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Sequencing-based genotyping and association analysis of the *MICA* and *MICB* genes in type 1 diabetes.

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Running Title: Analyses of *MICA* and *MICB* in type 1 diabetes.

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ABSTRACT

Objective: The non-classical major histocompatibility complex (MHC) class I chain related molecules (MIC), encoded within the MHC, function in immunity. The transmembrane polymorphism in *MICA* (*MICA-STR*) has been reported to be associated with type 1 diabetes. In this study we directly sequenced both of the highly polymorphic MIC genes (*MICA* and *MICB*) in order to establish if they are associated with type 1 diabetes independently of the known type 1 diabetes MHC class II genes, *HLA-DRB1* and *HLA-DQB1*.

Research Design and Methods: We developed a sequencing based typing method and genotyped *MICA* and *MICB* in 818 families (2,944 individuals) with type 1 diabetes from the UK and USA (constructing the genotype from single nucleotide polymorphisms in exons 2-4 of *MICA* and 2-5 of *MICB*), and additionally genotyped the *MICA-STR* in 2,023 type 1 diabetes cases and 1,748 controls from Great Britain. We analysed the association of the *MICA* and *MICB* alleles and genotypes with type 1 diabetes using regression methods.

Results: We identified known *MICA* and *MICB* alleles and discovered four new *MICB* alleles. Based on this large-scale and detailed genotype data, we found no evidence for association of *MICA* and *MICB* with type 1 diabetes independently of the MHC class II genes (*MICA P*=0.08, *MICA-STR P*=0.76, *MICB P*=0.03, after conditioning on *HLA-DRB1* and *HLA-DQB1*).

Conclusions: Common *MICA* and *MICB* genetic variations including the *MICA-STR* are not associated, in a primary way, with susceptibility to type 1 diabetes.

Introduction

Approximately 50% of the familial clustering of type 1 diabetes is attributable to the major histocompatibility complex (MHC) region on human chromosome 6p21 and the MHC class II genes, *HLA-DRB1* and *HLA-DQB1*, account for a large proportion of this clustering (1). However, association studies in the MHC region indicate other genes have effects in type 1 diabetes independently of the class II genes (2-4). Identification of these genes is complicated because genes in the MHC have multiple alleles, exhibit extensive linkage disequilibrium (LD) and the class II genes, *HLA-DQB1* and *HLA-DRB1*, show complicated dominance and epistatic effects. Importantly, any putative new effect must be distinguished from the effect of these class II genes (2; 3).

The non-classical MHC class I chain related molecule (MIC) genes *MICA* and *MICB* are located 46.4 kb and 141.2 kb centromeric of *HLA-B* and ~2 Mb telomeric of *HLA-DRB1* (5) and have been associated both with immunity and with autoimmune diseases (5). In common with most MHC HLA genes, "alleles" of *MICA* and *MICB* are named based on haplotypes of nonsynonymous single nucleotide polymorphisms (nsSNPs). However, the most studied polymorphism in either of these genes is a short tandem repeat (STR) in the transmembrane domain of *MICA* (*MICA-STR*). Whether or not these genes are associated with type 1 diabetes is uncertain with multiple conflicting reports in the literature (3; 6-14). Most of these studies failed to demonstrate convincingly that the association is independent of the MHC class II genes and all were restricted to analyses of the *MICA-STR*. The *MICA-STR* is a GCT repeat microsatellite in *MICA* exon 5 (alleles of the *MICA-STR* are named based on the number of repeat units). In European populations the most common allele of the *MICA-STR* is *MICA-STR*A5.1* (frequency 0.50), which contains five GCT repeats and a G insertion that causes a frame shift leading to a premature stop codon and the truncation of the cytoplasmic tail (*MICA-STR*A5* contains five GCT repeats but not the G insertion and is in frame). Here, we genotyped the *MICA-STR* in a large case control collection. However, different alleles of the *MICA-STR* associate with multiple alleles of other polymorphisms in *MICA* (5). Therefore, to capture the allelic variation of *MICA* more comprehensively, we designed a sequencing based typing (SBT) method that yields the genotype information for both *MICA* and *MICB* for exons 2-5 and used it to genotype two collections of type 1 diabetes families. Various approaches have been described to obtain a complete genotype of the *MICA* and *MICB* genes (15; 16). Our method is similar to other SBT methods but incorporates a novel scoring method that increases throughput.

MATERIALS AND METHODS

MICA and *MICB* share 83% DNA sequence homology. Each gene consists of six exons: exon 1, which codes for a signal peptide, exons 2-4 for three extracellular domains (α1, α2 and α3), exon 5 for a transmembrane domain and exon 6 for a carboxy-terminal cytoplasmic tail (5).

Genotyping of the MICA-STR. Genotyping of the *MICA-STR* was done using PCR (Online-Only Appendix Note 1) followed by capillary electrophoresis of the amplified product (17). We used primers CCTTTTTTTCAGGGAAAGTGC (FAM-labelled) and CCTTACCATCTCCAGAAACTG.

Sequencing-based typing of the MICA and MICB genes. Our aim was to maximise throughput in order to enable analysis of large sample sets, and therefore, we used a system that gave the most genotype information while minimising the amount of sequencing and the cost. For this reason we limited ourselves to the information that could be captured with a single pair of external primers. In both genes exon 1 is ~8.5 kb from exon 2, exons 2-5 are in close proximity to each other in a ~2 kb segment and exon 6 is ~2.25 kb centromeric of exon 5. Our *MICA* primers amplified a 1.9 kb fragment containing exons 2, 3 and 4; the *MICA-STR* is at the beginning of the exon 5 of *MICA*, and therefore, it was not possible to sequence through exon 5. Our *MICB* primers amplified a 2.1 kb fragment containing exons 2, 3, 4 and 5. PCR (Online-Only Appendix Note 1) was done using *MICA* primers: CCCCCTTCTTCTGTTCATCA (forward) and TGACTCTGAAGCACCAGCAC (reverse) and *MICB* primers: GGACAGCAGACCTGTGTGTTA (forward) and AAAGGAGCTTTCCCATCTCC (reverse). *MICA* sequencing primers: TCCTGCCCAGGAAGGTT and

CCTGCTGAGTTCCACTGAC for exon 2, AGGAATGGGGGTCAGTGGAA and GAGGGTTTCCCTGGACACAT for exon 3 and CTGTTCCTCTCCCCTCCTTA and CCATCCCTGCTGTCCCTAC for exon 4. *MICB* sequencing primers: GGACAGCAGACCTGTGTGTTA and GCCTCCCTGACCCTATTCC for exon 2, GAGTAATGGGAGGCCTTCT and TGCATCCATAGCACAGGG for exon 3, and CAGGAGTCCACCCTTGACAT, CGTTGACTCTGAAGCACCAG and AAAGGAGCTTTCCCATCTCC for exons 4 and 5. For sequencing conditions see Online-Only Appendix Note 1.

We aligned and scored sequence reads using the Gap4 program from the Staden package (http://www.mrc-lmb.cam.ac.uk/pubseq/). To increase throughput we included a consensus trace in the alignment. Sequence data is not phased and, therefore, a method was needed to determine which pair of alleles was present. While there are many nsSNPs in the MIC genes, they give rise to relatively few alleles per gene. The common alleles are listed on the IMGT/HLA Sequence Database (http://www.anthonynolan.org.uk/HIG/data.html) (18). Since the possible genotypes were limited and known, we used in-house software similar to the Helmberg Score software (19). Positions that varied were extracted from the static alignments of the IMGT/HLA Sequence Database. The alleles were reduced to just those SNPs that are present in the regions we sequenced. A file was then constructed of all possible pairs of *MICA* or *MICB* alleles. Our sequence data was extracted in the same format, and the two files compared. Ambiguity created by missing SNP values was clarified by examining familial relationship and the *MICA-STR* data.

We validated this method in a reference panel of 40 cell lines received from the International Histocompatibility Working Group (IHWG) International Cell and Gene Bank (http://www.ihwg.org/cellbank/dna/refpan.html) and eight artificial heterozygotes made by pooling DNA from different homozygous cell lines. We obtained 100% concordance with the published *MICA* genotypes.

Sample Populations. Using our SBT method we genotyped both *MICA* and *MICB* in 818 type 1 diabetes affected families. These families were from two collections: the Diabetes UK Type 1 Diabetes Warren collection (478 four member families comprised of an affected sib-pair and both parents) (20) and the USA Human Biological Data Interchange (HBDI) collection (340 four and five member families including more than one affected sibling and both parents) (21). In addition we genotyped the *MICA-STR* in 2,023 UK type 1 diabetes cases and 1,748 geographically matched controls (22). All collections comprised subjects of white European origin. *HLA-DRB1* and *HLA-DQB1* had been genotyped previously in these subjects using Dynal RELI SSO assays (Invitrogen Ltd, Paisley, UK) (2).

Statistical analysis. Statistical analyses were carried out in the statistical package STATA v9 (http://www.stata.com) and the rpart library in R was used for the recursive partitioning (23; 24).

Each *MIC* locus was first tested for association with type 1 diabetes without conditioning on the MHC class II loci. Sets of cases and matched pseudo-controls were generated from the affected sib-pair (ASP) families, and analysed using conditional logistic regression (25) with Huber-White sandwich estimators, to correct for the non-independence of siblings. In the case-control collection, cases and controls were matched to 12 broad geographical regions across Great Britain(Northern, East and West Ridings, North Midland, Eastern, Southeastern, Southern, Southwestern, Wales, Midlands, Scotland, London, Northwestern) (22) and analysed using logistic regression (22). The alleles of the *MIC* loci were modelled assuming a multiplicative mode of inheritance.

To establish whether any observed associations of *MICA* and *MICB* were

independent of, and not due to LD with, the class II genes, *HLA-DRB1* and *HLA-DQB1*, the effects of these highly associated loci must be taken into account in the analysis of *MICA* and *MICB*. The overall number of class II alleles, which includes many rare alleles, and the well-established non-multiplicative effects of these loci, complicates their modelling. We found recursive partitioning (rpart) was an effective method to model the genotypes of *HLA-DRB1* and *HLA-DQB1*(2). This is a risk-based grouping method that categorises individuals as affected or unaffected based on their MHC class II genotypes (2). The resulting groups of MHC class II genotypes define strata within which additional, non-class II, associations can be tested, using either conditional logistic regression (families) or logistic regression (case/control) (2). The rpart method was performed both in the case/controls and in the families (cases/pseudo-controls). The additional MHC class II independent effect of each *MIC* locus, was tested by adding the alleles of the test locus to the logistic model and testing their independent effects by a Wald test (families) or a likelihood ratio test (case/control). Note that all the alleles of *MICA* or of *MICB* were included in the logistic model, which allows testing of the conditional association of the locus and calculation of the conditional relative risks (RR)/odds ratios (ORs) of the individual alleles simultaneously, relative to a common reference allele.

For the most common allele of the *MICA-STR* (*MICA-STR*A5.1*), which has a minor allele frequency of 0.5, our power to detect an effect size of OR 2 is greater than 98% at $\alpha = 10^{-5}$.

RESULTS

Alleles and haplotypes. In *MICA* 40 SNPs plus the *MICA-STR* yielded 55 distinct alleles in IMGT/HLA release 2.8 (Jan 2005) (18). We limited ourselves to SNPs in exons 2-4 so, *MICA*009* and *MICA*049* were indistinguishable, as were *MICA*027* and *MICA*048*. Genotypes of the three loci were in Hardy-Weinberg equilibrium in unaffected parents and controls $P > 0.05$. Frequencies for the *MICA* and *MICB* alleles were consistent in the UK and USA families and similar to those reported in other white populations (9; 10; 15; 16). In Online Only Appendix Table 1 we list the combinations of *MICA* gene alleles and *MICA-STR* alleles observed in this data set.

Novel alleles. In *MICB* we found four novel alleles that were due to nsSNPs in common alleles: at position 800 in *MICB*004*, 813 in *MICB*008*, 508 in *MICB*010* and position 641 in *MICB*014* (Table 1). These SNPs were, however, extremely rare (each was found in only one family). We also identified a synonymous SNP (in 64 individuals) in *MICB* (G > T at position 762), which is not present on the IMGT/HLA alignment.

Association with type 1 diabetes. We analysed the *MICA* and *MICB* alleles for association with type 1 diabetes in the family collections and the *MICA-STR* in the family and case/control collections. For each locus we calculated two statistics: the *P*-value of association for each locus overall and the RR/OR for each individual allele. When the *MIC* loci are analysed without conditioning on *HLA-DRB1* and *HLA-DQB1* several alleles of both genes showed a strong association with type 1 diabetes (*MICB* $P=4.21\text{x}10^{-19}$, *MICA* $P=3.74\text{x}10^{-15}$, *MICA-STR* $P=5.44\text{x}10^{-9}$). However, if the effect of these genes is conditioned on *HLA-DRB1* and *HLA-DQB1* the association with type 1 diabetes disappears (*MICB* $P=0.03$, *MICA* $P=0.08$, *MICA-STR* $P=0.76$). Note that $P=0.03$ cannot be considered as evidence of association as it is produced as a result of using a Wald test that is artificially biased towards the alternative hypothesis with rare data (rare alleles, below 0.01, were analysed together, the resulting group had frequency 0.014). Use of a likelihood ratio test without robust variance estimates produces a less significant *P*-value, $P=0.1$.

In Table 2 we present the RR for the alleles of *MICA* and *MICB* in the UK and USA families, using the most common

alleles (*MICA*008* and *MICB*005*) as reference, as they give the tightest 95% confidence intervals. Since none of the other alleles were significantly different to the reference, the reference alleles themselves are also not associated. The *MICA-STR* was also not associated with type 1 diabetes after conditioning on *HLA-DRB1* and *HLA-DQB1* neither in the case/control collection (P=0.65 for the individual alleles and P=0.91 for the genotypes), nor in the families (P=0.76) (Table 3). We also tested the association of *MICA-STR*A5.1* genotypes against all the other genotypes grouped together. They were not associated with type 1 diabetes risk after conditioning (Table 4). We found that all *MICA* and *MICB* alleles were in strong LD ($D' > 0.9$) with one or more *HLA-DRB1* or *HLA-DQB1* alleles (Online-Only Appendix Table 2 and Table 3).

DISCUSSION

Our SBT method allows for large-scale and detailed genotyping of the full *MICA* and *MICB* alleles. SBT also allows discovery of novel alleles and, indeed, we found four new, rare, variants for *MICB*. These results illustrate the high level of allelic variability found in the *MICA* and *MICB* genes; implying that this variability has yet to be fully characterised.

Previous and ongoing studies of type 1 diabetes associations (including the Type 1 Diabetes Genetics Consortium (www.t1dgc.org) and the IHWG (http://www.ncbi.nlm.nih.gov/projects/mhc/ihwg.cgi?cmd=DS&ID=11)) have either omitted *MICA* and *MICB* or focused on the *MICA-STR*, because of the costs and complications associated with obtaining the full genotypes of *MICA* and *MICB*. Many published reports have identified one or more of the alleles or genotypes of the *MICA-STR* as being associated with type 1 diabetes. However, some of these studies did not condition adequately on the MHC class II genes (12-14), while others used subgroup analysis to analyse the *MICA-STR* in samples chosen to have particular class II genotypes (7-9; 11). This sub-grouping causes a multiple testing problem and reduces the sample set, and hence the power to detect MHC class II independent effect. We conclude that, in the European populations analysed here, the common *MICA* and *MICB* alleles are unlikely to affect type 1 diabetes susceptibility nor are they markers for the strong, independent, associations of the common alleles of *HLA-B* (*HLA-B39*) and *HLA-A* (2).

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TABLE 1. *MICB* novel nonsynonymous SNPs found in one parent and one or more children, ++ transmitted to both children, + transmitted to one child

Position*	Nucleotide change	Protein Change	Transmitted
508	A > G	Lys > Glu	++
641	G > T	Ser > Ile	+
800	G > A	Trp > stop	+
813	C > G	Ser > Arg	+

*Correspond to nucleotide position at http://www.anthonynolan.org.uk/HIG/seq/nuc/text/micb_nt.txt

TABLE 2. Association of *MICA* and *MICB* alleles with type 1 diabetes, without conditioning on *HLA-DRB1* and *HLA-DQB1* ($P = 3.74 \times 10^{-15}$ and 4.21×10^{-19} respectively) and with conditioning ($P = 0.08$ and 0.03 respectively). Relative risk (RR). 95% confidence interval (95% CI).

MICA alleles	Frequency*†	RR [95% CI] not conditioned on class II	RR [95% CI] conditioned on class II	*MICB* alleles	Frequency*†	RR [95% CI] not conditioned on class II	RR [95% CI] conditioned on class II
**001*	0.03	2.63 [1.76-3.92]	1.55 [0.99-2.43]	**002*	0.16	1.48 [1.19-1.84]	0.83 [0.64-1.09]
**002*	0.08	0.77 [0.57-1.04]	1.01 [0.72-1.43]	**003*	0.02	0.21 [0.10-0.42]	0.68 [0.33-1.41]
**004*	0.05	0.49 [0.34-0.70]	0.69 [0.48-1.00]	**004*	0.17	0.99 [0.80-1.23]	1.06 [0.83-1.37]
**007*	0.04	1.04 [0.72-1.48]	0.92 [0.61-1.37]	**005*	0.31	1.00 [reference]	1.00 [reference]
**008*	0.48	1.00 [reference]	1.00 [reference]	**008*	0.17	2.15 [1.74-2.66]	1.04 [0.81-1.35]
**009/ *049*	0.05	0.78 [0.55-1.11]	1.17 [0.81-1.70]	**013*	0.01	0.70 [0.33-1.49]	1.05 [0.37-2.98]
**010*	0.09	1.87 [1.41-2.48]	1.08 [0.79-1.47]	**014*	0.01	0.74 [0.46-1.19]	0.64 [0.38-1.06]
**011*	0.02	0.66 [0.35-1.25]	0.83 [0.43-1.61]				
**012*	0.01	0.47 [0.25-0.89]	0.58 [0.27-1.24]				
**016*	0.01	0.80 [0.37-1.74]	0.60 [0.21-1.68]				
**017*	0.02	0.23 [0.12-0.44]	1.00 [0.54-1.86]				
**018*	0.02	0.50 [0.28-0.92]	0.61 [0.29-1.27]				

**019*	0.01	0.98 [0.65-1.47]	1.46 [0.85-2.48]				
**021*	0.01	0.80 [0.21-2.99]	0.98 [0.37-2.60]				
**027/ *048*	0.01	0.44 [0.24-0.81]	0.81 [0.36-1.83]				
**051*	0.01	2.00 [0.12-32.06]	0.28 [0.04-1.87]				

*Frequencies in unaffected parents

†Only alleles with frequency > 0.01 are shown

TABLE 3. Association of the *MICA-STR* alleles with type 1 diabetes, in the family and the case-control collections, both without conditioning on *HLA-DRB1* and *HLA-DQB1* ($P = 5.44 \times 10^{-9}$ families and $P = 9.1 \times 10^{-9}$ case-control) and with conditioning on *HLA-DRB1* and *HLA-DQB1* ($P = 0.76$ families and $P = 0.65$ case-control). Odds ratio (OR)

	Family collection		Case-Control	
Allele	RR [95%CI] not conditioned on class II	RR [95%CI] conditioned on class II	OR [95%CI] not conditioned on class II	OR [95%CI] conditioned on class II
**A4*	1.02[0.83-1.26]	1.01[0.79-1.28]	1.05[0.91-1.23]	1.08[0.81-1.43]
**A5*	1.24[1.01-1.53]	0.87 [0.66-1.14]	1.47[1.26-1.71]	0.85[0.65-1.12]
**A5.1*	1.00[reference]	1.00 [reference]	1.00[reference]	1.00 [reference]
**A6*	0.62[0.50-0.77]	0.94 [0.72-1.24]	0.60[0.53-0.70]	0.90[0.7-1.17]
**A9*	0.59[0.46-0.75]	1.11 [0.84-1.47]	0.71[0.61-0.83]	1.03[0.78-1.37]

TABLE 4. Comparison of *MICA-STR*A5.1* genotypes with other *MICA-STR* genotypes

Genotypes	RR [95%CI] not conditioned on class II	RR [95%CI] conditioned on class II
X/X	1.00[Reference	1.00[reference]
*X/*A5.1*	1.22 [0.99-1.51]	0.94[0.72-1.24]
**A5.1/*A5.1*	1.43 [1.08-1.90]	1.05 [0.75-1.48]