is separate from the role played by genetic factors (polymorphisms in the cationic trypsinogen (PRSS1), cystic fibrosis transmembrane conductance regulator (CFTR), and serine protease inhibitor, Kazal type 1 (SPINK1) genes) in both hereditary and idiopathic pancreatitis.[3–9] This is supported by several studies reporting associations of particular HLA polymorphisms with both alcohol and non-alcohol related pancreatitis in diverse ethnic groups.[10–13] Further evidence for the importance of immune processes in the development of chronic pancreatitis is derived from studies showing that the disease is associated with increased concentrations of several cytokines within serum (interleukin 1β; IL-1β[14]), pancreatic juice (transforming growth factor β; TGFβ[15]), and involved pancreatic tissue. In situ hybridisation, northern blotting, and immunohistochemical methods have revealed increases in TGF-β1 mRNA and protein,[16–18] and the proinflammatory cytokine IL-8 mRNA and protein[19 20] in human chronic pancreatitis tissue. Similar tissue increases in mRNA for the proinflammatory cytokines tumour necrosis factor α (TNFα), IL-6, and interferon γ and the profibrotic cytokine TGFβ1 have been detected using reverse transcriptase polymerase chain reaction (PCR), in situ hybridisation, and immunohistochemistry in rat models of chronic pancreatitis.[21 22]

> "The presence of T cells and HLA class II upregulation within chronic pancreatitis strongly suggests that inflammatory processes are important in the development and progression of this disease"

A considerable body of research undertaken in recent years[23] has identified polymorphisms in many cytokine genes, especially within the upstream promoter sequences, which may be functionally relevant because they may influence the levels of expression of these genes. Some of these polymorphisms have been shown to be associated with susceptibility to several immune mediated diseases and malignant diseases.[24] Based on evidence for upregulation of several cytokines in chronic pancreatitis, along with earlier reports of HLA associations with both alcohol and non-alcohol associated pancreatitis, we sought to determine whether genetic polymorphisms associated with differential levels of cytokine expression are important in determining susceptibility to chronic pancreatitis. More specifically, we aimed to determine whether single nucleotide polymorphisms (SNPs) associated with differential expression of IL-1β, IL-6, IL-8, TNFα, and TGFβ1 are associated with susceptibility to chronic pancreatitis in a case–control study. In addition, two SNPs in the promoter region of the vascular endothelial growth factor

Abbreviations: ARMS, amplification refractory mutation system; HLA, human leucocyte antigen; ICAM-1, intercellular cell adhesion molecule 1; IL, interleukin; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TGFβ1, transforming growth factor β1; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor

(VEGF) gene were also studied, because upregulation of the VEGF cytokine (which promotes angiogenesis) and increased blood vessel counts have been reported in chronic pancreatitis.[25] Finally, two SNPs associated with amino acid substitutions in the intercellular cell adhesion molecule 1 (ICAM-1)[26] were also included in our study, because ICAM-1 expression is increased in chronic pancreatitis.[25]

METHODS

Patients

The patient group consisted of 34 white patients recruited at Southampton University Hospitals and a further 19 patients from Leeds, UK. Twenty one of the Southampton patients and all of the Leeds patients were identified within chronic pancreatitis clinics and consented to provide peripheral blood samples for our study. The remaining 13 Southampton patients were identified from the cellular pathology department computer files as a consecutive series of patients undergoing pancreatectomy for severe chronic pancreatitis. Archival formalin fixed and paraffin wax embedded tissue was available from these resection specimens for study. The mean age of these patients (36 men and 17 women) was 50.1 years (range, 20–80) and a clear alcohol related aetiology was indicated in 31 patients (history of longterm alcohol consumption in excess of UK national guidelines, with no further identifiable risk factors for chronic pancreatitis). Full local research ethics committee approval was gained for our study (Southampton and SW Hampshire LREC application reference 125/00).

Controls

The control group used in our project comprised genomic DNA samples from 266 white bone marrow and solid organ donors, collected by the molecular pathology laboratory of Southampton University Hospitals. The mean age of these controls (140 males and 126 females was 39.2 years (range, 3–69). All DNA samples and/or cytokine genotyping results[27 28] were available within the laboratory.

DNA extraction

Stored DNA samples were available from all control individuals and were provided by the 19 Leeds patients. DNA was extracted from peripheral blood samples from 21 Southampton patients with chronic pancreatitis by a standard salt precipitation technique[29] or a commercial extraction system (Whatman SNAPs; Whatman, Brentwood, Middlesex, UK). For the remaining 13 Southampton patients, DNA was extracted from archival formalin fixed and paraffin wax embedded histopathological biopsies, using a technique previously developed in our laboratory.[30]

Genotyping methodology

IL-1β −35 (C/T), IL-1β −511 (C/T), IL-6 −174 (G/C), IL-8 −251 (A/T), TNFα −308 (G/A), TGFβ1 +915 (G/C), VEGF −1154 (G/A), and VEGF −2578 (C/A) SNPs were genotyped by the amplification refractory mutation system (ARMS) PCR technique using one reaction for each allele of each SNP. All primer sequences and PCR conditions for genotyping these SNPs have been published previously.[27 28 31] All PCR reactions were performed in 10 µl reaction volumes and final reagent concentrations were: AS reaction buffer (ABgene, Epsom, Surrey, UK), 200 µM each dNTP, 12% (wt/vol) sucrose, 200µM cresol red, 1µM each specific/common primer, 0.2µM each internal control primer (see table 1 for sequences), 0.25 units Thermoprime^{PLUS} DNA polymerase (ABgene), and 50–100 ng/µl DNA. $MgCl_2$ concentrations were optimised for each SNP. Internal control primers were omitted when genotyping DNA from archival biopsies and ICAM-1 SNPs. PCR reactions were performed using a DNA engine, (MJ Research Inc, Watertown, Massachusetts, USA) or an MWG primus 96 plus Thermal Cycler (MWG Biotech UK Ltd, Milton Keynes, UK). PCR products were loaded directly on to 2% agarose gels (containing 0.5 mg/ml ethidium bromide), electrophoresed, and visualised by photography under ultraviolet transillumination.

ICAM-1 +241 (G/A) and +469 (G/A) SNPs were genotyped using the 5′ nuclease assay for allelic discrimination. Primers and TaqMan® probes were designed using Primer Express version 2.0 software (sequences shown in table 1) and synthesised and supplied by Applied Biosystems (Warrington, Cheshire, UK). The reporter dyes chosen were 6-FAM and VIC™. PCR reactions (5 µl) containing 10 ng of DNA, 0.9 µm primers, and 0.2 µm probes (final concentrations) were performed in 384 well plates using the Applied Biosystems allelic discrimination PCR protocol and run on the Applied Biosystems 7900HT sequence detection system. Each genotyping plate contained no DNA template (water) controls and randomly selected duplicate samples. SDS version 2.0 software was used to analyse real time and end point fluorescence.

Statistical analysis

Patient–control comparisons were made using 2 × 2 contingency tables and χ^2 analysis or Fisher's exact test when any value in a 2 × 2 table was less than 5. Uncorrected p values of less than 0.05 were considered significant and p values of 0.05–0.1 were considered indicative of non-significant trends towards association. Probability values were subsequently corrected for multiple comparisons (number of SNPs analysed = 10) as a more conservative criterion for significance. Odds ratios with 95% confidence intervals were also calculated for all patient–control genotype comparisons (Statistical Solutions Inc, Chicago, Illinois, USA). Power calculations were performed using Epi Info™ (US Department of Health Centers for Disease Control and Prevention, Atlanta, Georgia, USA).

RESULTS

Using the ARMS PCR technique, between 49 and 52 patients were successfully typed for each SNP. Control genotyping data were available from the following numbers of individuals: 164 for IL-1β −35, 261 for IL-1β −511, 224 for IL-6 −174, 235 for IL-8 −251, 214 for TNFα −308, 160 for TGFβ1 +915, 266 for VEGF −2578, 263 for VEGF −1154, 257 for ICAM-1 +241, and 226 for ICAM-1 +469. The number of patients and controls genotyped for each SNP was based on DNA availability. Fewer patients were genotyped by the 5′

Table 1 ICAM-1 genotyping 5′ nuclease (TaqMan®) primer and probe sequences

Primer/Probe	Sequence
ICAM-1 +241 Forward	5′CAGGGGACCGTGGTCTGTT3′
ICAM-1 +241 Reverse	5′CATAGGTGACTGTGGGGTTCAA3′
ICAM-1 +241 G allele probe	FAM-TGGGAACAGCCcGTC-MGB-NFQ
ICAM-1 +241 A allele probe	VIC-TGGGAACAGCCtGTC-MGB-NFQ
ICAM-1 +469 Forward	5′TCGAGATCTTGAGGGCACCTA3′
ICAM-1 +469 Reverse	5′CGGCTCACTCACAGAGCACAT3′
ICAM-1 +469 G allele probe	FAM-ACGGTCACCTcGCGGGTGA-TAMRA
ICAM-1 +469 A allele probe	VIC-CACGGTCACCTtGCGGGTGA-TAMRA

Positions of polymorphisms are marked in lower case.
FAM, reporter gene; ICAM-1, intercellular cell adhesion molecule 1; MGB, minor groove binding probe; NFQ, non-fluorescent quencher; TAMRA, fluorescence quencher labelled probe; VIC, reporter gene.

Table 2 Cytokine and ICAM-1 genotype frequencies in patients with CP and controls

Genotype	CP patients N (%)	Controls N (%)	p Value*	OR (95% CI)
IL-1β −35				
CC	11 (21.2)	22 (13.4)	0.26	1.73 (0.79 to 3.82)
CT	25 (48.1)	76 (46.3)	0.95	1.07 (0.58 to 1.99)
TT	16 (30.8)	66 (40.2)	0.29	0.66 (0.34 to 1.28)
	N = 52	N = 164		
IL-1β −511				
CC	17 (34.0)	87 (33.0)	1.00	1.03 (0.55 to 1.94)
CT	25 (50.0)	135 (51.7)	0.95	0.93 (0.51 to 1.70)
TT	8 (16.0)	39 (14.9)	1.00	1.08 (0.48 to 2.45)
	N = 50	N = 261		
IL-6 −174				
GG	18 (34.6)	79 (35.3)	1.00	0.93 (0.52 to 1.82)
GC	25 (48.1)	101 (45.1)	0.81	1.13 (0.62 to 2.05)
CC	9 (17.3)	44 (19.6)	0.85	0.86 (0.40 to 1.86)
	N = 52	N = 224		
IL-8 −251				
AA	6 (11.5)	54 (22.9)	0.10†	0.44 (0.18 to 1.06)
AT	28 (53.9)	105 (44.7)	0.30	1.44 (0.79 to 2.63)
TT	18 (34.6)	76 (32.3)	0.88	1.11 (0.59 to 2.08)
	N = 52	N = 235		
TNFα −308				
GG	38 (76.0)	146 (68.2)	0.37	1.48 (0.73 to 2.97)
GA	11 (22.0)	56 (26.2)	0.67	0.80 (0.39 to 1.64)
AA	1 (2.0)	12 (5.6)	0.47	0.34 (0.04 to 2.71)
	N = 50	N = 214		
TGFβ1 +915				
GG	44 (88.6)	133 (83.1)	0.97	1.12 (0.48 to 2.59)
GC	6 (11.5)	26 (16.3)	0.55	0.67 (0.27 to 1.70)
CC	2 (4.2)	1 (0.6)	0.17	6.36 (0.56 to 71.63)
	N = 52	N = 160		
VEGF −2578				
CC	15 (30.6)	82 (30.8)	1.00	0.99 (0.52 to 1.90)
AC	19 (38.8)	115 (43.2)	0.67	0.83 (0.45 to 1.54)
AA	15 (30.6)	69 (25.9)	0.61	1.26 (0.65 to 2.44)
	N = 49	N = 266		
VEGF −1154				
GG	21 (42.0)	120 (45.6)	0.75	0.86 (0.47 to 1.58)
GA	20 (40.0)	109 (41.4)	0.97	0.94 (0.51 to 1.74)
AA	9 (18.0)	34 (12.9)	0.47	1.48 (0.67 to 3.27)
	N = 50	N = 263		
ICAM-1+241				
GG	41 (85.4)	210 (81.7)	0.90	1.15 (0.51 to 2.60)
GA	8 (19.5)	40 (15.6)	1.00	1.06 (0.47 to 2.39)
AA	0 (0.0)	7 (2.7)	0.60	0.00 (0.00 to 2.86)
	N = 49	N = 257		
ICAM-1+469				
GG	5 (11.9)	48 (21.4)	0.24	0.50 (0.19 to 1.31)
GA	27 (64.3)	109 (48.7)	0.08†	1.93 (0.98 to 3.79)
AA	10 (23.8)	69 (29.9)	0.49	0.71 (0.34 to 1.51)
	N = 42	N = 226		

All p values were non-significant.
*Uncorrected p value, corrected p value is ×10 uncorrected value; †non-significant trend (p = 0.05–0.1).
CI, confidence interval; CP, chronic pancreatitis; ICAM-1, intercellular cell adhesion molecule 1; IL, interleukin; OR, odds ratio; TGFβ1, transforming growth factor β1; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor.

nuclease assay for the ICAM-1 +469 SNP because of limited DNA availability.

All genotype frequencies in both patient and control groups were distributed in accordance with the Hardy-Weinberg equilibrium at $p = 0.05$, except for the distribution of ICAM-1 +241 genotypes in the control group ($0.001 < p > 0.01$). Table 2 shows the results of the patient–control comparisons. From this table it can be seen that there were no significant associations between SNPs in the promoter regions of the IL-1β, IL-6, and TNFα proinflammatory cytokines genes and susceptibility to chronic pancreatitis, and neither were there significant associations between SNPs in the TGFβ1 and VEGF genes and chronic pancreatitis. All associated p values were non-significant both before and after correcting for multiple comparisons. However, a non-significant decrease in the IL-8 −251 AA genotype was seen in the chronic pancreatitis patient series (11.5% *v* 22.9% in controls). In addition, there was a non-significant increase in frequency of the ICAM-1 +469 GA genotype among the patients with chronic pancreatitis compared with controls (64.3% *v* 48.7%). Again, these trends were non-significant both before and after correction for multiple comparisons.

DISCUSSION

In this preliminary study, a panel of 10 cytokine SNPs was studied, namely IL-1β −511 and −35, IL-6 −174, IL-8 −251, TNFα −308, TGFβ +915, VEGF −2578 and −1154, and ICAM-1 +241 and +469. These SNPs were selected because they have all been reported to be associated with differential expression of their respective gene product in vitro, or may affect its biological function.[23 32 33] For example, numerous (but not all) studies report that the TNFα −308 AA genotype is associated with higher expression of TNFα in vitro, compared with the GG and GA genotypes.[34] In addition, increased expression of all gene products has been reported in chronic pancreatitis. Despite this, we found that the SNPs in the promoter regions of the IL-1β, IL-6, and TNFα proinflammatory cytokine genes showed no significant

associations with chronic pancreatitis compared with the larger control group. Furthermore, an SNP (+915) associated with differential expression of TGFβ1, a regulator of extracellular tissue remodelling in the pancreas, which may promote the pathogenesis of chronic pancreatitis,[35] showed no significant association with chronic pancreatitis. The same was true for two SNPs in the VEGF promoter. This is of particular interest, because VEGF is a very potent angiogenic factor and VEGF, blood vessel count, and other angiogenic growth factors have been shown to be upregulated in both chronic pancreatitis and pancreatic cancer.[25]

However, the IL-8 −251 AA genotype (associated with high expression of IL-8[32]) showed a non-significant decrease in frequency among patients with chronic pancreatitis compared with controls, suggesting that genetically raised concentrations of IL-8—a proinflammatory and angiogenic cytokine—may be protective for chronic pancreatitis. This is unexpected, because several studies have reported increased expression of IL-8 in chronic pancreatitis.[19 20] Because of the small number of subjects studied, these results must be treated with caution, but IL-8 polymorphisms merit further study in a larger patient series.

> ''Because of the small number of subjects studied, these results must be treated with caution, but interleukin 8 polymorphisms merit further study in a larger patient series''

With regard to ICAM-1, the SNP at position +241 showed no association with chronic pancreatitis. However, at position +469 the GA genotype showed a non-significant increase in frequency among patients with chronic pancreatitis compared with controls. This preliminary result is of particular interest, because ICAM-1 expression is increased in chronic pancreatitis, especially in ductal cells compared with endothelial cells.[25] Cell adhesion molecules, including ICAM-1, are known to play an important role in cell–cell interactions, such as leucocyte–endothelium adhesion and subsequent migration of inflammatory cells into tissue or adhesion dependent immune responses.[36 37] In particular, the role of ICAM-1 in the adhesion of neutrophils and lymphocytes to endothelium and cells of epithelial origin suggests that they may be important in the immunological response in chronic pancreatitis. The functional relevance of the ICAM-1 +469 SNP, which encodes an amino acid substitution in immunoglobulin-like domain 5 of the expressed protein,[26] remains unclear.[38] However, it is interesting to note that the +469 G allele has been shown to be associated with chronic allograft failure in renal transplantation, in which upregulation of adhesion molecules has also been reported,[39] and is also associated with susceptibility to Behcet's disease.[38] The authors of this last paper speculate that the ICAM-1 +469 SNP may exert a subtle influence on the strength and duration of the interaction of ICAM-1 with its LFA-1 (leucocyte function associated antigen 1) ligand, so facilitating an inflammatory response.

The lack of significant associations of all other SNPs with chronic pancreatitis in our study suggests that it is unlikely that these SNPs contribute to genetic susceptibility to chronic pancreatitis to any major extent. However, because of the small number of subjects studied, these results must be treated with caution. For example, a power calculation indicates that this study has 80% power to detect significant differences in genotype frequencies between cases and controls, conferring an odds ratio of 2.7 for the IL-6 −174 and 3.0 for the VEGF −1154 rarest genotypes, with 95% confidence. These power calculations (performed using the Epi Info Revision 2 (2003) statistical package), are based upon the number of subjects and controls examined and the frequency of the rarest genotype for the given SNP among the control subjects. These calculations were performed for all SNPs and the above are included as representative examples. Genetic associations conferring smaller odds ratios than those quoted above for the SNPs in question would not be expected to demonstrate significance at $p = 0.05$ in our study, even before correction for multiple comparisons. However, despite these limitations, our present study suggests that the role of IL-8 and ICAM-1 polymorphisms in conferring susceptibility to chronic pancreatitis requires further exploration in a larger patient series. A role for other genetic polymorphisms in these genes cannot be excluded at present. Chronic excess alcohol consumption was the dominant aetiological factor in most of our patients, but a comparative study of cytokine and growth factor SNPs in alcohol and non-alcohol related chronic pancreatitis would require significantly larger sample groups.

Take home messages

- This preliminary study suggests that genetic polymorphism within several cytokine genes is unlikely to influence susceptibility to chronic pancreatitis
- However, the possible role of polymorphisms within the interleukin 8 and intercellular cell adhesion molecule 1 genes in the development of this disease requires further investigation in a larger patient series

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