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Abnormal messenger ribonucleic acid (mRNA) transcribed from a mutant insulin receptor gene in a patient with type A insulin resistance

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Summary. In a previous report on a 16-year-old Japanese girl with type A insulin resistance, we found that one allele of the insulin receptor gene was inherited from her mother and contained a 1.2 kilobase pair deletion which removed the 14th exon in the β subunit. We extended investigation of the proband and found the deletion between two Alu sequences. To determine the effect of the deletion on the level of transcription and the splicing pattern of messenger ribonucleic acid (mRNA), we synthesized the complimentary DNA and used the polymerase chain reaction to amplify the region which included the deleted area. The deletion shifted the reading frame, resulting in a termination codon after amino acid 867 (Glu), thereby producing a truncated insulin receptor without a transmembrane region and cytoplasmic domain. We also sequenced each of 22 exons of the insulin receptor gene but found no mutation in exons of the insulin receptor gene, except for deletion of exon 14 of the maternal allele. Thus, the proband is a heterozygote for a single mutant allele. Abnormal mRNA transcribed from the mutant allele resulted in a decrease in insulin binding.

Key words: Insulin receptor, type A insulin resistance, deletion, polymerase chain reaction, insulin receptor gene, direct sequence, mRNA.

Cloning of complementary DNA (cDNA) of the human insulin receptor has facilitated analysis of the insulin receptor gene [1, 2]. The structure of this gene has also been characterized [3], it is composed of 22 exons and contains over 100 kilobases (kb). We examined a patient with type A insulin resistance and found evidence for a 1.2 kb deletion in the insulin receptor gene (IR-Yamanashi) [4, 5]. Several types of mutations in the insulin receptor genes have been identified in patients with insulin resistance [6–13]. These reports concerned a point mutation in the insulin receptor gene, but a deletion mutation was noted in only two cases [4, 6]. As deletions in the insulin receptor gene are rare, it was of interest to determine how the deletion affects the level of transcription and splicing of messenger ribonucleic acid (mRNA). We determined the partial sequence of the insulin receptor mRNA of the proband, using synthesis of cDNA and amplification by polymerase chain reaction (PCR).

The proband's mutant allele was inherited from her mother and the pedigree analysis indicated that four other members of her family (the mother, maternal grandfather and two aunts), were heterozygotes for the same mutation and have mild insulin resistance but normal or impaired glucose tolerance. Only the proband showed the diabetic pattern. Thus, we considered that the proband may have inherited from her father an allele with a different mutation. We also sequenced each of the 22 exons of the proband's insulin receptor gene.

Subjects and methods

Patient

A 16-year-old Japanese girl presented with fasting hyperinsulinaemia (337 pmol/l), acanthosis nigricans, decreased insulin binding and obesity (body mass index, 31.2). The oral glucose tolerance test showed a diabetic pattern. The plasma glucose level was 11.2 mmol/l and HbA_{1c} 8.8%. We identified a normal 12 kb and abnormal 10.8 kb restriction fragment, using the procedure of digestion with restriction enzyme EcoRI and hybridization with 4.4 kb insulin receptor cDNA. Cloning of the normal 12 kb and abnormal 10.8 kb fragments revealed that one allele of the insulin receptor gene inherited from her mother contained a 1.2 kb deletion, including the 14th exon, and that the proband was a heterozygote of the mutant allele. Four other members of her family with the same mutation presented neither acanthosis nigricans nor hirsuitism and only the proband was obese [4, 5].

Genomic sequencing of the deletion joint

To determine boundaries of the deletion in the mutant allele, cloned fragments from the normal and mutant alleles of the proband were digested with restriction enzyme, then were subcloned into M13 vec-

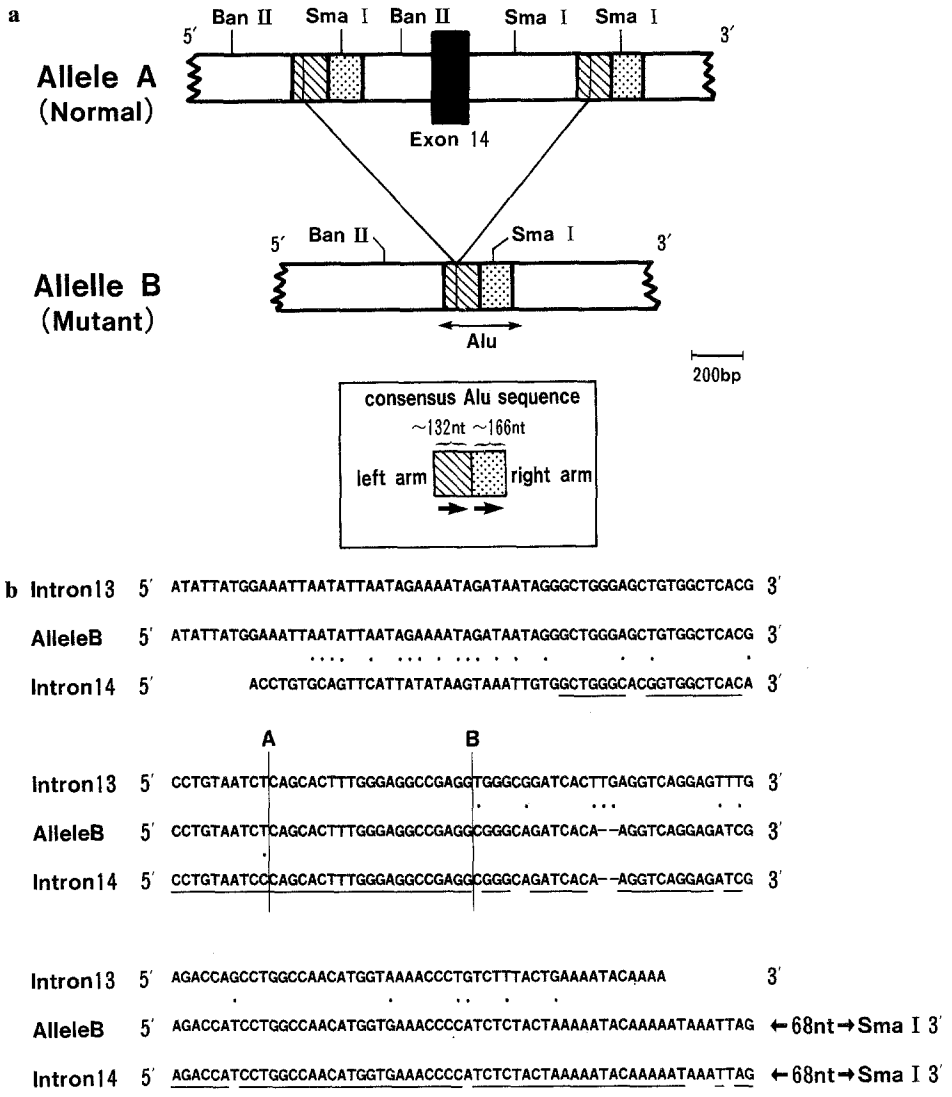

Fig. 1. a Restriction map and schematic representation of the deletion joint. Allele A and allele B indicate the normal allele and mutant allele, respectively. The exon is shown by the solid box and the introns by open segments. The position of known Alu sequences with right arms are indicated by dots and left arms by diagonal lines. **b** DNA sequence of the deletion joint. The top and bottom sequences are derived from introns 13 and 14 of the normal allele. The middle sequence is the deletion joint in allele B (mutant allele). Vertical lines designate limits of the deletion joint. Dots between the sequence indicate positions where the sequence differs. Gaps are introduced to optimize alignment and the underline below the sequence of intron 14 indicates homology between intron 14 and the consensus Alu sequence

tors mp18 and mp19. The nucleotide sequences were determined using dideoxy termination methods [14].

Northern blotting

Total ribonucleic acid (RNA) was extracted from Epstein-Barr virus (EBV) transformed lymphocytes using guanidine thiocyanate, as described elsewhere [15]. Poly(A)$^+$ RNA was purified and separated by formaldehyde agarose gel electrophoresis, blotted onto nitrocellulose filter and hybridized with ^{32}P-labelled probe, as described [16].

cDNA synthesis and PCR

Synthetic oligonucleotide YE81 (nucleotide number 3059→3041: 5′-CTTCCAATCACAACACTGA-3′) and YE82 (nucleotide number 2518→2538: 5′-GAGCACAGGCCTTTTGAGAAG-3′) were designed according to the published normal sequence of the human insulin receptor cDNA. Poly(A)$^+$ RNA (1 µg) was heated at 70 °C for 10 min and slowly cooled to room temperature. The synthesis of cDNA was carried out in a 50 µl reaction volume containing poly(A)$^+$ RNA, 1 µmol/l oligonucleotide primer YE81, reverse transcriptase from Avian myeloblastosis virus (Bethesda Research Laboratories, Gaithersburg, Md., USA), 50 mmol/l Tris HCl PH 8.5, 40 mmol/l KCl, 6 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, deoxyribonucleotide triphosphate (deoxyadenine triphosphate, deoxyguanine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, each 1 mmol/l) and 40000 units of RNasine (Promega, Madison, Wis., USA) at 42 °C for 1 h. The reaction mixture was extracted with phenol/chloroform, precipitated with ethanol and diluted in 10 µl of TE (10 mmol/l Tris HCl pH 7.5, 1 mmol/l EDTA pH 8.0).

Amplification of cDNA was performed as previously described [17]. Fifty percent of the cDNA mixture was subjected to 30 cycles of PCR with *Thermus aquaticus* (Taq) DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn., USA) in 1 µmol/l YE81 and YE82, using an automated DNA thermal cycler (Iwaki, Funabashi, Japan). Each cycle comprised 1 min at 95 °C for denaturing, 1 min at 48 °C for the primer anneal and 3 min at 72 °C for extension of the DNA strand. After this amplification, the reaction mixture was analysed by electrophoresis in a low-melting agarose gel (Bio Rad, Richmond, Calif., USA). Bands containing amplified products were excised and eluted, as described [16]. Each fragment was subcloned into M13 vector mp18 and sequenced according to the dideoxy chain termination method [14].

Sequencing of exons

Exons of the insulin receptor gene were amplified by PCR using sets of primers, as described by Seino et al. [18]. We modified the 5′end of each primer to create a restriction enzyme EcoRI site that could be used for subcloning. Amplified PCR products were directly se-

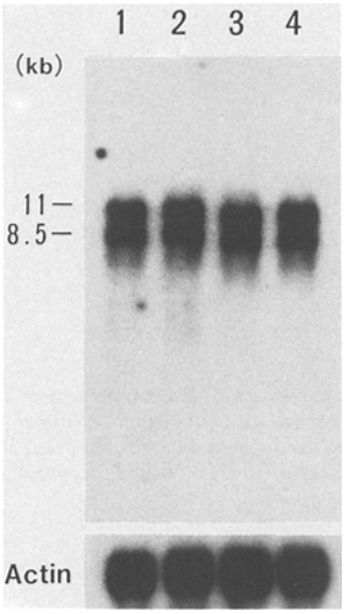

Fig. 2. Northern blot analysis of mRNA. mRNA was isolated from Epstein-Barr virus transformed lymphocytes from a normal control subject (lane 1), the proband (lane 2), her father (lane 3) and her mother (lane 4). Poly(A)$^+$ RNA was separated in each lane and hybridized with the ^{32}P-labelled insulin receptor cDNA (upper). The blot was then hybridized with actin cDNA (bottom), using the same filter. Numbers on the left mean size of the mRNA calculated from ribosomal RNA

quenced. Some exons (exon 1,18–20, 22) were also sequenced by subcloning into phage vector M13, mainly because the sequence ladder of a direct sequence is obscure. When using M13, we sequenced at least five clones with each exon. Direct sequencing was done as described elsewhere [13], but with modification. First PCR and second PCR (synthesis of single strand DNA) were done as described. We used a G50 spin column (Boehringer Mannheim, Mannhein, FRG) to separate the single-strand DNA. The amplified single-stranded DNA was sequenced using sequenase sequencing kits (United States Biochemical Corporation, Cleveland, Ohio, USA). The amplified single-stranded DNA was mixed with primer and polymerase buffer, heated at 95 °C for 3 min then cooled on ice and annealed at 60 °C for 15 min. The sequencing was done under the recommended conditions, using [α-^{35}S] deoxycytidine triphosphate (1000 Ci/mmol). The sequencing reaction was carried out at 48 °C for 30 min.

Results

We cloned normal and abnormal restriction fragments from the patient and prepared a restriction map. Exon 14 was deleted in the mutant allele [4]. Using these cloned fragments, a restriction map near the deleted joint was prepared (Fig. 1 a). To determine the boundary of the deletion in the mutant allele, we sequenced the BanII-SmaI fragment of the mutant allele and the corresponding region of the normal allele (BanII-SmaI and SmaI-SmaI, respectively). The sequence is shown in Figure 1 b. The sequence at the 5′ end of the deleted joint in the mutant allele was exactly the same as the sequence of the Alu element [19] in the normal intron 13 to the left of the point marked by vertical lines and designated B. The sequence at the 3′ end of the mutant allele was the same as the Alu sequence in intron 14 to the right of point A. Between points A and B, the sequence of mutant allele was the same as the Alu sequence of both introns. Thus, breakage and repair of the DNA strand occurred between points A and B.

As schematically shown in Figure 1 a, normal introns 13 and 14 contained the Alu sequence and were oriented in the same direction. The deletion occurred between the left arm of the Alu sequence in intron 13 and the left arm of the Alu sequence in intron 14.

Figure 2 shows Northern blot analyses of the mRNA isolated from EBV-transformed lymphocytes from a normal control subject, the proband and her parents. There is no gross difference in the level of mRNA after normalization to the actin signal. Multiple hybridization bands were detected but there was no difference in size of bands between the normal subject and the proband.

We then searched for abnormal mRNA from the mutant allele, using PCR. Primers YE81 and YE82 were derived from the sequence of exons 15 and 12. This primer set was expected to amplify the 542 base pair (bp) fragment in a normal subject. As the proband is a heterozygote of the mutant allele, amplification of cDNA was expected to produce two fragments if the deletion did not affect the splice junction, one fragment was 542 bp and the other was 382 bp which is 160 bp shorter than normal

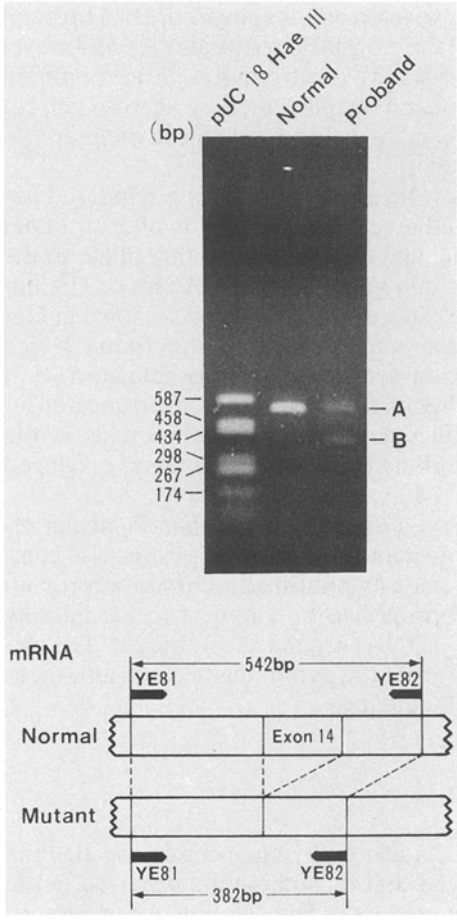

Fig. 3. Agarose gel electrophoresis of the product amplified by polymerase chain reaction. *Upper panel,* cDNA synthesized from a normal control subject and the proband's mRNA were amplified by polymerase chain reaction and separated in a 1.5% agarose gel, which was then stained with ethidium bromide. Restriction enzyme HaeIII digested plasmid pUC18 was also electrophoresed, as a size marker. *Lower panel,* Schematic representation of the amplified portion of the mRNA from normal allele and mutant allele

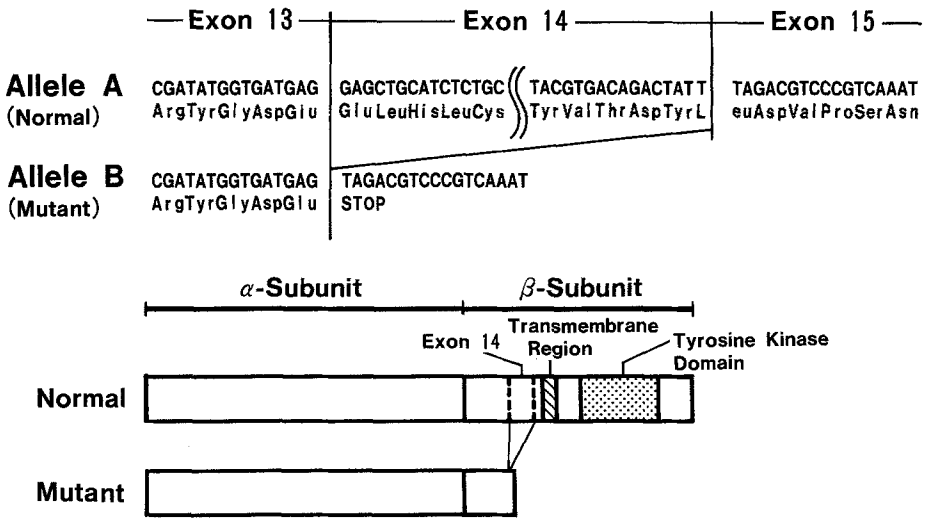

Fig. 4. Nucleotide and amino acid sequence caused by the deletion *(upper panel)* and schematic representation of the putative mutant insulin receptors *(lower panel)*

bands, as shown in Figure 3. Agarose gel electrophoresis of the product amplified by PCR is also shown in Figure 3. In the normal subject, the 542-bp band appeared, and in the proband, two expected bands appeared, a 542 bp band corresponding to the normal band (band A) and novel 382 bp band (band B). We electrophoresed the amplified product of the proband on a low melting agarose gel, cut these two bands from the gel and sequenced the preparations (data not shown).

The sequences from the normal 542 bp band and abnormal 382 bp band revealed that deletion of exon 14 did not affect the splicing pattern of the mutant allele so the same splice donor and acceptor sites were used. The nucleotide sequence caused by the deletion is shown in Figure 4. The deletion altered the reading frame which caused a termination codon (TAG) after Glu (GAG) at amino acid 867. Thus, in the present case, the truncated insulin receptor with deletion of 488 amino acids in the transmembrane and the cytoplasmic domain is produced (Fig. 4).

We then sequenced exons of the proband's insulin receptor gene. The nucleotide sequence of exons was compared with the previously published normal receptor sequence [1, 2] and proved to be identical to the initially reported normal cDNA sequence (Table 1). The sequence of exon 14 from the paternal allele was also identical to the normal sequence.

Discussion

Examination of the nucleotide sequence surrounding the deleted area showed that recombination occurred in the left arm of the Alu sequence. The deletion occurring with the recombination between two Alu elements was also noted for LDL receptors [20]. Sequencing of the PCR amplified product from cDNA revealed that the deletion resulted in a premature termination codon. Mutations which cause a premature translation termination are often associated with decreases in the amount of mRNA [13, 21, 22], yet can be associated with a normal level of mRNA [8]. While the deletion altered the reading frame and produced a premature termination codon, the level of transcription of mRNA was not reduced and the mRNA lacking exon 14 was stable.

Other investigators indicated that the sequence of introns near the splice site is important for a normal splicing pattern [23, 24]. In our patient, the 1.2-kb pair deletion conserved the intron exon junction (5′ end of intron 13 and 3′ end of intron 14) and had no untoward effect on the splicing site. The result was that the abnormal mRNA transcribed from the mutant allele was 160 bp (corresponding to the length of exon 14) shorter than that transcribed from the normal allele. Because of this slight difference, we detected no difference in mRNA size by Northern blotting.

As the termination codon is premature, truncated insulin receptors probably lack transmembrane and cytoplasmic domains. We found no truncated insulin receptors in culture medium from the EBV-transformed lymphocytes of the proband, following purification of the receptor using a wheat germ agglutinin column and cross-linkage with ^{125}I-insulin. Presumably the truncated insulin receptors are unstable [25]. However, even if the truncated insulin receptor was translated and stable, it is unlikely that such receptors would be functional or would locate on the cell surface [25, 26]. This mutation in the proband resulted in a decreased insulin binding to the cells [4].

Southern blotting of the DNA from the father of the proband showed a normal pattern, determined using restriction enzymes EcoRI, StuI, BglII, PvuII, BanII (data not shown). Thus, it is unlikely that the paternal allele of the insulin receptor gene contained a large deletion or insertion. The normal pattern on Northern blots, and PCR amplification of the cDNA indicated that transcription and splicing of the proband's insulin receptor mRNA is probably normal. The sequence of each exon of the proband was as normal except for exon 14 of the maternal allele which was deleted. There was no apparent additional mutation in the insulin receptor gene of the proband.

Insulin binding to erythrocytes, fibroblasts and EBV-transformed lymphocytes from the father was normal, but

Table 1. Comparison of insulin-receptor nucleotide differences in healthy subjects and the proband

Amino acid codon No.	Ebina et al. [1]	Ullrich et al. [2]	Proband
144	His (CAC)	Tyr (TAC)	Tyr (TAC)
276	Gln (CAA)	Gln (CAG)	Gln (CAG)
421	Thr (ACC)	Ile (ATC)	Ile (ATC)
465	Lys (AAG)	Gln (CAG)	Gln (CAG)
519	Asp (GAT)	Asp (GAC)	Asp (GAC)
523	Ala (GCG)	Ala (GCA)	Ala (GCG/GCA)
873	Val (GTC)	Asp (GAC)	Val (GTC)
874	Ser (TCC)	Thr (ACC)	Ser (TCC)
1058	His (CAC)	His (CAC)	His (CAC/CAT)[a]
1251	Lys (AAG)	Asn (AAC)	Lys (AAG)

[a] His-1058, CAT is reported by Moller et al. [9]

autophosphorylation and kinase activity of the insulin receptor were moderately decreased in the proband and in her parents [5]. Decrease in kinase activity and autophosphorylation events in the proband and her mother were presumably due to deletion of the insulin receptor gene [5]. While we have no explanation for the decrease in autophosphorylation and kinase activity of the father, this decrease was moderate, hence probably in the lower limit of the normal range. Other investigators have found a decrease in autophosphorylation and kinase activity in freshly isolated cells from a patient with Type 2 diabetes [27–30]. Thus, the existence of genetic or environmental factors which suppress the autophosphorylation and kinase activity of the insulin receptor may have a role.

Obesity may have contributed to the onset of diabetes in the proband because only the proband showed marked obesity. In addition to mutation of the insulin receptor gene, undetermined genetic factors related to the paternal allele may be linked to the diabetes, the father had mild impaired glucose tolerance and family history of diabetes [4, 5]. Our investigation suggests that the onset of diabetes in the proband relates to obesity and to genetic factors and is not solely caused by a mutation of the insulin receptor gene.

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