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ORIGINAL ARTICLE

Gene Polymorphisms of the Macrophage Migration Inhibitory Factor and Acute Pancreatitis

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ABSTRACT

Context Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that is released by macrophages and lymphocytes and plays an important pathogenetic role in acute pancreatitis. It is present in large amounts in the serum and ascitic fluid in rats with experimental pancreatitis and its levels are elevated in humans with pancreatitis. Polymorphisms associated with inflammatory joint diseases exist in the promoter region of the macrophage migration inhibitory factor gene that alter its expression.

Objective We investigated the association of macrophage migration inhibitory factor polymorphisms with acute pancreatitis in a population in the UK.

Participants A cohort of 164 patients with acute pancreatitis and 197 healthy controls.

Main outcome measures The -173 G to C single nucleotide polymorphism and the -794 (CATT) n repeat microsatellite were investigated. Restriction fragment length polymorphism (RFLP) was used to assay the -173 polymorphism and PCR followed by polyacrylamide gel electrophoresis (PAGE) was used for the microsatellite.

Results The microsatellite did not show any significant differences in distribution between patients and controls. The -173 GG genotype showed a trend towards reduced frequency seen in patients (P=0.056) and the C allele was significantly over expressed in patients (P=0.025). No differences were observed in subgroups based on severity or aetiology of pancreatitis.

Conclusions The -173 C allele is over expressed in acute pancreatitis, however studies are needed to explore this further. Our distribution of the microsatellite alleles was quite different to a previously reported Caucasian population and needs further study from viewpoint of population genetics.

INTRODUCTION

Acute pancreatitis is a commonly encountered intra-abdominal catastrophe. The disease course is variable and usually results in a benign outcome; however in about 20-25% patients the disease follows a more severe course with pancreatic necrosis, organ dysfunction and septicaemia [1]. The initial clinical response to pancreatitis is a systemic inflammatory response syndrome (SIRS) which, if abnormally persistent develops into a worsening scenario of tissue damage and sepsis resulting in multiple organ dysfunction syndrome (MODS) [2]. The primary humoral mediators of this process are the cytokines. The proinflammatory cytokines, such as interleukin-1 (IL-1), tumour necrosis factor (TNF), and interleukin-8 (IL-8), are

responsible for the tissue damage as well as organ dysfunction, while the anti-inflammatory factors such as interleukin-1 receptor antagonist (IL-1ra) are the protective arm of the immune system [3].

Macrophage migration inhibitory factor (MIF) is a T-cell derived lymphokine that inhibits the migration of macrophages and contributes to delayed type hypersensitivity [4, 5]. For a number of years, MIF was thought to be a T-cell product that acted on macrophages. More recently, it has been discovered that in addition to being a target, the macrophage itself is an important source of MIF [6]. MIF is released from monocytes/macrophages upon stimulation by TNF-alpha, interferon-gamma, lipopolysaccharide and streptococcal exotoxin [6, 7].

MIF has been demonstrated to potentiate the lethal effects of lipopolysaccharides (LPS) in mice, thereby increasing mortality from 15% to 85%. Anti-MIF antibody has been shown to protect mice from lethal sepsis in two different models: caecal ligature and puncture (CLP) as well as intraperitoneal injections of *E.coli*. In the CLP model, treatment with anti-MIF antibody immediately post-event increased survival from 0% to 62%. When repeated in TNF knockout (TNF--/--) mice, anti-MIF monoclonal antibody reduced mortality from 100% to 38% [8].

More relevantly, in humans, elevated concentrations of MIF have been detected in the alveolar airspace of patients with adult respiratory distress syndrome (ARDS), which is a frequent complication of severe acute pancreatitis. The alveolar cells from patients with ARDS show that MIF augments TNF-alpha and IL-8 secretion, while anti-MIF antibody attenuates TNF-alpha and IL-8 production [9].

In human acute pancreatitis, mononuclear cells play an important role in generation of the inflammatory response [10]. Serum and ascitic fluid MIF levels have been shown to be elevated in severe acute pancreatitis in Wistar rats, as well as in severe acute pancreatitis as compared to mild acute pancreatitis and healthy controls [11].

The human MIF gene situated on chromosome 22 (q11.23) has a 2-kb structure with three exons and two introns [12]. A ‘G’ to ‘C’ single nucleotide polymorphism at the -173 position has been described in a population-sample drawn from Caucasian systemic-onset juvenile idiopathic arthritis (JIA) patients [13]. This polymorphism creates a binding site for activator protein-4 (AP-4), which is involved in intracellular transport activities. Luciferase studies in human T lymphoblast cell line have shown increased promoter activity of MIF-173C-Luc compared to MIF-173G-Luc [14]. The polymorphism has been correlated with increased susceptibility to JIA [13] and sarcoidosis [15]. In addition, a CATT repeat microsatellite has also been described at the -794 position with repeats varying from 5-8 [16]. Luciferase assays on this have shown this to have functional significance on MIF secretion *in vitro*. The 5-CATT allele has the lowest *in vitro* promoter activity and is associated with low disease severity in rheumatoid arthritis [16]. There is no evidence to show linkage dysequilibrium between the 5-CATT allele and -173C allele.

The aim of our study was to investigate the association between polymorphisms of the MIF gene with acute pancreatitis as well as disease severity.

PATIENTS AND CONTROLS

One hundred and sixty four patients admitted with a clinical diagnosis of acute pancreatitis were identified and recruited for the study. All patients with known chronic pancreatitis were excluded from this study. The criteria for diagnosis of acute pancreatitis were abdominal pain and hyperamylasaemia greater than 3 times normal (reference range: 0-100 U/mL) as well as supportive radiology. Patients were classified as having mild or severe acute pancreatitis based upon outcome criteria set out by the Atlanta convention of 1992 [17]. Maximal organ failure scores were calculated for these patients [1]. Aetiology of the attack was classified as being due to gallstones (in the presence of corroborative

appropriate laboratory or radiological parameters), alcohol (if the patients consumed more than 80 g/day for more than 6 months) or idiopathic if no cause could be found. The patient details are displayed in Table 1. Of the 164 patients in this study, 116 had mild disease while 48 went on to have severe disease manifested by either organ dysfunction or local complications such as necrosis, pseudocyst or fluid collections. Twelve patients had local complications without evidence of any organ dysfunction.
One hundred and ninety seven Caucasoid cord blood samples following a normal healthy delivery were used as healthy controls to obtain representative population samples.

METHODS

Genomic DNA was extracted from peripheral venous blood samples of patients.

MIF -173G/C Typing

This was carried out as previously described by Donn *et al*. [13]. Polymerase chain reaction was used to amplify a 366 bp fragment. Forward primer was 5'-ACT-AAG-AAA-GAC CCG-AGG-C-3' and the reverse primer was 5'-GGG-GCA-CGT-TGG-TGT-TTA-C-3'. The annealing temperature used was 59°C. The resulting fragment was digested with *Alu I* restriction endonuclease (New England Biolabs, Ipswich, MA, USA) overnight at 37°C and the digest was resolved on 2.5% agarose gel stained with 10% ethidium bromide and visualised using UV light. The 366 bp PCR product had a consistent restriction site resulting in a 98 bp and a 268 bp fragment. The GG genotype did not have a second cutting site for *Alu I*. The CC genotype had a second cutting site resulting in 3 fragments: 205 bp, 98 bp, 63 bp size. The heterozygous GC genotype was characterised by 4 bands: 268 bp, 205 bp, 98 bp, 63 bp. Twenty percent of the samples were duplicated as internal quality control to avoid sample or reading errors.

Table 1. Patient details.

Age	Median: 57 years Range: 20-95 years
Sex	
- Male	83 (50.6%)
- Female	81 (49.4%)
Aetiology	
- Gallstone related	93 (56.7%)
- Alcohol related	25 (15.2%)
- Idiopathic	46 (28.1%)
Severity	
- Mild	116 (71.3%)
- Severe	48 (28.7%)
Local complications	
- Absent	134 (81.7%)
- Present	30 (18.3%)
Organ dysfunction	
- Absent	129 (78.7%)
- Present	35 (21.3%)
Respiratory	12
Renal	8
Multiple organ	15

MIF Microsatellite Typing

Oligonucleotide primers were designed to amplify a 150 bp segment of the MIF promoter region containing the microsatellite repeat sequence. The forward primer was 5'-AAT-CTC-TGA-GGA-CCT-GGC-CTG-TGA-TCC-AGT-3' and the reverse primer was 5'-CAT-CTA-GCA-GGT-GCC-AGG-CAT-ATC-AAG-AGA-3'. The forward primer was radiolabelled with gamma ^{32}P (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using T4 polynucleotide kinase (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The PCR reaction mix contained 100-500 ng DNA, 1.0 μM of each amplimer (radiolabelled forward), 3.0 mM $MgCl_2$, 300 μM of each nucleotide, 0.8 units Taq polymerase (Invitrogen, Paisley, United Kingdom), 2 μL PCR buffer minus Mg (Gibco BRL, Gaithersburg, MD, USA) in a total reaction volume of 25 μL. PCR was performed in a Cyclogene® thermocycler (Techne, Cambridge, UK). The conditions were: 95°C for 12 minutes then 30 cycles of 95°C for 1 minute, 68°C for 1 minute, 72°C for 1 minute followed by 10 minute extension at 72°C. Amplification products (6 μL) were

mixed with 3 μL stop solution containing formamide (Amersham Life Science, Bucks, United Kingdom) were then separated on a 6% polyacrylamide gel at 1,800 V for 4 hours. After drying the gel, products were exposed to Kodak imaging film (Scientific Imaging Systems, Cambridge, United Kingdom) at -80°C for 18 hours. The films were then developed and genotyping done based on the developed bands. Fifteen percent of the samples were repeated as internal quality control and a representative number of amplimers were sequenced by MWG Biotech (Ebersberg, Germany), these being used as markers for all subsequent assays.

ETHICS

Local regional ethics committee approval was obtained prior to initiating the study and patients gave written informed consent.

STATISTICS

Frequencies were used as descriptive statistics. The SPSS 11.01 for Windows was used as the package for computerised analysis. Comparison of allelic and haplotype frequencies was made using the Pearson chi-squared and the Fisher's exact tests while the hierarchical log-linear models were applied to compare the observed *vs*. the expected frequencies. Statistical significance was considered for two-tailed P value below 0.05.

RESULTS

-173 Polymorphism

Allele frequencies were measured in 160 patients and 197 controls for this biallelic polymorphism. Hardy-Weinberg equilibrium was maintained in both patients and controls. Table 2 shows the genotype and allele distribution among patients and controls. A significant different genotype distribution was found between acute pancreatitis patients and healthy controls (P=0.046): in particular, there was a trend towards lower frequency of GG genotype among patients (106/160, 66.3%) when compared with healthy controls (151/197, 76.6%), however this did not attain the statistical significance (P=0.056). Moreover, there was a significantly (P=0.025) higher frequency of the C allele in acute pancreatitis patients (58/320, 18.1%) than in healthy controls (47/394, 11.9%). Subgroup analysis did not show any significant differences between patients of varying severity or aetiology.

Table 2. Distribution of MIF -173 genotypes and alleles.

	Acute pancreatitis (n=160)	Healthy controls (n=197)	Severity of acute pancreatitis: Mild (n=112)	Severity of acute pancreatitis: Severe (n=48)	Etiology of acute pancreatitis: Gallstones (n=90)	Etiology of acute pancreatitis: Alcohol (n=24)	Etiology of acute pancreatitis: Idiopathic (n=46)
Genotypes:							
- GG	106 (66.3%)	151 (76.6%) P=0.056 [a]	70 (62.5%)	36 (75.0%) P=0.974 [a]	62 (68.9%) P=0.669 [a]	12 (50.0%) P=0.520 [a]	32 (69.6%) P=0.895 [a]
- GC	50 (31.3%)	45 (22.8%) P=0.604 [a]	40 (35.7%)	10 (20.8%) P=0.076 [a]	26 (28.9%) P=0.630 [a]	12 (50.0%) P=0.279 [a]	13 (28.3%) P=0.740 [a]
- CC	4 (2.5%)	1 (0.5%) P=0.198 [a]	2 (1.8%)	2 (4.2%) P=0.273 [a]	2 (2.2%) P=0.808 [a]	0 P=0.429 [a]	1 (2.2%) P=0.748 [a]
Overall P	0.046 [b]		0.140 [b]			0.334 [b]	
Alleles:							
- G	262 (81.9%)	347 (88.1%)	180 (80.4%)	82 (85.4%)	150 (83.3%)	36 (75.0%)	77 (83.7%)
- C	58 (18.1%)	47 (11.9%)	44 (19.6%)	14 (14.6%)	30 (16.7%) P=0.382 [a]	12 (25.0%) P=0.151 [a]	15 (16.3%) P=0.415 [a]
Overall P	0.025 [c]		0.343 [c]			0.368 [b]	

[a] Hierarchical log-liner model
[b] Pearson chi-squared test

-794 Microsatellite

Allele frequencies were measured in 164 patients and 156 healthy controls. We observed 3 out of the 4 known alleles, the 8-CATT allele being absent in our population. Hardy-Weinberg equilibrium was maintained in both patients and controls. Table 3 shows the allelic frequencies of the microsatellite in patients and controls. There was no significant difference in the distribution of genotypes (P=0.367) or alleles (P=0.342) among patients and controls. Subgroup analysis did not show any significant difference in genotype and allele frequency when assessed by severity (P=0.737, and P=0.357, respectively) or aetiology (P=0.296, and P=0.377, respectively) of acute pancreatitis although significantly (P=0.038) lower frequencies of the 5,5 and 5,6 genotypes seem to be present in the gallstone and alcohol related acute pancreatitis, respectively. Because of the non-significant overall evaluations and the low number of cases, these data require further confirmation in a larger population.

Haplotype analysis did not show any differences in distribution between patients and controls (data not shown). A preliminary analysis shows no evidence of linkage dysequilibrium between the -173 and the -794 polymorphisms in our population (data not shown).

DISCUSSION

This study investigates the association between acute pancreatitis and functionally active polymorphisms in the MIF gene.

In case of the -173 allele, there was a trend

Table 3. Distribution of MIF-794 microsatellite genotypes and alleles.

	Acute pancreatitis	Healthy controls	Severity of acute pancreatitis		Etiology of acute pancreatitis		
			Mild	Severe	Gallstones	Alcohol	Idiopathic
	(n=164)	(n=156)	(n=116)	(n=48)	(n=93)	(n=25)	(n=46)
Genotypes:							
- 5,5	11 (6.7%)	7 (4.5%)	8 (6.9%)	3 (6.3%)	2 (2.2%)	3 (12.0%)	6 (13.0%)
	P=0.585 [a]		P=0.771 [a]		P=0.038 [a]	P=0.492 [a]	P=0.104 [a]
- 6,6	53 (32.3%)	49 (31.4%)	34 (29.3%)	19 (39.6%)	33 (35.5%)	9 (36.0%)	12 (26.1%)
	P=0.623 [a]		P=0.202 [a]		P=0.196 [a]	P=0.602 [a]	P=0.575 [a]
- 7,7	3 (1.8%)	3 (1.9%)	3 (2.6%)	0	1 (1.1%)	1 (4.0%)	0
	P=0.760 [a]		P=0.488 [a]		P=0.896 [a]	P=0.262 [a]	P=0.448 [a]
- 5,6	57 (34.8%)	71 (45.5%)	41 (35.3%)	16 (33.3%)	35 (37.6%)	5 (20.0%)	17 (37.0%)
	P=0.073 [a]		P=0.718 [a]		P=0.087 [a]	P=0.038 [a]	P=0.398 [a]
- 6,7	28 (17.1%)	19 (12.2%)	21 (18.1%)	7 (14.6%)	15 (16.1%)	5 (20.0%)	8 (17.4%)
	P=0.541 [a]		P=0.983 [a]		P=0.724 [a]	P=0.705 [a]	P=0.931 [a]
- 5,7	12 (7.3%)	7 (4.5%)	9 (7.8%)	3 (6.3%)	7 (7.5%)	2 (8.0%)	3 (6.5%)
	P=0.451 [a]		P=0.914 [a]		P=0.626 [a]	P=0.800 [a]	P=0.883 [a]
Overall P	0.367 [b]		0.737 [b]			0.296 [b]	
Alleles:							
- 5	91 (27.7%)	92 (29.5%)	66 (28.4%)	25 (26.0%)	46 (24.5%)	13 (26.0%)	32 (34.8%)
	P=0.306 [a]		P=0.893 [a]		P=0.479 [a]	P=0.436 [a]	P=0.118 [a]
- 6	191 (58.2%)	188 (60.3%)	130 (56.0%)	61 (63.5%)	116 (61.7%)	28 (56.0%)	49 (53.3%)
	P=0.354 [a]		P=0.180 [a]		P=0.196 [a]	P=0.595 [a]	P=0.609 [a]
- 7	46 (14.0%)	32 (10.3%)	36 (15.5%)	10 (10.4%)	24 (13.8%)	9 (18.0%)	11 (12.0%)
	P=0.148 [a]		P=0.300 [a]		P=0.729 [a]	P=0.275 [a]	P=0.376 [a]
Overall P	0.342 [b]		0.357 [b]			0.377 [b]	

[a] Hierarchical log-liner model

[b] Pearson chi-squared test

towards GG genotype being under-represented among patients, however this relationship did not attain statistical significance (P=0.056). When assessing allele frequency, the 'C' allele is more frequent among patients than controls (P=0.025). This suggests that a difference may exist between the two, however, further studies with larger numbers would be required to develop this relationship to its fullest extent. This is keeping with Donn's results, where serum MIF levels were significantly higher in subjects with the MIF-173C allele compared to the MIF-173GG genotype [14]. Subgroup analysis did not reveal any further differences, however it must be kept in mind that only a small fraction of patients go on to develop severe disease and this has an effect on the numbers required for the adequacy of statistical analysis.

In our population, there was no difference in the distribution of the CATT alleles between acute pancreatitis and controls, or between mild and severe disease. Looking at subgroups with/without organ dysfunction or local complications, we were unable to demonstrate a differential genotype or allele distribution pattern for either MIF polymorphism studied. Baugh *et al*. did not have controls that were closely matched for geographic and ethnic origin [16]. Our controls had significant differences in genotypic and allelic distribution compared to the North American controls used by Baugh *et al*. and this is a difference is worth pursuing in the interests of population genetics.

MIF plays an important role in the inflammatory cascade and has been implicated in the pathogenesis of other inflammatory diseases such as rheumatoid arthritis, ulcerative colitis, Crohn's disease and iritis. It appears to have a counter-regulatory role to glucorticoid induced cytokine suppression. MIF is emerging as an important player in the pathogenesis of acute pancreatitis in both experimental systems as well as in humans. In 2 models of experimentally induced pancreatitis in rats (taurocholate infusion and cerulein infusion), MIF levels in ascitic fluid as well as serum increased [11]. In taurocholate induced pancreatitis, MIF levels are elevated in the lung and ascitic fluid and survival is improved by pre-treatment with anti-MIF antibody [11]. Among humans with acute pancreatitis, MIF levels are elevated compared to healthy volunteers and this is especially true for severe acute pancreatitis [11].

This study has not shown any clear-cut influence of MIF polymorphisms on disease outcome and this can be due to a number of reasons such as redundancy within the inflammatory system or that MIF activity may follow a sigmoidal curve rather than a linear curve resulting in full effect even at low concentrations. In addition, the MIF gene has a number of putative transcription factor binding sites in the promoter region that may have competing influences and thereby affect the outcome of this study.

In summary, we have shown that the -794 microsatellite polymorphism does not affect severity or occurrence of acute pancreatitis. The -173 functionally active polymorphism does seem to have some association with the causation of acute pancreatitis, however further studies will be required for this association to be elucidated.

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Abbreviations AP-4: activator protein-4; ARDS: adult respiratory distress syndrome; CLP: caecal ligature and puncture; IL-1ra: interleukin-1 receptor antagonist; JIA: juvenile idiopathic arthritis; LPS: lipopolysaccharides; MIF: macrophage migration inhibitory factor; MODS: multiple organ dysfunction syndrome; SIRS: systemic inflammatory response syndrome

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