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FULL PAPER

The EIF2AK3 gene region and type I diabetes in subjects from South India

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Mutations in the EIF2AK3 gene underlie susceptibility to the Wolcott–Rallison syndrome, which is a monogenic disease associated with insulin-deficient neonatal diabetes. Furthermore, suggestive evidence of linkage between type 1 diabetes (T1DM) and the EIF2KA3 chromosomal region has been reported in Scandinavian families. We have investigated the hypothesis that polymorphic variants in and around the EIF2AK3 gene might partially account for susceptibility to T1DM in South Indian subjects. Excess transmission of the common alleles of two polymorphic markers (D2S1786 and 15INDEL, located within the gene) downstream of EIF2AK3, either singly (D2S1786, $P = 0.01$) and 15INDEL ($P = 0.02$) or as a combination ($P < 0.001$), were found in 234 families with a T1DM proband. There was also a clear paternal effect for the 15INDEL marker ($P = 0.005$) on disease susceptibility. The presence of the common allele of both markers was found in decreased frequency in the subjects with normal glucose tolerance compared to probands with T1DM (both $P \leqslant 0.0001$). Major common mutations of the EIF2AK3 gene in T1DM were excluded. In conclusion, this pilot study demonstrates an association between the region around the EIF2AK3 locus and T1DM susceptibility.
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Introduction

A large number of chromosomal regions and genes have been found to be associated with type 1 diabetes mellitus (T1DM) by various approaches, including candidate gene testing (most consistently HLA and the insulin gene), genome scans and studies of human homologues of mouse susceptibility genes.[1] The major contribution to familial clustering of T1DM comes from genes located to the major histocompatibility region on chromosome 6, although the specific haplotypes and the strength of the associations vary between ethnic groups. HLA genotype plays an important role in Southern India, with HLA-DQB01*0302 and *0201 haplotypes being positively associated with T1DM.[2] Although over 90% of T1DM subjects possess either HLA-DQB01*0302 or *0201 in most ethnic groups studied, this is not the case in South India, where the presence of one or both of these alleles was found in only 79% of patients.[2] Furthermore, whereas an association between the insulin gene hyper-variable region and T1DM has been found in most ethnic groups, this has not been found in South Indian subjects.[1,3] It is therefore conceivable that individuals with T1DM in South India might have input from a greater range of different susceptibility loci compared to other ethnic groups.

Another approach towards the elucidation of susceptibility genes in multifactorial disease is to identify genes associated with monogenic syndromes and then to investigate the relationship of the identified gene with the more common form of the disease. The Wolcott–Rallison syndrome (WRS; MIM 226980), inherited as an autosomal recessive trait, has been classified as a rare cause of early-childhood insulin-dependent diabetes.[4,5] The syndrome is characterised by a short trunk compared to arm span, multiple epiphyseal dysplasia, bone demineralisation, multiple fractures, tooth discoloration, abnormal skin, hepatosplenomegaly and renal insufficiency, in addition to insulin-dependent diabetes. The onset of diabetes in the probands of WRS families is mainly below the age of 3 months and permanent severe insulin deficiency was present in the 15 families that have been reported to date.[5–10] In those families examined, with the exception of one,[5] islet cell antibodies were not detected in the diabetic subjects and in the single case of WRS that came to autopsy the pancreas

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was found to be hypoplastic, with interstitial fibrosis and poor staining for insulin.[6,8] It is therefore likely that the diabetes of WRS is due to beta cell dysfunction, which in turn contributes to the aetiology of T1DM. Mutations of the gene-encoding translation initiation factor 2-alpha kinase 3 (*EIF2AK3*) were found to account for WRS in all but one of the families published.[5] Of the 15 reported mutations, approximately two-thirds are located to the Ser/Thr protein kinase domains.[5,9,10] To further support the candidacy of the *EIF2AK3* gene, 'suggestive' evidence for linkage was found between the chromosomal region (2p12), encoding this gene, and T1DM in Scandinavian families; a single-point lod score of 2.1 was reported for the marker D2S113, which is 3′ to *EIF2AK3*.[11]

Results

In order to investigate whether there is an association between the *EIF2AK3* gene and T1DM, we used a family-based approach (234 South Indian families), and studied markers from the 3′ flanking region of the locus: D2S113 (the linked marker in the T1DM Scandinavian genome scan),[11] D2S1786 and an AT insertion/deletion at intron 15 (15INDEL) (Table 1). The locations of the two microsatellite markers in the Celera database with respect to the *EIF2AK3* gene are *EIF2AK3* (88714627)–D2S1786 (90978323)–D2S113 (93261377), and hence the decision to study only the 3′ flanking region in the initial stages. The families had been ascertained through a proband with T1DM, with both parents also available for study.

The D2S1786 marker was found to be significantly transmitted to the affected offspring (overall allele-wise ETDT; $P = 0.0098$ uncorrected; $P = 0.03$ corrected; Table 1); this was partly attributable to allele 4 (215 bp) being transmitted 142 times compared to 102 nontransmissions ($P = 0.010$) and decreased transmission of allele 5 (219 bp) (46 transmissions *vs* 71 nontransmissions; $P = 0.021$). There was a small parental effect dependent on maternal meiosis ($P = 0.04$). Excess transmission was also found of the common allele of 15INDEL (Table 1). Allele 1 was transmitted 99 times and not transmitted 69 times (allele-wise ETDT; uncorrected $P = 0.02$; $P = 0.06$ corrected); this could be largely explained by a paternal effect ($P = 0.005$; allele 1, 33 transmissions *vs* 11 nontransmissions). No association was found between T1DM and D2S113 (13 alleles) in the South Indian families. D2S113 is located on a separate YAC (WC 2.8) to the disease-associated marker D2S1786 (WC2.7 that also includes *EIF2AK3*).

TRANSMIT was used to analyse the potential effects on diabetes risk of the D2S1786 and 15INDEL marker combinations. Of 14 possible combinations, only four were found to have a frequency greater than 5%. There was evidence for an association between T1DM and particular *EIF2AK3* marker combinations (overall global $P < 0.001$), with excess transmission of both the 15INDEL allele 1/D2S1786 allele 4 and 15INDEL allele 1/D2S1786 allele 3 combination (both $P < 0.0001$), and decreased transmission of 15INDEL allele 1/D2S1786 allele 5 combination ($P < 0.001$). Since there were a large number of D2S113 alleles, it was not possible to perform an accurate TRANSMIT analysis of all the three markers (15INDEL two alleles, D2S1786 five alleles and D2S113 13 alleles), because of the large number of haplotypes generated.

Since we had evidence for an association between markers from the *EIF2AK3* region and T1DM, we proceeded to compare allele frequencies of the disease-associated 15INDEL and D2S1786 markers in ethnically matched unaffected subjects, with normal glucose tolerance[12] and the previously typed probands with T1DM. The frequencies of the 15INDEL genotypes (1/1, 1/2 and 2/2) in 286 subjects without diabetes were 40.9, 47.9 and 11.2%, respectively, and the corresponding figures in the 225 probands with T1DM were 68.9, 30.2 and 0.9% (Fisher's exact two-sided $P < 0.0001$; odds ratio for 1/1 genotype 3.3, 95% CI 2.2–4.7). Genotype frequencies in the subjects were in Hardy–Weinberg equilibrium. Supporting the family data, allele 4 of D2S1786 was present in 82.4% of the probands compared to 60.9% of subjects without diabetes ($P < 0.0001$). Similarly, there were fewer subjects with allele 5 of D2S1786 in the probands (22.5%) compared to subjects without diabetes (35.4%; $P = 0.001$). The allele frequencies in the control subjects therefore support an association between T1DM susceptibility and *EIF2AK3* in this ethnic group. The allele frequency of the 15INDEL in South Asians is more frequent to that previously reported in Europeans[7] (Table 2). In order to validate our typing, we therefore sequenced 48 subjects (30% possessing the uncommon allele); in all cases, the

Table 1 Transmission of *EIF2AK3* gene polymorphisms from heterozygous parents to T1DM offspring in 234 parent–offspring trios

Polymorphism/and marker order	*Allele*	*Location*	*Number of transmissions*		*P-value**		
			Transmitted	*Not transmitted*	*Overall P-value*	*Paternal*	*Maternal*
SNP E	Allele 1	5′ of EIF2AK3	102	123	0.162		
SNP C	Allele 1	5′ of EIF2AK3	87	100	0.342		
SNP 2	Allele 1	Exon 2	125	114	0.476		
SNP 3	Allele 1	Exon 3	141	132	0.586		
SNP 10	Allele 1	Exon 10	83	89	0.647		
SNP 11	Allele 1	Exon 11	67	60	0.534		
15INDEL	Allele 1	Intron 15	99	69	0.020	0.005	0.138
D2S1786	Overall	3′ of EIF2AK3	—	—	0.0098	0.102	0.040
D2S113	Overall	3′ of EIF2AK3	—	—	0.204		

*Paternal and maternal transmissions only calculated if raw data are significant.

Table 2 Variations identified from the sequencing of *all 15 EIF2AK3* exons and flanking intronic regions

Exon/intron	*cDNA position*	*Amino acid*	*Genomic position*	*Allelic frequencies*	*European alleles*
Exon 1	112–132(CTG)7/8	14–20(L)7/8	112–132(CTG)7/8	0.85/0.15*	0.75/0.25
Exon 1	242G/T	57Arg/Leu	242G/T	0.99/0.01*	—
Exon 2	476C/G	135Ser/Cys	1141C/G	0.63/0.37	0.68/0.32
Exon 3	566G/A	165Arg/Gln	978G/A	0.46/0.54	0.62/0.38
Intron 6	—	—	2661A/G	0.93/0.07*	—
Intron 10	—	—	811A/T	0.66/0.34	0.74/0.26
Exon 11	1860G/A	596Gln/Gln	707G/A	0.10/0.90	0.30/0.70
Exon 13	2179T/G	703Ser/Ala	1638T/G	0.80/0.20*	0.68/0.32
Intron 15	—	—	845A/C	0.97/0.03*	0.94/0.06
Intron 15	—	—	1217–1255 (CA)n	Not detected*	
Intron 15 (15INDEL)	—	—	1641–1642(AT/-)	0.88/0.12	0.95/0.05

Variants are positioned in relation to reference cDNA and genomic sequences.[7] Allelic frequencies have been estimated from parental chromosomes ($n = 468$); those with an asterisk (*) have been estimated from the 48 chromosomes sequenced. European allele frequencies were based on 190 chromosomes from the study of Delepine *et al*.

sequencing results were concordant with the PCR-RFLP assay.

Based on this evidence of a disease association with the two markers closest to the *EIF2AK3* gene, 24 subjects were selected for comprehensive sequencing studies of all 17 exons and exon/intron boundaries of the gene to identify any novel mutations and define haplotype blocks within the gene. These subjects comprised 10 South Indian T1DM probands with the disease-associated combination (allele 1 of 15INDEL and allele 4 of D2S1786, subsequently referred to as the 1:4 combination) that had been transmitted from a parent, 12 South Indian subjects (three parents, two probands and six controls), who did not possess the 1:4 combination, and two European controls (untyped). The previously reported mutations leading to WRS were not found.[5,9,10]

Nucleotide variations were found at 11 different residues of the *EIF2AK3* gene (Figure 1 and Table 2), corresponding to nine previously reported polymorphisms and two novel SNPs found in exon 1 (position 242 G/T; Arg57Leu) and intron 6 (position 2661 A/G), respectively. Inspection of the sequences in these subjects suggest linkage disequilibrium (LD) of all SNPs apart from the 15INDEL, and that five possible haplotypes were defined by four SNPs 2, 3, 10 and 11. We therefore proceeded to genotype the families for the four relevant SNPs by a PCR-RFLP assay.

LD between all markers used in this study was assessed using the PM (permutation and model-free analysis) and PM plus programmes,[13,14] and the results are presented in Table 3. These programs allow for both a permutational approach, as well as analysis between two loci and between haplotype blocks, and they confirmed the results from the 24 subjects we had sequenced (Table 2 and Figure 1). SNPs 2, 3, 10 and 11 were all in tight LD and formed a specific haplotype block. Of 16 possible haplotypes, only 14 were observed, and five of these were present at a frequency of greater than 5%. The haplotype block composed of SNPs 2, 3, 10 and 11 was in linkage equilibrium with all other surrounding markers. No individual marker (Table 1) or haplotype was transmitted in excess to the diabetic offspring (TRANSMIT global $P = 0.18$) from within the block. The two disease-associated markers in our study (D2S1786 and 15INDEL) were in LD with D2S113 (the marker linked to T1DM in the Scandinavian population), thereby placing

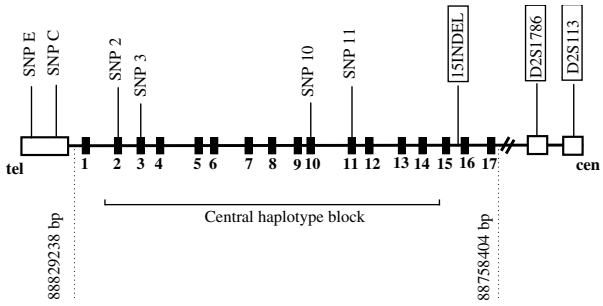

Figure 1 Genomic structure of the *EIF2AK3* gene and polymorphisms identified. The blocks represent the 17 exons and the vertical lines indicate the position of the polymorphisms (see Table 2). The labelled lines indicate those polymorphisms studied in the entire family data set, and the boxed labels those markers associated or linked to T1DM in either the South Indian or Scandinavian subjects. At present, only the exons and exon/intron boundaries have been elucidated and therefore the diagram is not to scale. Dotted lines indicate current map position of the *EIF2AK3* gene (http://genome.ucsc.edu/). cen = centromere, tel = telomere.

disease susceptibility in the 3′ flanking region of the EIF2AK3 locus.

To confirm our preliminary findings that disease susceptibility lies 3′ of the gene, we used a PCR-RFLP assay to investigate two other reported SNPs, SNP C (rs2364564) and SNP E (rs1606803) (located approximately 4 and 6 kb, respectively, 5′ of *EIF2AK3*) in our study population. No association was found between SNP C and SNP E and T1DM susceptibility (Table 1). Analysis using the PM plus programmes revealed that SNP C and E are in LD with each other and also with markers located to the central haplotype block (SNPs 2, 3, 10 and 11) (Table 3). The two SNPs located 5′ of the gene were not in linkage with our disease-associated markers, 15INDEL and D2S1786, which are 3′ of the gene, which further supports our hypothesis of disease susceptibility located either 3′ or downstream of the *EIF2AK3* locus in this population.

Discussion

We have demonstrated an association between the region around the *EIF2AK3* locus and T1DM susceptibility in

Table 3 Pairwise linkage disequilibrium values for markers flanking and within the *EIF2AK3* gene

	SNP E	*SNP C*	*SNP2*	*SNP3*	*SNP10*	*SNP11*	*15INDEL*	*D2S1786*	*D2S113*
SNP E	—	<0.001	<0.001	<0.001	<0.001	<0.001	0.0077	NS	NS
SNP C	0.853	—	<0.001	<0.001	<0.001	<0.001	NS	NS	0.021
SNP2[a]	0.802	0.829	—	<0.001	<0.001	<0.001	NS	NS	NS
SNP3[a]	0.919	0.734	0.877	—	<0.001	<0.001	NS	NS	NS
SNP10[a]	0.831	0.821	0.760	0.612	—	<0.001	NS	0.010	NS
SNP11[a]	0.913	0.778	1.000	0.831	0.661	—	NS	NS	NS
15INDEL[b]	0.245	0.246	0.035	0.051	0.001	0.370	—	NS	0.032
D2S1786[b]	0.094	0.064	0.042	0.043	0.028	0.073	0.162	—	0.008
D2S113[c]	0.033	0.153	0.035	0.156	0.100	0.261	0.249	0.193	—

PM+permutation *P*-values above diagonal; *D*′ calculated by 2LD below diagonal.
[a]Not disease associated.
[b]Disease associated by TDT (see Table 1).
[c]No disease association in South Indian data set but linkage with T1DM in Scandinavian data set.
NS = not significant.
SNP2, 3, 10, 11 and 15INDEL are all located within the *EIF2AK3* gene (Figure 1); the chromosomal order of D2S1786 and D2S113 is based on the Celera Map.

subjects from South India. Our data set has excluded common major coding region mutations of *EIF2AK3* leading to this disease; however, the associations between T1DM susceptibility and both 15INDEL and D2S1786 reinforce the observed linkage found in sibpairs on 2p12 in Scandinavian families.[11] The two datasets would therefore suggest that a T1DM susceptibility gene is located to this region. Whether there are one or more susceptibility determinants and whether the *EIF2AK3* gene is implicated in T1DM susceptibility remains to be determined. Further studies will require the elucidation and analysis of the regulatory regions and the full intronic structure of *EIF2AK3* (as yet unavailable in the public database), and a detailed analysis of other genes 3′ to *EIF2AK3*, D2S1786 and D2S113. It is not known whether the AT insertion deletion (15INDEL) in intron 15 is of functional significance. Inspection of the sequences around this polymorphism does not reveal any transcription factor-binding sites that might be altered, depending on the presence or absence of the polymorphism.

The *EIF2AK3* gene would appear to be a good candidate gene for T1DM for several reasons, including the fact that the gene is widely expressed in human fetal pancreas at 8 weeks postconception, while in the adult pancreas it is expressed extensively in the islets, with a predominance in β-cells.[10] There is 88% sequence homology between this gene and rat pancreatic eIF2-alpha kinase (otherwise known as *PEK*), a gene that has an essential role in protecting the cell from endoplasmic reticulum stress.[15,16] PEK phosphorylates eIF2α, which then inhibits eIF2β, leading to cellular events that inhibit mRNA translation. The highest expression of PEK is in the delta cells of the pancreas, which is the main site of secretion of somatostatin hormone.[15] One action of somatostatin is to inhibit the secretion of insulin, which could, in turn, lead to hyperglycaemia. Targeted mutations of the mouse *EIF2AK3* gene abolish phosphorylation of EIF2A, and the resultant phenotype in mice homozygous for the mutation is characterised by insulin deficiency, hyperglycaemia, growth retardation, steatorrhoea and high mortality between 2 and 4 weeks of life.[17] In contrast, heterozygous mice are phenotypically similar to wild-type mice, although they are glucose intolerant following intraperitoneal injection of glucose. Pancreatic islets are reduced in size at postnatal day 12, with a decrease in insulin-containing cells and an increase in glucagon-secreting cells. Furthermore, there is evidence of beta cell apoptosis in these animals. It could therefore be hypothesised that dysregulation of the *EIF2AK3* gene may cause insulin deficiency, leading to diabetes in some subjects with T1DM. Although the *EIF2AK3* gene has not been excluded as a susceptibility gene in polygenic type II diabetes (T2DM), no mutations of the *EIF2AK3* gene have been found in a study of French subjects with monogenic early-onset T2DM.[18] No studies to date have investigated the *EIF2AK3* gene in T1DM.

In summary, we have produced preliminary evidence of an association between T1DM and the *EIF2AK3* gene for the first time in any population, using a family-based approach and supported by the distribution of disease-associated alleles in an ethnically matched nondiabetic population. Further work is required to confirm if the disease susceptibility gene is indeed *EIF2AK3* and if so to elucidate the mechanisms whereby variants in (or around) the *EIF2AK3* gene might lead to diabetes.

Materials and methods

Study subjects

Southern Indian families were recruited from patients attending either the MV Hospital for Diabetes or the MV Diabetes Specialties Centre, Chennai, India. All affected subjects were ketosis prone and had acute onset of disease, which required immediate treatment with insulin. Cases of fibrocalculous pancreatic diabetes were excluded by radiology, ultrasonography and on clinical grounds. The mean age of onset in T1DM probands was 11.7 years ($\pm$5.8; range 1–29 years), the male to female sex ratio was 0.73 and the mean body mass index at time of venesection was 17.3 ($\pm$4.5) kg m^{-2}. Blood samples were taken from 234 probands and also from both parents in all families and an additional six affected siblings ($n = 708$ subjects).

DNA samples were available from 286 South Indian subjects with normal glucose tolerance, initially recruited as part of an urban population-based survey using a cluster analysis design across all socio-economic groups into the prevalence of T2DM and associated risk factors.[12] The mean age was 43 ($\pm$13; range 18–94) years, mean BMI 21.7 ($\pm$4.1) kg m^{-2} and 51% were males. Glucose tolerance was defined by the most recent WHO criteria.[12] The protocol was passed by the local ethical board and informed consent was obtained from all participants prior to venous sampling in Chennai. All samples were exported to the UK, either in or prior to 1997, with a valid permit issued by the Government of India.

Genotyping
SNP2, SNP3, SNP10, SNP11 and 15INDEL were studied by a PCR-RFLP assay, using restriction enzymes *Sty*1, *BsmA* 1, *Hinf*1, *AlwN*1 and *Nde*1, respectively. SNP10 was studied by acrylamide gel electrophoresis; all other markers were studied by agarose gel electrophoresis. D2S1786 and D2S113 were genotyped by PCR, using fluorescent primers with detection by either an ABI 377 or ABI3700 DNA sequencer (Applied Biosystems).

Nucleotide sequencing
Primers for exons 2–17 (including the exon–intron boundaries) were as described previously.[7] For exon 1, a two-step nested PCR was performed using primer pairs NE1F (5′-CGATGTCCGAGA-3′) and NE1R (5′-GCTCGCGCGCGT-3′), followed by the previously described exon 1 primer pair. Sequencing reactions were performed using the big-dye v2 terminator chemistry (Applied Biosystems), run on an ABI 3700 (Applied Biosystems) and analysed using Polyphred software.[19]

Statistical analysis
Transmission of individual markers and haplotypes (or marker combinations) were assessed by the extended transmission disequilibrium test (ETDT) and TRANSMIT, respectively. To assess LD between markers, the permutation and model-free analysis (PM) and PM plus programs were utilised. Differences in genotype distribution between groups were tested by χ^2 analysis (*SPSS for Windows* version 11).

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