Non-linkage of the islet amyloid polypeptide gene with Type 2 (non-insulin-dependent) diabetes mellitus

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Summary. Type 2 (non-insulin-dependent) diabetes is associated with the deposition of islet amyloid. The major formative peptide, islet amyloid polypeptide, has recently been characterised and an abnormality of the structure or expression of this gene is a possible candidate for the inherited component of Type 2 diabetes. A restriction fragment length polymorphism of the gene has been identified with Pvu II. To study the relationship between the islet amyloid polypeptide gene and Type 2 diabetes, two distinct genetic approaches have been undertaken. Firstly, non-linkage has been demonstrated in four pedigrees, with four normoglycaemic first degree relatives having an allele associated with diabetes in

other family members, and one affected relative not having the putatively associated allele. The LOD score taking age-related penetrance into account was -1.68 , making linkage unlikely ($p = 0.02$). Secondly, in a population-based restriction fragment length polymorphism survey, no linkage disequilibrium of the alleles was found between a population of unrelated Caucasian subjects with Type 2 diabetes and a normal population. A mutation in or near the islet amyloid polypeptide gene is thus unlikely to be a common cause of Type 2 diabetes.

Key words: Linkage, islet amyloid.

A major genetic predisposition to Type 2 diabetes is shown by the near 100% concordance found in identical twins [1, 2]. Whilst several genetic abnormalities probably contribute to the development of diabetes, a major single gene defect is suggested by the bimodality of glucose tolerance in three populations where the disease is common [3–5]. Similar bimodality has been shown in the first degree relatives of Caucasian Type 2 diabetic patients [6]. Köbberling and Tillil (1982) calculated that the ultimate prevalence of diabetes in the first degree relatives was 38% assuming that all relatives lived to age 80 years [7]. This is close to the 50% expected for an autosomal dominant disease. It is thought that other genetic and environmental factors contribute to the expression of the genetic abnormality [8] and some subjects may have a genetically determined increased risk of disease which is expressed at an early age, especially when associated with obesity [9].

The nature of the putative gene defect is unknown [10]. Insulin resistance is nearly always present in patients and contributes to the diabetes [11], but as it occurs in both glucose intolerant and normoglycaemic relatives, it may be an exacerbating rather than predisposing factor [12]. In Caucasian populations, impaired Beta-cell function appears to be the most characteristic abnormality of first degree relatives of subjects with Type 2 diabetes who have

glucose intolerance [13, 14]. Progression from glucose intolerance to diabetes is accompanied by diminished Beta-cell function in a number of ethnic groups [15, 16]. This has been interpreted to suggest that the major gene defect in Type 2 diabetes affects the Beta cells.

Deposition of amyloid is the most characteristic abnormality in the islets of Type 2 diabetic patients [17–19]. Amyloid deposition occurs in up to 90% of Caucasian Type 2 diabetic patients, and rarely in the normal population [20]. Amyloid deposition is also a prominent feature in diabetic Pima Indians, whereas it is rarely found in Pima Indians who were known to have normoglycaemia or impaired glucose tolerance [21]. The islet amyloid deposition occurs between the endocrine cells and the capillaries and it has been postulated that the deposition of islet amyloid may be a pathological factor interfering with the normal function of Beta cells [22], possibly eventually causing the moderate diminution of Beta cell numbers found in Type 2 diabetes [19].

The formative peptide of the islet amyloid of Type 2 diabetes is a 37 amino-acid polypeptide which has 43 and 46% homology with calcitonin gene-related peptide (CGRP) -I and -II, respectively [23, 24]. The islet amyloid polypeptide (IAPP) is a product of normal pancreatic Beta cells and is stored together with insulin in Beta cell

granules [25]. The DNA sequence of the human gene encoding IAPP has been described [26, 27]. It is a single copy gene located on chromosome 12 [26]. IAPP is synthesised as an 89 amino-acid residue prepropeptide which consists of a signal peptide followed by IAPP which is flanked by two short peptides [28]. The two flanking peptides are subsequently cleaved off at basic amino-acid residues. The availability of the c-DNA [27, 29] and the discovery of a restriction fragment length polymorphism (RFLP) [30] allows investigation of the association of the IAPP gene with Type 2 diabetes.

Subjects and methods

Pedigree study

The protocol was approved by the Central Oxford Research and Ethics Committee. Potentially informative families were obtained by examining DNA from 98 unrelated Type 2 diabetic patients to

identify probands who were heterozygous for an IAPP marker. Twenty-one were heterozygous for the Pvu II RFLP for IAPP [30] with bands at 24 kb and 16 kb. Blood samples for DNA analysis were obtained from their available first degree relatives. We describe the only four families in which a second member was diabetic or glucose intolerant and in whom sharing of a specific IAPP allele with the proband could be identified. The pedigrees are shown in Figure 1 and Table 1 gives details of the probands. The probands presented with Type 2 diabetes after 40 years of age. The clinical pattern of diabetes seen in these probands and their affected family members was indistinguishable from classic Type 2 diabetes. First degree relatives were screened with a 5 mg·kg ideal body weight·min⁻¹ continuous glucose infusion test [31]. Blood samples were obtained for glucose, insulin and C-peptide assay. Where glucose tolerance testing was not possible, fasting plasma glucose and insulin samples were obtained. Family members who were not available for investigation were excluded from the study. As expected, several family members who were asymptomatic were found to have diabetes or glucose intolerance on testing.

The following subjects were considered affected: (1) Diabetes diagnosed by WHO criteria, and on therapy with oral hypoglycaemic agents, insulin or a specific diet. (2) Fasting hyperglycaemia,

Fig. 1. Pedigrees of Type 2 diabetic families showing inheritance of islet amyloid polypeptide (IAPP) alleles and non-linkage with diabetes. **Family JA.** Type 2 diabetic subjects II 2 and II 4 share the "A" allele which is the putative IAPP diabetic gene in this family, but subject II 4 transmits the "A" allele to normoglycaemic offspring III 3 aged 40 years. **Family JB.** Affected subjects II 1, II 6 and II 8 share the "A" allele, but subject II 6 transmits it to her normoglycaemic offspring III 4 and III 8, aged 45 years and 40 years, respectively. **Family JC.** Type 2 diabetic subjects I 1 and II 4 share the "B" allele, which is the putative IAPP diabetic gene in this family. However, normoglycaemic offspring II 5 and II 6 aged 31 years and 29 years received the "B" allele. The affected subject II 3 did not inherit the "B" allele. **Family JD.** Subject II 3 had two affected parents and may have been homozygous for a predisposing gene for diabetes [9] and had three husbands unavailable for study. There was, however, no selective transmission of either the "A" or "B" allele to affected subjects III 4 and III 5. Subject III 3 shares the putative diabetic IAPP genotype "A" with her diabetic half-sister III 5, but at age 45 has normal glucose tolerance. **Key:** Roman numerals on the left of the Figure indicate generation number. The "A" and "B" alleles refer to the 24 kb and 16 kb bands -respectively, detected with IAPP cDNA probe on Southern blots of genomic DNA digested with the restriction endonuclease Pvu II

Table 1. Clinical details of diabetic probands

Family	JA	JB	JC	JD
Subject	II 4	II 6	I 1	II 3
Sex	M	F	F	F
Age at diagnosis (years)	57	58	64	45
Duration of diabetes (years)	3	8	3	23
Height (cm)	167	163	158	158
Weight (kg)	76	82	76	50
Body mass index (kg/m ²)	27.3	30.9	30.4	20.0
Presentation	thirst, polyuria	thirst, nocturia, pruritis vulvae	infection	thirst, pruritis vulvae
Initial therapy	diet	OHA	diet	diet
Time on initial therapy (months)	36	96	16	2
Current therapy	Diet	OHA	OHA	OHA
Complications	nil	nil	nil	CVA retinopathy

OHA – oral hypoglycaemic agents; CVA – cerebrovascular accident

Table 2. Age-related penetrance factors used in linkage analysis for the three putative genotypes (NN normal, ND heterozygote, DD homozygote) for Type 2 (non-insulin-dependent) diabetes

Liability class	Age (years)	NN	ND	DD
1	> 60	0	0.8	0.95
2	40–60	0	0.45	0.9
3	< 40	0	0.25	0.8

with a fasting plasma glucose concentration > 6 mmol/l, which is > 3 standard deviations above the mean of a normal population. (3) Glucose intolerance, defined as plasma glucose levels > 2 standard deviations above the mean of an age and body-weight matched normal population, one hour after a continuous infusion of glucose 5 mg · kg ideal body weight · min⁻¹. In the families studied only one member was classified as abnormal on this basis alone, the others also having fasting hyperglycaemia.

Population study

Eighty-eight unrelated Type 2 diabetic subjects were Caucasians from the Oxford UK Prospective Diabetes Study clinic [32]. Sixty-seven non-diabetic subjects included 15 normoglycaemic Caucasians aged 59–94 years, and 52 Oxford blood donors.

DNA studies

DNA was extracted from blood samples using standard methods. 5 µg of DNA was digested with a three-fold excess of the restriction endonuclease Pvu II (Anglian Biotech, Colchester, UK) in accordance with the manufacturer's instructions. The resulting fragments were electrophoresed in 0.8% agarose gels, denatured, neutralized

and transferred using Southern blotting on to nylon filters (Hybond-N Amersham, UK). The filters were subsequently hybridized with a ³²P-labelled islet amyloid polypeptide c-DNA probe (Sc IAP-8) by hexanucleotide labelling, using a commercial kit (Pharmacia, Milton Keynes, UK). Sc IAP-8 contains the 588 bp EcoR1 insert encoding λhIAPP-C1 [28]. Following hybridization the filters were washed and subjected to autoradiography. Pvu II digestion of genomic DNA identified a two allele polymorphism with bands at 24 kb (allele A) and 16 kb (allele B). The location of the Pvu II restriction site within the IAPP gene is unknown.

Linkage analysis

Linkage analysis was performed with version 4.8 of the LINKAGE computer programmes. The putative gene frequency used for Type 2 diabetes was 0.10, based on prevalence studies in a Caucasian population [33]. As the probe for a candidate gene was being examined, LOD score was calculated with the recombination fraction theta equal to zero. The age-dependent penetrance factors (Table 2) were derived from the studies of Köbberling and Tillil [7], O'Rahilly et al. [9] and Page et al. [35]. For example, the 5 mg · kg ideal body weight · min⁻¹ glucose infusion test in first degree relatives, aged 40–49 years, of Type 2 diabetic patients found 22% to have impaired glucose tolerance. On a dominant gene hypothesis this would represent 44% of those potentially affected [35].

Results

Pedigree data

The pedigrees and IAPP haplotypes for the four families are shown in Figure 1. Inspection of the pedigrees excludes close linkage between identified alleles of the IAPP gene and Type 2 diabetes. In each of the four families, a first degree relative with diabetes or glucose intolerance could be identified as having one of the two alleles of the proband, and this was assumed to be the putative IAPP gene associated with diabetes. In each family, normoglycaemic first degree relatives could be identified with the same allele, four of these subjects being aged more than 40 years (Table 3). Age-dependent penetrance factors allowed for uncertainty in the diagnosis of normality in these individuals. In the family JC, one affected family member did not share the same allele as two other affected family members. The formal LOD score (logarithm of the odds for linkage between the IAPP locus and Type 2 diabetes) of – 1.68 made it unlikely (*p* = 0.02) that a defect in the IAPP gene is the cause of diabetes in these families.

Population data

No significant difference in the frequency of the polymorphisms at 24 kb (A) and 16 kb (B) was demonstrated between the 88 unrelated Caucasian subjects with Type 2 diabetes, 15 Caucasian non-diabetic control subjects and 52 randomly-selected blood donors (Table 4).

Table 3. Normoglycaemic subjects with allele for islet amyloid polypeptide associated with diabetes in two or more members of the pedigree

Family	JA	JB	JC	JD
Subject	III 2	III 8	II 4	III 3
Age (years)	40	40	45	43
Gender	male	male	female	female
BMI (kg/m ²)	26.5	26.9	40.0	23.5
HbA _{1c} (%)	5.4	4.6	4.8	5.6
FPG (mmol/l)	4.5	4.4	5.2	4.3
APG (mmol/l)	–	–	7.7	8.4
Z (SD)	–	–	0.7	0.1
FPI (pmol/l)	–	–	55.3	33.7
API (pmol/l)	–	–	191.7	89.0

Abbreviations: BMI – body mass index; HbA_{1c} – glycosylated haemoglobin (normal range ± 2 SD 4.3–6.1); FPG – fasting plasma glucose; APG – achieved plasma glucose at 60 min of glucose infusion; Z – standardized residual from the prediction of an age and % ideal body weight regression model applied to a population of 105 normal subjects; FPI – fasting plasma insulin; API – achieved plasma insulin at 60 min of glucose infusion

Table 4. Islet amyloid polypeptide (IAPP) genotypes of diabetic and non-diabetic populations. Polymorphism for IAPP was demonstrated by a band at 24 kb (allele “A”) and 16 kb (allele “B”)

	(n)	AA	AB	BB
Type 2 diabetic subjects	88	74	14	0
Normoglycaemic control subjects	15	12	3	0
Blood donors	52	40	11	1

The frequency of alleles “A” and “B” in each group was tested by $\chi^2 = 2.72$ with 4 degrees of freedom (not significant)

Discussion

The demonstration of non-linkage of the IAPP gene with Type 2 diabetes in four Caucasian families together with the lack of linkage disequilibrium in the population study make it unlikely that a defect in or near the IAPP gene is a common cause of Type 2 diabetes. Linkage analysis in pedigrees is a powerful tool for examining the role of candidate genes in the aetiology of inherited diseases with a defined mode of transmission [37]. The linkage analysis assumes that Type 2 diabetes has a major single-gene autosomal dominant genetic component as suggested by bimodality in population studies [3–6]. The late age of onset and the frequency of subclinical disease have made it difficult to define the mode of inheritance. However, the suggestion that the common form of Type 2 diabetes results from an autosomal dominant trait with a penetrance dependent upon age and obesity is supported by results from family studies in which ultimate prevalence was estimated by statistical extrapolation [7] and in the prevalence of abnormality in first degree relatives of Type 2 diabetic patients [9]. This view is supported by a recent WHO expert committee [38].

When performing linkage analysis in Type 2 diabetes the complicating factors of high disease prevalence and late age of onset must be considered. Age-dependent penetrance allows for uncertainty in the exclusion of identification of diabetes and reduces the risk of spuriously elevated LOD scores, whether positive or negative. The penetrance factors used in this study were calculated

from the studies of first degree relatives of Type 2 diabetic subjects assuming dominant inheritance [7, 9, 35] and from studies of asymptomatic women who have had gestational diabetes and were studied by glucose tolerance tests [39]. The gene frequency for the disease in the normal population is assumed from the 10% prevalence of known diabetes in a Caucasian population in the 8th decade [33].

The biological rationale for population-based RFLP surveys relies on the concept of non-random association of alleles with putative disease susceptibility genes giving linkage disequilibrium [40]. If a mutated IAPP gene existed in Type 2 diabetic patients, it would probably be in linkage with DNA polymorphisms within or flanking the gene, as demonstrated with sickle cell anaemia and the thalassaemias [41]. The population study data demonstrated no significant difference in the frequency of the IAPP alleles between Caucasian Type 2 diabetic subjects, Caucasian non-diabetic control subjects and randomly-selected blood donors. The lack of linkage disequilibrium demonstrated in the population study provides no suggestive evidence for the presence of a mutated IAPP gene in Type 2 diabetic patients.

The deduced amino-acid sequence of the IAPP encoding gene [26] reveals that the normally occurring polypeptide is identical to the polypeptide isolated from pancreatic amyloid [23, 24]. This provides further evidence to suggest that amyloidogenesis is not due to the presence of a mutated form of IAPP. Similarly there is no identified gene defect of the formative amyloid peptide found in Alzheimer's disease [42]. This is in contrast to the molecular basis of amyloid deposition in familial amyloidotic polyneuropathy [43] and hereditary cerebral haemorrhage with amyloidosis [44] where the synthesis of a genetically abnormal protein is thought to facilitate the deposition of amyloid.

The mechanisms leading to islet amyloid formation in Type 2 diabetes are unknown: amyloidogenesis may result from altered levels of expression of the IAPP gene, overproduction or decreased catabolism of the peptide. Abnormal clearance of the IAPP precursor molecule may be an aetiological factor, analogous to the putative role of defective proinsulin processing in Type 2 diabetes [45]. In addition to the mature IAPP, the N-terminal flanking peptide has been identified in islet amyloid deposits [46].

The demonstration of non-linkage of the IAPP gene with Type 2 diabetes in four Caucasian families together with the lack of linkage disequilibrium in the population study make it unlikely that a mutation of the IAPP gene is associated with Type 2 diabetes. This does not exclude the excessive deposition of islet amyloid from being a major factor in the development of Type 2 diabetes.

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