An analysis of amplified insulin gene products in diabetics of Indian origin

G A Hitman, P K Kambo, M Viswanathan, V Mohan

Abstract
We have previously described an increased incidence of the class 3 allele of the hypervariable region (HVR) 5' to the insulin gene in south Indian non-insulin dependent diabetics; this association is absent in Punjabi Sikhs with this disorder. Using the polymerase chain reaction we have amplified parts of the insulin gene from 130 subjects to look for mutations which may be in linkage disequilibrium with the class 3 allele and hence explain its association with non-insulin dependent diabetes (NIDDM). In 23 south Indian subjects with NIDDM, using the restriction enzyme MboII, a B chain mutant (insulin Chicago) was excluded. Two patterns (α and β) were found, representing a PstI polymorphism in the 3' untranslated region of the insulin gene. In subjects homozygous for the class 1 allele, the allelic frequency for α was 0·94 (143/152) and for β was 0·06, in heterozygotes (1,3) α 0·63 (54/86) and β 0·37, and in homozygotes for the class 3 allele α 0·18 (4/22) and β 0·82 (p<0·001), thus establishing linkage disequilibrium between these two loci. No differences in allelic frequency were found in the south Indians or Punjabi Sikhs between controls and the different types of non-insulin requiring diabetes (NIDDM, fibrocalculus pancreatic diabetes and maturity onset diabetes of the young) when both groups were matched for insulin genotypes. Thus, although this polymorphism in the 3' untranslated region of the insulin gene is in linkage disequilibrium with the class 3 allele, it does not appear to be any better at predicting diabetes than the class 3 allele itself.

Despite strong evidence that non-insulin dependent diabetes (NIDDM) is a genetic disease, the progress in identifying the genes involved in its pathogenesis has been slow. This contrasts sharply with the recent advances in identifying genes involved in the susceptibility to insulin dependent diabetes. Several candidate genes for NIDDM have been investigated including those coding for insulin,2–7 the insulin receptor,8–10 the erythrocyte glucose transporter,11,12 and HLA,13–15 but no consistent association with NIDDM has been found. Furthermore, in pedigree studies, apart from the rare exception, linkage of proposed markers has not been found with NIDDM.1

Recent studies on diabetes from south India have indicated an association with non-insulin requiring diabetes (for example, NIDDM,7 maturity onset diabetes of the young (MODY) (unpublished), and fibrocalculus pancreatic diabetes (FCPD)7) of the class 3 allele of the hypervariable region in the 5' flanking region of the insulin gene on chromosome 11. Furthermore, the association was particularly marked in those with a family history of NIDDM. Similar results have also been reported in a Japanese study.5 However, in a separate study we were unable to find the insulin gene association in north Indians (Punjabi Sikhs).6 Associations of this marker have been described in several ethnic groups but are absent in others.1 Many interpretations of these studies exist including the following.

1. Spurious results leading to a false association.
2. Ethnic heterogeneity in study groups.
3. The primary association not being with NIDDM but an associated disorder (for example, atherosclerosis or hypertriglyceridaemia).16–18
4. A high rate of recombination around the hypervariable region of the insulin gene. This would reduce the usefulness of the HVR as a polymorphic marker for a linked disease associated mutation in and around the insulin gene locus. Indeed, meiotic recombination of this locus occurs more frequently than one expects from its physical length. Thus Chakravarti et al19 have calculated by analysing four insulin gene locus polymorphisms (including the HVR) that the recombination frequency is 24 times greater than would be predicted.
5. Linkage disequilibrium of the class 3 allele with either an aetiologically significant mutation within the insulin gene locus or a diabeticogenic gene near to the insulin gene on chromosome 11. This might also
explain the lack of an association of the hypervariable region of the insulin gene with NIDDM in some well defined ethnic groups, as one would postulate that the class 3 allele in these ethnic groups is no longer in linkage disequilibrium with the aetiological mutation. This situation would not be unique and would parallel findings in population studies of sickle cell disease. A polymorphism in the 3' flanking region (HpaI) of the β globin gene can be used to predict sickle cell disease in west but not east Africans, despite the aetiology of the disease being the same in the two ethnic groups, that is, a single amino acid substitution in the β globin molecule.20

The aim of our study was to test the hypothesis that in non-insulin requiring diabetes in south Indian subjects there is a nucleotide substitution in the coding regions of the insulin gene and this in turn would be in linkage disequilibrium with the class 3 allele of the insulin gene locus which would account for its association with diabetes. Furthermore, in population groups who do not show an association of NIDDM with the class 3 allele (for example, Punjabi Sikhs) the same insulin gene locus mutant might also be found as in the south Indians, although not linked to the class 3 allele. The strategy adopted was to amplify areas of the coding region of the insulin gene using the polymerase chain reaction,21 digest these fragments with restriction enzymes, and separate the resulting fragments by polyacrylamide gel electrophoresis. Among the possible intragenic polymorphisms to be studied would be one of the B chain determining insulin Chicago22 and another adjacent to the A chain in the 3' untranslated region.23 24

Methods

SUBJECTS STUDIED

South Indian (n=99) and Punjabi Sikh (n=31) subjects were selected for analysis according to the presence or absence of the class 3 allele (determined from previous studies).6 7 In south Indian subjects the following groups were analysed: healthy controls (n=26), NIDDM (n=23), MODY (n=24), and FCPD (n=26). In Punjabi Sikhs only healthy controls (n=17) and patients with NIDDM (n=14) were studied. Clinical groups were defined as previously described.7

EXPERIMENTAL METHODS

A total of 10 μl of 10 × polymerase chain reaction (PCR) buffer (500 mmol/l KCl, 100 mmol/l Tris-Cl, pH 8·5, 15 mmol/l MgCl2, 0·1% w/v gelatin), 16 μl of dNTP (1·25 mmol/l), 5 μl of each primer (Oswell DNA Service, Edinburgh), and 200 ng of target DNA was mixed together with double distilled water to give a final volume of 100 μl. DNA in the PCR mix was then amplified in 40 cycles using Taq polymerase, 2·5 units/assay (Cetus Corporation) in a Techne heating block. The exact conditions for each cycle were as follows. (1) The B chain: one minute denaturing at 99°C followed by a combined step of three minutes annealing and extension at 68°C using two primers of sequence 5' ATACACTGTCTTCTGCTGGGATGG 3' and 5' CCTGCA GGTCTCCTGCCTC CCC 3'. (2) The A chain and 3' untranslated region: one minute denaturing at 99°C, 18 seconds annealing at 55°C, and 2·5 minutes extension at 72°C using two primers of sequence 5' GCTGTTCAAGGGGGCTTATTC 3' and TGGGGCGGTGGAGCTGGGCG 3'.

One tenth of the polymerase chain reaction product was then mixed with ethidium bromide and separated out on a 1·5% agarose gel to check that a single sized product (218 bp for the A chain and 203 bp for the B chain) had been obtained. A total of 50 μl of PCR product was then subjected to phenol/chloroform extraction, precipitated using ethanol and sodium acetate, and redissolved in 10 μl of a Tris/EDTA buffer by previously described techniques.3 The A chain related PCR product in 51 subjects was digested with 50 U of PvuII and 50 U PstI. In the remaining subjects only PstI was used. The B chain related PCR product was digested with MboII and HaeIII and studied in 23 south Indian subjects, 12 of whom possessed the class 3 allele. The digested PCR products were separated according to size on 15% polyacrylamide gel (for 15 hours at 200 volts) and visualised after staining with ethidium bromide. Sizes of restriction fragments were deduced by comparison with a 1 kb DNA ladder (Bethesda Research Laboratories, Paisley, Scotland).

Results

B CHAIN RELATED AMPLIFICATION

Using MboII (to detect insulin Chicago21) and HaeIII (as a general screen for polymorphism) four fragments were resolved, sized 20, 39, 94, and 51 base pairs. Insulin Chicago would have resulted in three fragments of size 20, 39, and 146 base pairs. In all 23 south Indian NIDDM subjects, of whom half possessed the class 3 allele, no polymorphism was detected, thus ruling out mutations at these restriction sites as a common cause of NIDDM in south Indians.

A CHAIN RELATED AMPLIFICATION

No polymorphisms were detected with PvuII in 23 south Indian NIDDM subjects, 13 of whom possessed the class 3 allele. Two patterns were detected with PstI; pattern α consisted of fragments sized 150, 27, and 41 base pairs whereas in pattern β there was an extra PstI site with fragments sized 65, 85, 27, and 41 base pairs (figure). In subjects homozygous for the class 1 allele the allelic frequencies for pattern α and β
respectively were 0.94 (143/152) and 0.06, whereas in subjects homozygous for the class 3 allele the corresponding frequencies were 0.18 (4/22) and 0.82, and in heterozygotes (1,3) 0.63 (54/86) and 0.37 (2×3 \( \chi^2=78, p<0.0001 \), Cramer's V=0.55). Thus, patterns \( \alpha \) and \( \beta \) were in linkage disequilibrium with the class 1 and class 3 alleles respectively in both south Indian and Punjabi Sikh populations, and were no more informative for diabetes than the insulin gene HVR flanking region polymorphism. It was therefore not surprising that results in the south Indian population did not differ between the types of diabetes (NIDDM, MODY, and FCPD), nor between NIDDM and control subjects in the Punjabi Sikhs, although only small numbers were studied (table).

**Discussion**

Results from this study indicate (1) that at least one mutant insulin (insulin Chicago) is not a common cause of NIDDM in the south Indian population, and (2) in both the south Indian and Punjabi Sikh populations the class 3 allele in the 5' flanking region of the insulin gene is in linkage disequilibrium with a point mutation in the 3' untranslated region of the insulin gene (the two loci being physically separated by approximately 2 kb), which therefore provides no more additional information than studying the insulin gene HVR itself.

The *PstI* intragenic insulin gene polymorphism was first described by Bell et al\(^ {23} \) and Ullrich et al.\(^ {24} \) In the two alleles sequenced, a point mutation in the 3' untranslated region at position 1628 involving a C to A transversion accounted for the presence (gHI 12\(-5\)/\( \beta \) allele\(^ {24} \)) or absence (gHI 14\( \alpha \)/\( \alpha \) allele\(^ {24} \)) of the *PstI* cutting site. Subsequently it was studied by Elbein et al\(^ {25} \) by Southern blot analysis in 50 alleles each from Caucasoids, Pima Indians, and blacks; they found it to be non-polyomorphic and therefore concluded that the \( \beta \) type allele represented an isolated mutation.\(^ {25} \) From our studies this is clearly not the case, its frequency depending on the frequency of the class 3 allele in the study groups.

The next question which arises is could this point mutation which creates the extra *PstI* site in the 3' untranslated (3UT) region be a mutation which provides a small part of the genetic susceptibility to non-insulin requiring diabetes in some south Indian subjects. The function of the 3UT is not known, although it does form part of the primary mRNA transcript; some authors have postulated that this region may be important for mRNA stability.\(^ {26} \) Thus, a possible hypothesis is that this polymorphism may lead to less mRNA being translated and hence hypoinsulinaemia, thus causing NIDDM in south...
Indian subjects; this would be in keeping with recent thoughts on the aetiology of this disease.27 Alternatively, both the insulin gene HVR and the described PstI RFLP could be in linkage disequilibrium with sequences important to insulin regulation in the 5' flanking region of the insulin gene.

Finally, it is worth highlighting the ease of the methodology used in this project in detecting possible aetiologi
tional mutations within coding regions of a gene without the need for Southern blot technology and the use of radioactive probes.

PKK was funded by the Medical Research Council. We thank H Mather and E Kohner for allowing us access to their patients.

14 Omar M, Kammo, Viswanathan, Mohan
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doi: 10.1136/jmg.28.2.97

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