Polymorphisms of the macrophage migration inhibitory factor gene in a UK population with Type 1 diabetes mellitus

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Polymorphisms of the macrophage migration inhibitory factor gene in a UK population with Type 1 diabetes mellitus

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Abstract

Aims Macrophage migration inhibitory factor (MIF) is a potent pro-inflammatory cytokine whose production is transcriptionally regulated by glucose. Experimental data from both Type 1 diabetes mellitus (T1D) patients and animal models suggests a role for MIF in the development of T1D. The aim of this study was to employ gene resequencing to identify common DNA polymorphisms in the *MIF* gene and subsequently assess haplotype tagged single nucleotide polymorphisms (htSNPs) using a combination of case–control and family-based association analyses in order to assess the association of *MIF* htSNPs with the development of T1D in a white population.

Methods All exons, introns and approximately 3 kb upstream and downstream of the *MIF* gene were screened for DNA polymorphisms in 46 individuals using DNA sequencing. Genotyping of the htSNPs was performed in 432 cases, 407 control subjects and 290 T1D parent–offspring trios, using Taqman, Sequenom, Pyrosequencing and fluorescence-based microsatellite technologies.

Results Twenty-three polymorphisms (two novel) with a minor allele frequency > 10% were identified. Four *MIF* htSNPs (rs875643 G>A, rs7388067 C>T, rs5844572 –/CATT, rs6003941 T>G) were identified. Allele and haplotype frequencies were similar between case and control groups (*P* > 0.6 by permutation test) and assessment of allele transmission distortion from informative parents to affected offspring also failed to find an association. Stratification of these analyses for age-at-onset and human leukocyte antigen (HLA)-DR risk group (DR3/DR4) did not reveal any significant associations.

Conclusions It is unlikely that common polymorphisms in the *MIF* gene strongly influence susceptibility to T1D in the UK population.

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Keywords haplotype, macrophage migration inhibitory factor, polymorphism, Type 1 diabetes mellitus

Abbreviations HLA, human leukocyte antigen; htSNP, haplotype tagged SNP; HWE, Hardy–Weinberg equilibrium; LD, linkage disequilibrium; MAF, minor allele frequency; MIF, macrophage migration inhibitory factor; SNP, single nucleotide polymorphism; T1D, Type 1 diabetes mellitus; TDT, transmission disequilibrium test

Introduction

Type 1 diabetes mellitus (T1D) is an autoimmune disease mediated by Th1 lymphocytes resulting in T-cell mediated destruction of insulin-producing pancreatic B-cells [1]. The triggering of autoimmunity against the B-cells is probably caused by environmental agents acting in the context of a predisposing genetic background. Once activated, the immune cells invade the islets and exert their harmful effects on B-cells via mechanisms involving diverse mediators such as Fas/FasL, perforin/granzyme, reactive oxygen and nitrogen species and pro-inflammatory cytokines [2].

Macrophage migration inhibitory factor (MIF) is a potent pro-inflammatory cytokine and a key modulator of immune and inflammatory responses. It is expressed in numerous types of tissues, including pancreatic B-cells and cells of the immune system (T and B cells, monocytes, macrophages) [3,4]. A unique

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property of MIF is its release from macrophages after stimulation with glucocorticoid hormones. It then acts to counter-regulate the suppressive effects of these hormones on inflammatory cytokine production [5].

There is a close relationship between the role of MIF in the inflammatory/immune response and its role in glucose metabolism [6]. MIF production is transcriptionally regulated by glucose in a time- and concentration-dependent manner, with expression of *MIF* within B-cells following a circadian rhythm [7]. It is also secreted together with insulin from B-cells during stress or systemic inflammatory processes [8].

Reduction in MIF serum levels has been reported in multiple islet autoantibody-positive vs. -negative T1D patients at time of diagnosis [9]. Experimental data also suggest a role for MIF in the development of T1D. MIF mRNA expression is up-regulated during the development of cell-mediated diabetes in non-NOD mice and diabetes incidence is increased by 31% in NOD mice treated with recombinant MIF protein [10]. Animal experiments have also shown that anti-MIF antibody and knockout of *MIF* could prevent autoimmune diabetes in mice [11,12]. These findings suggest that *MIF* may play a key role in the pathogenesis of T1D.

The *MIF* (glycosylation-inhibitory factor) gene on 22q11.23 is remarkably small, covering less than 1 kb, and appears to be present as a single functional gene [13]. Polymorphic sites previously identified within the gene region include a five to eight allele repeat of the CATT element at position −794 (rs5844572), −173 G>C (rs755622) in the 5′ flanking region and two intronic polymorphisms at +254 T>A (rs2096525) and +656 C>G (rs2070766) [14].

MIF genetic polymorphisms are associated with a number of autoimmune diseases. Baugh and colleagues reported association of the short CATT repeat (CATT$_5$) with less severe rheumatoid arthritis [15], while a specific *MIF* promoter haplotype composed of CATT$_7$-MIF-173*C was reported to be associated with susceptibility to adult inflammatory polyarthritis [16] and juvenile idiopathic arthritis [17].

Both the *MIF* CATT$_{5–8}$ repeat and −173 G>C polymorphisms are functional variants *in vitro*. Variation in reporter gene luciferase activity for the different CATT alleles has been described, with the CATT$_5$ allele having the lowest level of basal and stimulated *MIF* promoter activity in human lung epithelial (A549) and fibroblast cell lines [18]. In contrast, higher reporter gene luciferase activity is found for the *MIF*-173*C allele in human T lymphoblast cell line (CEMC7A) and for *MIF*-173*G in human A549 lung epithelial cells [14]. This work suggests that *MIF* promoter activity is altered in different cell types by binding of several transcription factors to the CATT and *MIF*-173G>C polymorphic sites. There are sequences within the proximal promoter of *MIF* which resemble cAMP responsive elements; however, no tissue-specific regulatory elements have been identified [18]. These important gene regulatory elements can map to distant sites both 5′ and 3′ of the gene coding region [19], therefore it is necessary to comprehensively assess the genomic region surrounding the *MIF* gene for such sequences.

The aim of this study was to investigate the role of common *MIF* polymorphisms in T1D. In order to address this, we have screened all exons, introns and 3 kb both 5′ and 3′ of the *MIF* gene by dideoxy sequencing in order to identify common polymorphisms and allow construction of common haplotypes. Haplotype tagged single nucleotide polymorphisms (htSNPs) were investigated using a combination of case–control and family-based association analyses in order to rigorously assess the association of *MIF* polymorphisms with development of T1D in a white population.

Patients and methods

Subjects

Ethical approval was obtained from the Queen's University of Belfast Research Ethics Committee and written, informed consent was granted by subjects prior to conducting this study. The diagnosis of T1D was based upon elevated plasma glucose levels above diagnostic threshold values defined for Type 1 diabetes and absolute dependency of insulin. Cases with Type 1 diabetes secondary to another condition were excluded. Probands were derived from a Northern Ireland register of patients with T1D, diagnosed before their 15th birthday [20].

For the case–control study, 432 probands (54.6% male) were recruited; parents and grandparents of each proband were born in Northern Ireland. DNA from both parents was also available for 290 of the 432 probands. These trios were employed for the family-based association study. Control subjects ($n = 407$, mean age 13.4 years, 49.1% male) were derived from the Young Hearts 2000 Study collection; a group of children selected from a random sample of Northern Ireland schools [21].

Single nucleotide polymorphism (SNP) discovery

Gene regions of interest were divided into ∼500-bp fragments and amplified by PCR using HotStar Taq DNA polymerase (Qiagen, Crawley, UK). Polymorphisms were identified in 46 of the Young Hearts 2000 Study participants [21] by DNA sequencing. The genotype information from observed SNPs provided data for haplotype construction and identification of htSNPs. All sequencing was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK) using BigDye® terminator v3.1 cycle sequencing kits (Applied Biosystems) according to the manufacturer's instructions.

Genotyping

All subjects were genotyped for rs875643 using Taqman 5′-nuclease assays (C_2448248_1) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Genotyping for *MIF* rs738807 was performed using the Sequenom® MassARRAY iPLEX technology™ (Sequenom, Hamburg, Germany) according to manufacturer's instructions. Fluorescence-based microsatellite genotyping was performed

for rs5844572 using Qiagen Multiplex PCR kits (Qiagen). Amplification products were resolved by capillary electrophoresis using an ABI 3730 Genetic Analyser (Applied Biosystems) and alleles scored using GENOTYPER software (Applied Biosystems).The rs6003941 SNP was genotyped by Pyrosequencing using a PSQ™ HS 96A instrument (Biotage, Uppsala, Sweden). Pyrosequencing reactions were performed according to the manufacturer's instructions. Primer sequences are available on request.

Stringent quality control measures were employed for all assays which included placing case and control samples on the same plate, negative controls, sample duplicates, DNAs of known genotype and father-mother-proband trios used to assess quality by checking for Mendelian errors. Genotypes were scored independently by two trained personnel. Genotypes were also confirmed in a subset of samples ($n = 24$) for rs875643, rs738807 and rs5844572 variants by sequencing on an ABI 3730 Genetic Analyser.

Statistical analysis

The extent of linkage disequilibrium (LD) between pairs of SNPs was quantified using Lewontin's D' value [22]. Using the genotyping data from the 46 control samples, haplotypes across the entire genetic region encompassing *MIF* [using all SNPs with minor allele frequency (MAF) ≥ 10%] were inferred using SNPHAP software [23]. The htSNPs were then identified using the htSNP2 package, with the r^2 threshold set to ≥ 0.8 [24].

Genotype frequencies were assessed for Hardy–Weinberg equilibrium (HWE) using a χ^2 goodness-of-fit test with the level of significance set to $P < 0.001$. The χ^2-test for contingency tables was used to compare genotype and allele frequencies between case and control subjects with the level of significance set to $P < 0.05$. Subgroup analyses were also performed by stratifying cases for gender, age at diagnosis of diabetes (< 5 years and ≥ 5 years) and for human leukocyte antigen (HLA)-DR risk group (DR3/DR4 heterozygotes $n = 115$, other carriers of DR3 $n = 84$, other carriers of DR4 $n = 77$). Assessment of allele transmission distortion from the expected 50 : 50 transmission from informative parents to offspring was performed using the transmission disequilibrium test (TDT). Haplotype analyses for both case–control and family data were obtained using the Haploview [25]. The case–control and TDT study results were combined after removal of cases used in TDT analysis.

Results

Subjects

Of the 432 cases analysed, 236 (54.6%) were male and 155 (35.9%) developed Type 1 diabetes before 5 years of age. The

Table 1 *MIF* polymorphisms identified

No.	Variant*	Location	Description†	Minor allele	MAF‡
1	rs5760090	5′ upstream	3624161 C>T	T	0.398
2	ss141323267	5′ upstream	3624289 TGCACA/-	TGCACA	0.261
3	ss141023271	5′ upstream	3624296 A>G	A	0.261
4	rs12628766	5′ upstream	3624483 T>G	G	0.189
5	**rs875643**	5′ upstream	3624567 G>A	A	0.413
6	rs738806	5′ upstream	3624741 A>G	A	0.261
7	**rs738807**	5′ upstream	3624814 C>T	T	0.13
8	rs5751759	5′ upstream	3625062 A>G	G	0.136
9	rs2012124	5′ near gene	3625233 T>C	T	0.216
10	rs2012133	5′ near gene	3625303 C>G	C	0.216
11	rs12483859	5′ near gene	3625376 T>C	C	0.136
12	rs12485058	5′ near gene	3625428 A>G	G	0.136
13	rs11428310	5′ near gene	3625683 −/A	A	0.13
14	**rs5844572**	5′ near gene	3626319 −/CATT	5 CATT	0.307
15	rs755622	5′ near gene	362696 G>C	C	0.12
16	rs2096525	intron	3627388 T>C	C	0.122
17	rs2070767	3′ near gene	3628032 T>C	T	0.244
18	rs2000466	3′ near gene	3628431 T>G	G	0.136
19	rs10433309	3′ downstream	3629243 C>T	T	0.348
20	rs10433310	3′ downstream	3629392 G>A	A	0.348
21	rs10433311	3′ downstream	3629399 T>C	C	0.348
22	**rs6003941**	3′ downstream	3629426 T>G	T	0.228
23	rs1007889	3′ downstream	3629812 T>G	G	0.348

*The htSNPs are in bold.
†Based on NT_011520.11.
‡MAF based on sequencing up to 46 individuals.
htSNP, haplotype tagged single nucleotide polymorphism; MAF, minor allele frequency; *MIF*, macrophage migration inhibitory factor.
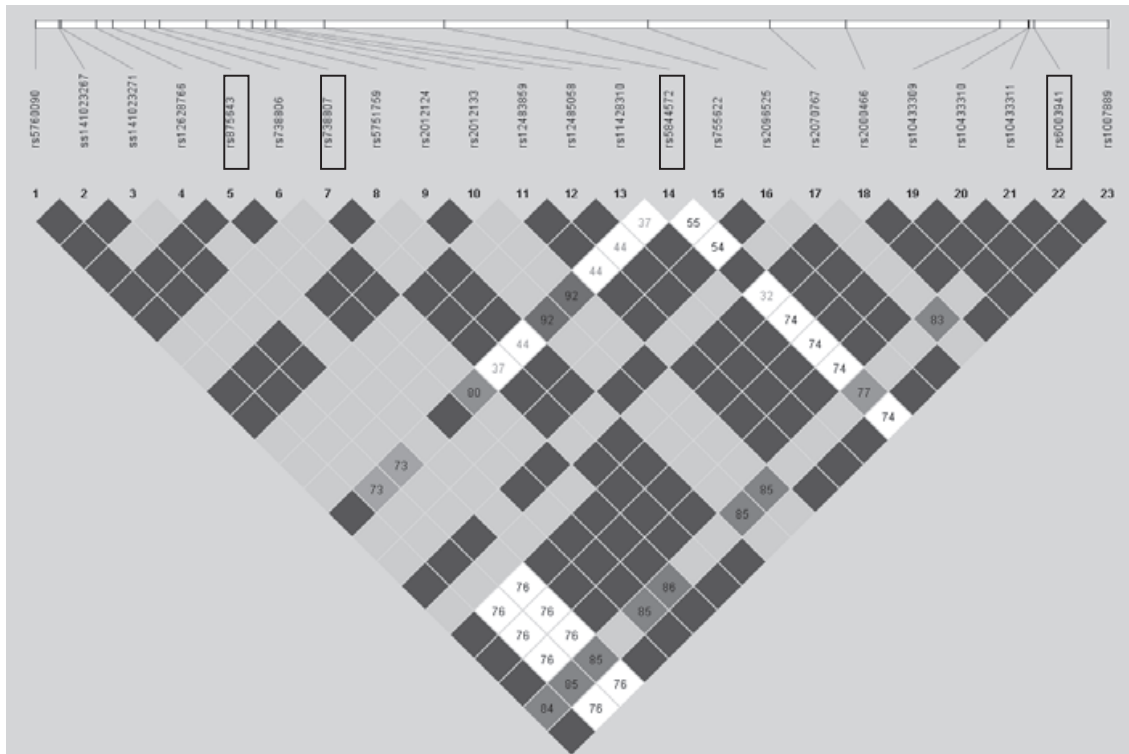
FIGURE 1 *MIF* LD pattern by D′ value between the 23 SNPs identified with a MAF ≥ 10% from sequencing 46 individuals. Loci are listed in linear order proceeding from upstream to downstream with respect to *MIF*. The htSNPs are boxed. SNP details are given in Table 1. Black cells D′ = 1 LOD ≥ 2, dark grey cells D′ < 1 LOD ≥ 2, pale grey cells D′ = 1 LOD < 2, white cells D′ < 1 LOD < 2. Informative values of D′ < 1 are specified within each cell. htSNP, haplotype tagged SNP; LD, linkage disequilibrium; LOD, logarithm of odds; MAF, minor allele frequency; *MIF*, macrophage migration inhibitory factor; SNP, single nucleotide polymorphism.

407 control subjects had an average age at recruitment of 13.4 years and 200 (49.1%) were male.

htSNP identification

All three exons, exon–intron boundaries, introns and approximately 3 kb upstream and downstream of the *MIF* gene were screened for genetic polymorphisms by DNA sequencing. In total, approximately 6.7 kb of DNA sequence was screened for each of the 46 individuals. Twenty-three (20 SNPs, 2 indel, 1 CATT$_{5-8}$) polymorphisms, two of which were novel, were identified with a MAF > 10% (Table 1). Two rare variants NT_011520.11 3627157 A>T and 3627758 C>T were identified in only one of the 46 individuals screened (MAF = 1%).

Strong LD was detected between the majority of the *MIF* gene variants examined (D′ = 1) with the exception of rs5844572 (Fig. 1). The seven most common haplotypes with estimated frequency > 1% could be identified by genotyping four htSNPs. The haplotypic r^2 value associated with this htSNP selection was r^2 = 0.86. The htSNPs consist of the 5′ sequence SNPs rs875643 G>A, rs738807 C>T, the CATT$_{5-8}$ repeat polymorphism rs5844572 and the 3′ SNP rs6003941 T>G.

Polymorphism analysis

As it has been reported previously that the transcriptional activity of the *MIF* promoter with the CATT$_5$ repeat was lower than the larger alleles CATT$_{6-8}$ [10], we reduced the genotyping data to three groups: subjects with the CATT$_5$ allele (5/5, 5/X) and those without the CATT$_5$ allele (X/X where X indicates CATT alleles 6, 7 or 8), as described previously [26].

The distribution of genotypes was in HWE for all variants identified during screening and in htSNPs in both case and control groups. Genotype frequencies for the individual htSNPs did not differ significantly in cases compared with control subjects (Table 2). Using the Haploview permutation test (100 000 permutations), there was no evidence of variation in either allele or haplotype frequencies between case and control groups (P > 0.6; Tables 2 and 3).

TDT analysis was performed for *MIF* rs875643, rs738807, rs5844572 and rs6003941 polymorphisms on 290 trios (cases from case–control study and parents where available). No significant distortion in allele transmissions were observed for the *MIF* polymorphisms in informative families (Table 4). Transmissions of *MIF* haplotypes from heterozygous parents to diabetic offspring all failed to reach statistical significance (Table 5). Case–control and TDT study results were combined

Table 2 Association analysis of *MIF* htSNP alleles and genotypes in 432 cases and 407 control subjects

Polymorphism	Case, *n* (%)	Control, *n* (%)	*P*-value	OR (95% CI)
rs875643				
G	495 (57.3)	477 (58.6)	0.59	0.95 (0.78–1.16)
A	369 (42.7)	337 (41.4)		
GG	131 (30.3)	137 (33.6)	0.48	
GA	233 (53.9)	203 (49.9)		
AA	68 (15.8)	67 (16.5)		
rs738807				
C	750 (86.8)	708 (87.0)	0.92	0.98 (0.73–1.32)
T	114 (13.2)	106 (13.0)		
CC	322 (74.5)	305 (74.9)	0.99	
CT	106 (24.6)	98 (24.1)		
TT	4 (0.9)	4 (1.0)		
rs5844572				
5	199 (23.0)	184 (22.6)	0.83	1.02 (0.81–1.30)
X	665 (77.0)	630 (77.4)		
55	13 (3.0)	24 (5.9)	0.03	
5X	173 (40.0)	136 (33.4)		
XX	246 (57.0)	247 (60.7)		
rs6003941				
T	206 (23.8)	196 (24.1)	0.91	0.99 (0.78–1.24)
G	658 (76.2)	618 (75.9)		
TT	19 (4.4)	30 (7.4)		
TG	168 (38.9)	136 (33.4)	0.08	
GG	245 (56.7)	241 (59.2)		

CI, confidence interval; htSNP, haplotype tagged single nucleotide polymorphism; *MIF*, macrophage migration inhibitory factor; OR, odds ratio.

Table 3 Estimated haplotype frequencies in cases and control subjects

Haplotype*	Case frequency (%)	Control frequency (%)	χ^2	*P*-value
ACXG	38.1	36.0	0.78	0.38
GCXG	21.7	23.2	0.56	0.45
GC5T	17.9	17.9	0.001	0.97
GTXG	11.7	12.3	0.14	0.71
GC5G	3.5	3.5	0.0	0.99
ACXT	2.8	4.1	1.91	0.17
GCXT	1.4	1.1	0.22	0.64

*Haplotype defined on the rs875643, rs738807, rs5844572 and rs6003941 markers.

Table 4 Transmissions of *MIF* alleles from heterozygous parents to diabetic offspring in 290 parent–offspring trios

Polymorphism	Allele	Transmission : non-transmission to affected offspring	χ^2	*P*-value
rs875643	A	148 : 141	0.17	0.68
rs738807	C	72 : 65	0.36	0.55
rs5844572	5-CATT	112 : 110	0.02	0.89
rs6003941	G	120 : 111	0.35	0.55

MIF, macrophage migration inhibitory factor.

for both allele and haplotype analyses but none was significant (data not shown). Furthermore, stratification for the age at diagnosis of diabetes (< 5 years and ≥ 5 years), gender and for the case's HLA-DR risk group (DR3/DR4 heterozygotes, other carriers of DR3, other carriers of DR4) revealed no statistically significant differences in case–control and TDT studies (data not shown).

Discussion

MIF is a potent pro-inflammatory cytokine whose production is transcriptionally regulated by glucose [3,4,6,7]. Experimental

data from T1D patients and animal models also suggests a role for MIF in T1D development [9–12]. We have performed the most comprehensive screening of the *MIF* gene and its surrounding genomic region to date to identify variants and obtain allele frequencies for our population. The data allowed us to estimate common haplotype frequencies, thereby allowing optimal selection of htSNPs for our main association studies. Donn *et al.* have previously screened the *MIF* gene and 1 kb of the 5′ flanking region in 32 unrelated white subjects using denaturing HPLC identifying a CATT repeat and three SNPs with an MAF ≥ 10% [14]. By extending the region examined to 3 kb up- and downstream of the gene, we identified 23 polymorphisms, including two novel variants [14]. The novel variants (ss141323267, ss141023271) have been submitted to

Table 5 Transmissions of major *MIF* haplotypes from heterozygous parents to diabetic offspring in 290 parent–offspring trios

Haplotype*	Frequency	Transmission : non-transmisson to affected offspring	χ^2	*P*-value
ACXG	35.4	138.9 : 127.0	0.53	0.47
GCXG	20.8	95.1 : 95.4	0.001	0.98
GC5T	19.4	87.3 : 85.2	0.03	0.87
GTXG	11.3	56.0 : 56.2	0.0	0.99
GC5G	3.5	21.7 : 19.4	0.12	0.73
ACXT	3.2	17.0 : 17.6	0.01	0.91
GCXT	2.2	10.1 : 19.0	2.73	0.10

*The seven most common haplotypes defined on the rs875643, rs738807, rs5844572 and rs6003941 markers.
MIF, macrophage migration inhibitory factor.

dbSNP to facilitate further study of the *MIF* gene. Comparison of our resequenced data with that available via HapMap (assessed 21 July 2009) revealed that HapMap is not optimal to investigate *MIF* variants in a white population, as less than half of the resequenced common SNPs (MAF ≥ 10%) in our study are recorded in this resource [27]. Indeed, the latter 5.4 kb of DNA screened in this study detected 13 variants, while HapMap only presents data for two SNPs within this region.

Strong LD was found between the majority of *MIF* variants and, as previously reported, rs755622 and rs2096525 mutant alleles were highly correlated [D′ = 1, logarithm of odds (LOD) = 9.17, r^2 = 0.898] [14]. Four htSNPs were identified which captured all haplotypes with greater than 1% frequency. The MAFs of the polymorphisms identified were in agreement both with previous publications [14,28], the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/projects/SNP/) and recent meta-analysis data [29].

In this case–control and family-based association study to examine the role of common polymorphisms of the *MIF* gene in the development of T1D, no positive associations were identified. Both haplotype and TDT analyses failed to reach statistical significance despite stratification for the age at diagnosis of diabetes (< 5 years and ≥ 5 years), gender and for the case's HLA-DR risk group. Examination of eight of the SNPs (rs5760090, rs12628766, rs875643, rs738806, rs738807, rs5751759, rs2012124, rs20000466) in a recent genome-wide association scan and meta-analysis of Type 1 diabetes loci performed by the Type 1 Diabetes Genetic Consortium also failed to provide any evidence of association with T1D [29]. It is unlikely therefore that this region contributes strongly to the genetic risk of T1D in white populations. The two-point promoter haplotype *MIF* CATT$_7$/−173*C is three times more common in patients with inflammatory polyarthritis compared with control subjects [16] and there is excess transmission of this haplotype to the proband in juvenile idiopathic arthritis [17]. However, no significant difference was detected with this functionally interactive haplotype [17] between case and control groups in our study (7.8 vs. 8.9%, respectively; χ^2 = 0.653, *P* = 0.419).

The merits of our study include the relatively homogeneous population examined and the use of TDT, in addition to our case–control analysis, which minimizes the possibility of spurious association as a result of population heterogeneity. The sample sizes employed in the case–control comparison of this study were sufficient to give approximately 80% power to detect as significant (*P* < 0.05) an odds ratio of 1.8 for a minor allele whose frequency was at least 10% in the control population. This study is underpowered, however, to detect the rather small odds ratios which are now being found by genome-wide association scans. Strict genotyping quality control measures were employed. There was a high genotyping success rate and 100% concordance rate between duplicate samples, including those genotyped on two different platforms.

Given the negative results obtained in our study, it is unlikely that common polymorphisms of the *MIF* gene have a major influence on susceptibility to T1D in our population. However, we cannot rule out the possibility that unexamined rare variants or common polymorphisms which reside outside the region screened may have an influence on T1D risk. Indeed, the C allele of the SNP rs1007888 (3.8 kb 3′ of the translation termination codon and consequently just outside the region investigated here) has been associated with both increased circulating MIF and increased risk of Type 2 diabetes in women [30]. In conclusion, common polymorphisms of the *MIF* gene are not strongly associated with increased risk of Type 1 diabetes in a UK population.

Competing interests

Nothing to declare.

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