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

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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, fibrocalculous 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, the erythrocyte glucose transporter, and HLA, 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. Recent studies on diabetics from south India have indicated an association with non-insulin requiring diabetes (for example, NIDDM, maturity onset diabetes of the young (MODY) (unpublished), and fibrocalculous pancreatic diabetes (FCPD)) 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. However, in a separate study we were unable to find the insulin gene association in north Indians (Punjabi Sikhs). Associations of this marker have been described in several ethnic groups but are absent in others. 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).
(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 al 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 diabetogenic 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 poly-
morphisms 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 8.3, 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' ATACCTGCTCTTCGCCATGG 3'
and 5' CCTGCA GGTCTGCTGCCCTCCC 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' GCTGGTCTCAAGGGCCTTATTCT 3'
and TGGGGCAAGTGGAGCTGGGCG 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 Laborato-
ries, Paisley, Scotland).

Results

B CHAIN RELATED AMPLIFICATION

Using MboII (to digest 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 frag-
ments 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
Frequencies of insulin gene $\alpha$ and $\beta$ alleles in study populations.

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<th>Population</th>
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<td>South Indian</td>
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<td>Punjabi Sikhs</td>
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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. and Ullrich et al. 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 of the HGI 12-S/\beta allele or absence of the HGI 14-S/\alpha allele of the PstI cutting site. Subsequently it was studied by Elbein et al. by Southern blot analysis in 50 alleles each from Caucasoids, Pima Indians, and blacks; they found it to be non-polymorphic and therefore concluded that the $\beta$ type allele represented an isolated mutation. 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 (3’UT) 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 3’UT 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. 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. 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 aetiological mutations within coding regions of a gene without the need for Southern blot technology and the use of radioactive probes.

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