Insulin receptor and insulin-responsive glucose transporter (GLUT 4) mutations and polymorphisms in a Welsh Type 2 (non-insulin-dependent) diabetic population

S. O'Rahilly¹, A. Krook¹, R. Morgan², A. Rees², J.S. Flier³ and D.E. Moller³

¹ Departments of Medicine and Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge, ² Department of Medicine, University Hospital of Wales, Cardiff, UK and ³ Department of Medicine, Beth Israel Hospital, Boston, Massachusetts, USA

Summary. We have recently examined the exons encoding the insulin receptor tyrosine kinase domain and GLUT 4 in 30 subjects with Type 2 (non-insulin-dependent) diabetes mellitus using a molecular scanning approach. The variant sequences Val-Met⁹⁸⁵ and Lys-Glu¹⁰⁶⁸ of the insulin receptor and Val-Ile³⁸³ of GLUT 4 were each separately found in three different diabetic subjects. In a study of a Welsh population, the GLUT 4³⁸³ variant was found in three of 160 diabetic and none of the 80 control subjects. In this study, the same group of Welsh Type 2 diabetic and control subjects was analysed using allele-specific oligonucleotide hybridisation, single nucleotide primer extension and allele-specific restriction digestion to ascertain the frequency of the two insulin receptor mutations. The Val-Met⁹⁸⁵ mutation was found in none of the 160 Welsh Caucasian Type 2 diabetic subjects and two of 80 control subjects. The Lys-Glu¹⁰⁶⁸ mutation removes a Sty 1 site and digestion of amplified exon 18 with Sty 1 confirmed the presence of this mutation in the heterozygous state in the original subject. None of the Welsh diabetic or control sub-

jects had the Glu¹⁰⁶⁸ mutation. The discovery of a very common silent polymorphism at codon 130 of GLUT 4 allowed examination of the association of this locus with Type 2 diabetes using allele-specific oligonucleotide hybridisation in a subset of the Welsh subjects. The genotypic frequencies (homozygous wild-type and heterozygous polymorphic (poly) sequences) were not significantly different between diabetic and control subjects (Type 2 diabetic subjects: wild-type/wild-type 40%, wild-type/poly 46%, poly/poly 14%; Control subjects: wild-type/wild-type 37%, wild-type/poly 45%, poly/poly 18%; $p > 0.05$). In conclusion, in a British Caucasian population the examined insulin receptor tyrosine kinase domain mutations are uncommon. Also the GLUT 4 locus does not appear to be strongly associated with Type 2 diabetes.

Key words: Genetics, Type 2 (non-insulin-dependent) diabetes mellitus, insulin receptor, glucose transporters.

Recently, several subjects with syndromes of severe insulin resistance such as leprechaunism, Rabson Mendenhall syndrome and type A insulin resistance have been found to have insulin receptor mutations [1]. In certain cases heterozygous relatives of such patients have had no obvious clinical features except moderate to marked hyperinsulinaemia and sometimes, impairment of glucose tolerance [2]. This has led to increased interest in the possibility that a certain proportion of subjects with "typical" Type 2 (non-insulin-dependent) diabetes mellitus may harbour such mutations and that these might contribute to the inherited insulin resistance component of the disease. Studies of the insulin receptor locus in Type 2 diabetes and control populations by restriction fragment length polymorphisms (RFLP) have produced conflicting results while the sequence of the receptor has been found to be normal in the small number of Type 2 diabetic patients in whom it has been established [3]. We

have recently employed a "molecular scanning" technique, namely single stranded conformation polymorphism (SSCP) analysis, for the analysis of the insulin receptor and insulin-responsive glucose transporter genes in Type 2 diabetic patients [4, 5]. We established that the method has a high specificity and sensitivity for the detection of variant sequences and, when applied to the screening of 30 Caucasian Type 2 diabetic subjects, it detected two novel insulin receptor variants. The first aim of this study was to establish the frequency of these variants in a large well-matched group of Type 2 diabetic and control subjects in order to assess the potential importance of these variant receptors to the larger Type 2 diabetic population.

In a prior study of the GLUT 4 locus by SSCP we detected a common silent polymorphism at codon 130 in exon 4a [5]. As RFLP's of GLUT 4 have not been frequent or informative, and as preliminary data from other

Fig. 1. **A** Allele specific oligonucleotide (ASO) hybridisation of wild-type (above) and mutant (below) probes for the detection of insulin receptor (IR) mutant Val (GTG)-Met (ATG)⁹⁸⁵. Subject 6 is a heterozygote and other subjects are homozygous for the wild-type sequence. **B** Single nucleotide primer extension (SNUPE) analysis of the insulin receptor 985 mutant. Lane 3 is blank. Lanes 1 and 4 contain amplified exon 17 from a subject heterozygous for the G to A mutation at the 985 locus. Lanes 2 and 5 contain amplified exon 17 from a subject homozygous for the wild-type sequence. Lanes 1 and 2 show incorporation of the ³²P-labelled wild-type nucleotide (G) in both subjects after the SNUPE reaction. Lane 4 shows incorporation of the ³²P-labelled mutant nucleotide (A) in the heterozygous subject but not in the homozygous wild-type subject in lane 5

workers suggested an association of a marker in the exon 3–5 region of GLUT 4 with Type 2 diabetes [6] we used allele-specific oligonucleotide (ASO) hybridisation to examine the frequency of the polymorphic sequence in a subset of the Welsh Type 2 diabetic and control populations.

Subjects, materials and methods

The subjects studied by ASO and allele specific restriction fragment length polymorphism (ASRFLP) analysis were all Welsh Caucasians recruited at the University Hospital of Wales. Subjects gave informed consent to the studies. Diabetic subjects ($n = 165$) conformed to World Health Organisation (WHO) criteria. Normal subjects ($n = 85$) had no family history of diabetes and had normal random blood glucose levels (WHO criteria) and HbA_{1c} less than 8.5%. Mean age was 64.9 ± 9.5 years and mean body mass index was 24.6 ± 4.5 kg/m².

Allele specific oligonucleotide hybridization

ASO was performed as previously described [5] using the following probes: for insulin receptor codon 985: wild-type – TGTGTAACG TGCCGGAC; mutant – TGTGTAACATGCCGGAC; for insulin receptor codon 1068: wild-type – TGGTGTCCAAGGGCCAG; mutant – TGGTGTCCGAGGGCCAG; for GLUT 4 codon 130: wild-type – CCTGGCCAACGCTGGTG; mutant – CCTGG CCAIGCTGGTG.

Allele specific restriction digestion

The Lys-Glu¹⁰⁶⁸ mutation results in the loss of a Sty 1 site in exon 18. Exon 18 of the insulin receptor was amplified from 100 ng of genomic DNA from each subject using the polymerase chain reaction (PCR) as previously described [5]. Restriction digests were performed at 37°C for 3 h using 18 µl of the PCR reaction and 10 U of Sty 1 with the NaCl concentration adjusted to 0.1 mol/l. Ten µl of the unpurified digest was subjected to electrophoresis on a 1.7% agarose minigel at 150 V for 15 min and the DNA bands were visualized by staining with ethidium bromide.

Single nucleotide primer extension (SNUPE)

A 17-mer oligonucleotide (TTCCATGCTCTGTGTAC) was designed which hybridised to the 17 nucleotides immediately 5' to the site of the GTG-ATG mutation in codon 985 of the insulin receptor. Fifty nanograms of amplified exon 17 from three subjects with the wild-type sequence only and three subjects who were heterozygous for the mutant sequence by ASO, were examined. Using amplified exon 17 material as template, duplicate SNUPE reactions were set up for each subject as described above for PCR reactions except for the absence of deoxynucleotide tri-phosphates (dNTPs) and the presence in one tube of α -³²P-labelled deoxy ATP (wild-type) and the presence in the other tube of α -³²P-labelled deoxy GTP (mutant). The samples were subjected to one cycle consisting of 2 min denaturation at 94°C, 2 min annealing at 55°C and 2 min primer extension at 72°C. Five microlitres of sample was mixed with 5 ml of gel loading buffer (80% formamide/50 mmol/l Tris Borate pH 8.3/1 mmol/l EDTA, 0.1% bromophenol blue) heated for 2 min at 90°C and loaded onto a 6% polyacrylamide gel containing 8 mol/l urea. Gels were run for 2 h at 250 V and then subjected to autoradiography using Kodak X-AR5 film and exposed for 1–2 h.

Statistical analysis

Statistical analysis of the GLUT 4 polymorphism data was performed using the Chi-square test.

Results

Insulin receptor mutations

The Met⁹⁸⁵ variant was detected by ASO analysis (Fig. 1a) in two of 85 control subjects and none of the 160 Welsh diabetic subjects. Both subjects were heterozygotes. Single nucleotide primer extension was used to confirm the results of the ASO studies. Gel-purified exon 17 from the index patient and the two control subjects with the mutation were studied in parallel with three subjects in whom the ASO studies revealed only wild-type sequence. SNUPE analysis confirmed the ASO findings (Fig. 1b).

Fig. 2. Allele specific restriction digestion to detect the exon 18 insulin receptor mutation Lys-Glu¹⁰⁶⁸. Polymerase chain reaction (PCR) amplification of exon 18 results in a 214 base pair (bp) fragment (Lane 1). Digestion of the wild-type PCR product with Sty 1 normally results in two fragments of 134 and 80 bp. The 1068 mutation removes the Sty 1 site and in a heterozygous individual (lane 2) three bands are seen, as a result of digestion of the amplified wild-type allele only

The Lys-Glu¹⁰⁶⁸ mutation in the index case resulted in the expected ASRFLP pattern with the 314 base pair (bp) band representing the mutant allele and the 214 and 70 bp bands representing the wild-type allele (Fig. 2). This mutation was not detected in any of the Welsh diabetic or control subjects.

GLUT 4 polymorphism

A randomly selected subgroup of diabetic ($n = 86$) and control ($n = 76$) subjects were studied for the presence of the silent polymorphic variant in codon 130 of GLUT 4, exon 4a. ASO hybridisation distinguished between subjects who were known to have homozygous wild-type (wt) and homo- and heterozygous polymorphic (poly) sequences. The genotype frequencies in both the diabetic and control populations did not deviate significantly from Hardy-Weinberg equilibrium. There was no significant difference in allelic (number of polymorphic alleles in Type 2 diabetic vs control subjects: 64/172 vs 62/152; NS) or genotype frequencies (Type 2 diabetic subjects: wt/wt 34/86, wt/poly 40/86, poly/poly 12/86; Control subjects: wt/wt 28/76, wt/poly 34/76, poly/poly 14/76; NS) between diabetic and control populations.

Discussion

The development of PCR-based techniques such as direct sequencing and SSCP analysis has facilitated the analysis of the fine structure of candidate genes in Type 2 diabetes and other disorders. Having used these techniques to detect a variant sequence in an affected subject, the relationship of the mutation to the disease remains to be established. Studies of the co-segregation of the mutation with metabolic abnormalities in families members and examination of the functional properties of the variant molecule when expressed *in vitro* are important steps in this evaluation. As Type 2 diabetes is likely to be a complex polygenic disorder and the metabolic alterations induced by one variant molecule in isolation may be subtle, studies

of the prevalence of the mutation in large populations of Type 2 diabetic and control subjects are helpful in the assessment of its potential pathogenic role. In addition, this type of analysis can also provide information regarding the potential significance of this mutation for the broader Type 2 diabetic population.

In this study we have used three complementary techniques for the detection of known mutations. ASO hybridisation is applicable to any mutation but clear distinction between wild-type and mutant sequence can occasionally be difficult when the melting temperatures of the wild-type and mutant probes are similar. ASRFLP analysis is simpler and quicker, but is only applicable when the mutation results in a change in a convenient restriction site. The recently developed technique of SNUPE [7] requires the time consuming step of gel-purification of each PCR product to remove all contaminating dNTPs and is therefore unsuitable, in this form, for large-scale population studies. It is very useful as a confirmatory technique and may be applicable to pooled genomic samples thereby making it a potentially powerful tool for population genetic studies.

The Val⁹⁸⁵ residue of the insulin receptor is highly conserved between species and is also present in the analogous position in the insulin-like growth factor I receptor [8]. The variant residue Met⁹⁸⁵ represents a conservative amino acid substitution. Its presence in two elderly non-diabetic subjects suggests that it is unlikely to cause significant disturbance of glucose metabolism. In addition we have recently detected this variant in a young normoglycaemic subject with normal fasting insulin levels (unpublished observations). It seems likely that this is a normal variant of the insulin receptor, although it remains possible that, in combination with other genetic or environmental factors, it may contribute in some way to the glucose intolerance of the one diabetic patient carrying the mutation.

Lysine¹⁰⁶⁸ is also highly conserved and the Lys-Glu mutation is, *a priori*, more likely to be significant as it represents a change from a basic to an acidic amino acid. We detected this mutation solely in our index case and further family and *in vitro* studies will be necessary to establish its pathological significance.

Reduced insulin-mediated glucose disposal is a major feature of Type 2 diabetes and may be partially genetically determined [9]. GLUT 4 is therefore a strong candidate gene. One earlier study using denaturing gradient gel electrophoresis of genomic DNA had suggested an association between a polymorphism in the region of exon 3 to 5 of GLUT 4 and Type 2 diabetes in an American Caucasian population [6]. Other genetic studies to date have been limited because of the relative paucity of informative RFLPs. The finding of a common silent polymorphism at codon 130 enabled us to perform a simple case/control study which found no association between the polymorphism and Type 2 diabetes. The ability to detect this polymorphism by SSCP or ASO analysis will allow this to be a useful marker for linkage studies.

In conclusion we have applied a variety of methodologies to the assessment of the frequencies of specific insulin receptor tyrosine kinase domain mutations in a large

population of Welsh Type 2 diabetic and control subjects and found these mutations to be uncommon. No association was found between a common silent polymorphism in exon 4a of GLUT 4 and Type 2 diabetes.

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Dr. S. O'Rahilly
Department of Medicine
Addenbrooke's Hospital
Cambridge CB2 2QR
UK