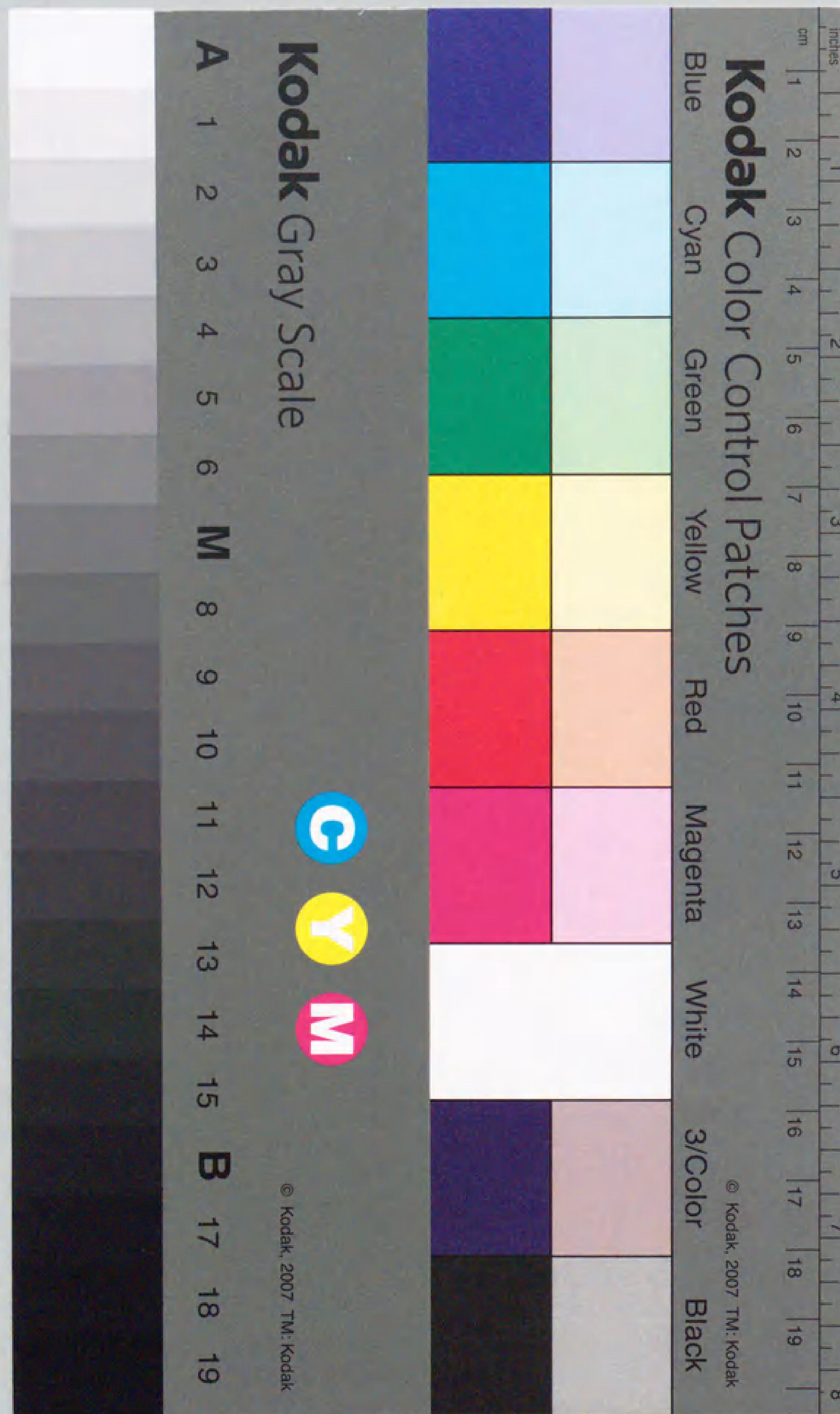

論　文　目　録

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主論文

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2）New Gaucher disease mutations in exon 10 : a novel L444R mutation produces a new *Nci*I site the same as L444P　1冊
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（主任：折 居 忠 夫）

Identification of a Nonsense Mutation in ALD Protein cDNA From a Patient with Adrenoleukodystrophy

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IDENTIFICATION OF A NONSENSE MUTATION IN ALD PROTEIN cDNA FROM A PATIENT WITH ADRENOLEUKODYSTROPHY

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SUMMARY : The molecular basis of X-linked adrenoleukodystrophy (ALD) was investigated. Six (A to F) fragments of cDNA for ALD protein (Mosser et al. Nature 361 : 726-730, 1993) from an adult patient with adrenomyeloneuropathy were amplified by PCR and mutations were screened by Mutation Detection Enhancement gel electrophoresis. A single base substitution (2154 C → T), which resulted in the formation of a termination codon for glutamine (Q590STOP) and deletes Pst I site (CTGCAG→CTGTAG), was detected. Eight other ALD patients did not have this mutation. A family study revealed the presence of both the mutant and normal alleles in the mother, a sister and a niece, indicating that these individuals were carriers. A nephew with childhood ALD who died 10 years earlier had the same mutant allele as detected by Pst I restriction assay. This report is the first description of a mutant allele for ALD, at the cDNA level, and presents confirmatory evidence of ALD protein as the primary etiology of ALD. © 1994 Academic Press, Inc.

Adrenoleukodystrophy (ALD) is an inborn error of saturated very long chain fatty acids (VLCFA), with X-linked recessive inheritance (1, 2). Patients with childhood ALD, the most common phenotype, manifest progressive neurologic deficits including visual disturbance, spastic paraplegia, dementia and adrenocortical insufficiency, and the average age at onset is 5 to 10 years. Extensive demyelination of white matter in the central nervous system leads to a vegetative state within a few years. Adrenomyeloneuropathy (AMN) is a typically adolescent-onset disease characterized by spastic paraparesis, subclinical peripheral neuropathy and variable degrees of adrenal insufficiency. There are both phenotypes in a pedigree.

The primary etiology of ALD has been considered to be related to dysfunction of lignoceroyl-CoA ligase, a peroxisomal enzyme (3). This defect leads to an accumulation of saturated VLCFA, including lignoceric acid (C24:0) and cerotic acid (C26:0) (4). However a putative gene for ALD, which was cloned by Mosser et al. (5), encodes a peroxisomal membrane protein (ALD protein) and shares unexpected homology with an ATP-binding cassette superfamily of transporters. Although some patients with ALD have a large deletion in this gene (5), point mutation in the cDNA has not been reported.

Abbreviations used in this paper : ALD, adrenoleukodystrophy; VLCFA, very long chain fatty acids; AMN, adrenomyeloneuropathy; PCR, polymerase chain reaction; MDE, mutation detection enhancement.

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We report here an exonic point mutation in ALD protein cDNA in a patient with AMN, which resulted in the formation of a termination codon for glutamine. A family study revealed that a nephew with childhood ALD had the same mutation, and that the mother, a sister and a niece were the carriers. This report is apparently the first description of a mutant allele for ALD at the cDNA level.

MATERIALS AND METHODS

Patients and cell lines. An adult patient with AMN (patient 1) and 8 patients with childhood ALD (patients 2 to 9) from 9 pedigrees, members of their families and control subjects were studied. All these subjects were Japanese living in Japan. Fibroblasts from these persons were cultured in Eagle's minimal essential medium supplemented with 10 % fetal calf serum.

Amplification of cDNA for ALD protein. Messenger RNA was extracted from cultured fibroblasts using Quick Prep mRNA Purification Kits (Pharmacia). cDNA for ALD protein was synthesized with the use of M-MLV reverse transcriptase (Gibco BRL) with antisense specific primers for ALD protein, designed based on the sequence of normal cDNA for ALD protein (5). Primer for fragments A and B: 5'-ACCACACGCGAGTGCATGTA-3' (1227-1246 base); primer for fragments C, D, E and F: 5'-GGGTGGGTGCTGCTGTCT-3' (2676-2693 base). Overlapping 6 fragments of cDNA for ALD protein (A, B, C, D, E and F-fragment) were amplified by 40 cycles of PCR (1 min at 94 ℃, 1 min at 60 ℃, and 2 min at 72 ℃), using 50 pmol of each primer, 2.5 units of Taq DNA polymerase (Wako), and a DNA thermal cycler (Perkin-Elmer Cetus). The primer sequences for each fragment are listed in Table 1.

Mutation Detection Enhancement (MDE) gel electrophoresis. 2.5 μl of PCR products for each patient and an equal volume from control subjects were mixed, denatured at 94℃ for 3 min, renatured gradually and subjected to MDE gel (AT biochem) electrophoresis at 700 volts for 20 hours, according to the manufacturer's instructions.

Sequence analysis of PCR amplified fragments. Amplified fragments, subcloned into pT7Blue T-vector (Novagen), were sequenced by the dideoxy chain termination method of Sanger et al. (6) with the use of a Sequenase Version 2.0 DNA Sequencing Kit (USB).

Pst I restriction assay. Ten μl of PCR products, purified by phenol / chloroform extraction and ethanol precipitation, were digested with 15 units of Pst I. The digested DNA was separated on 2 % agarose gel or 5 % acrylamide gel, and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Screening of exonic point mutation. Six fragments (A to F) of ALD protein cDNA from patient 1 could be synthesized using the primers listed in Table 1. Screening of exonic point mutation was performed by MDE gel electrophoresis of the amplified cDNA. To detect heteroduplex DNA consisting of mutated and normal fragments, PCR products of the patient and of an equal volume from controls were mixed and subjected to MDE gel electrophoresis. The slower moving heteroduplex DNA became visible (see Fig. 1, lane 1), when fragment E of patient 1 was mixed with that of control. No heteroduplex was detected in other fragments from patient 1 (data not shown). This observation suggests the presence of a point mutation in fragment E from patient 1. Heteroduplexes were also detected in 2 of 8 other patients (data not shown). Investigations are under way to clarify whether these mutations

Table 1. Primer sequences for PCR amplification of ALD protein cDNA

Fragment	Primer sequence
A (518bp)	S = 5'-[321]CCACCTGCCTCAACTGCTGC A = 3'-[818]ATGGAAGCAGTTGTCACGGTA
B (429bp)	S = 5'-[760]AGGACCCGCGGGCTTTTGGCT A = 3'-[1187]AAGAGCGGGTTCAAGCCCCTCG
C (389bp)	S = 5'-[1134]GTGGTGTTCCTCACGGCCAA A = 3'-[1508]TTCCTCCTCCTCGACCACTC
D (421bp)	S = 5'-[1481]GAAGAAGGCAGCCTTGGAAA A = 3'-[1882]TTCCGTACGTAGACGAGTAG
E (426 p)	S = 5'-[1831]GTGGTGGCCAGCCTCAACAT A = 3'-[2257]TCATGCGGGAGGACCTACTT
F (444bp)	S = 5'-[2229]ATGGCCCGCATGTTCTACCAC A = 3'-[2652]GGGGTTCGAGCCTAGTGTACT

S: sense (5' to 3'), A: antisense (3' to 5').

are pathogenic or simply indicate polymorphism. Some fragments were difficult to amplify, probably due to high GC content of the cDNA. Fragments D and E from all patients were amplified. No abnormally shorter or longer fragment was detected.

Identification of a point mutation in an AMN patient. When fragment E of patient 1 was sequenced, a single base substitution of T for C at position 2154 led to replacement of glutamine (CAG) at position 590 with a termination codon (CAG→TAG) (Fig. 2).

Analysis of the primary structure of ALD protein revealed that this protein consists of 745 amino acids and shares homology with the ATP-binding cassette superfamily of transporters, which include 70 KDa peroxisomal membrane protein (5, 7). This nonsense mutation creates the truncated ALD protein which lacks the second ATP-binding motif (^{617}Arg ∼ ^{630}Glu) and highly conserved region (^{662}Leu ∼ ^{676}Ala), thereby suggesting that the mutant ALD protein is non-functional.

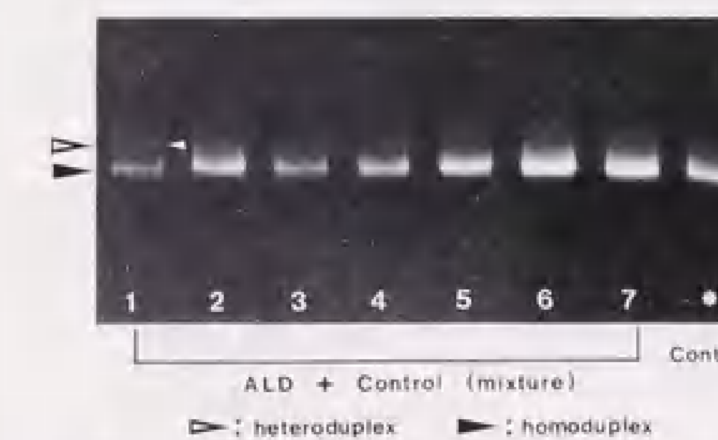

Figure 1. Screening of exonic point mutation by MDE gel electrophoresis. Amplified fragment E from each patient and an equal volume from controls were denatured at 94 ℃ for 3 min, renatured gradually and subjected to MDE gel electrophoresis. Lanes 1, 2, 3, 4, 5, 6 and 7 : patients 1, 2, 3, 4, 5, 6 and 7 + control, respectively; * : control. The slower moving heteroduplex DNA (open arrowhead) is visible in lane 1. Closed arrowhead indicates homoduplex DNA.

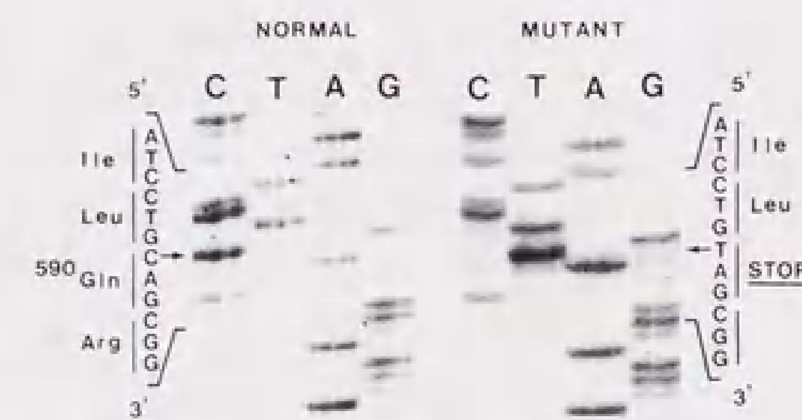

Figure 2. Partial nucleotide sequences of the normal and the mutant ALD protein cDNA (patient 1). A single base substitution of T for C at position 2154 resulted in replacement of Glutamine (CAG) at position 590 with stop codon (TAG).

Pst I restriction assay. The point mutation detected in cDNA of patient 1 has a deleted Pst I site. Amplified fragments E from 9 patients, including patient 1, were digested with Pst I and electrophoresed on a 2 % agarose gel. As shown in Fig. 3, the normal cDNA fragment was digested to 306 and 120 bp fragments, while the mutated cDNA was not digested. Thus only patient 1 had this nonsense mutation.

To investigate whether this mutation was present in the family members of patient 1, mRNA was extracted from either white blood cells or autopsied liver. Fragment E was amplified, digested with Pst I and electrophoresed (Fig. 4). The same mutation was detected in a nephew with childhood ALD and who died 10 years earlier, using mRNA from the autopsied liver (lane 2). This observation supports the hypothesis that both childhood ALD and AMN are caused by the same mutation; an unknown modifier gene may influence the clinical phenotype (8). The mother, a sister and a niece had both the mutant (426 bp) and normal (306 and 120 bp) alleles, thereby suggesting that these individuals are obligate carriers of ALD. Serum VLCFA levels were high in the mother and the sister, and were within normal range in the niece. DNA analysis in combination with conventional VLCFA analysis is essential for the precise diagnosis of a carrier and for genetic counseling of the family.

Figure 3. Pst I restriction analysis of the mutation site. Amplified fragment E was electrophoresed on a 2 % agarose gel after digestion with Pst I. The cDNA samples were derived from three controls (∗), patient 1 (lane 1) and eight patients with childhood ALD (lanes 2 to 9). Molecular size marker (Mr: 123 base ladder) was electrophoresed (left side). Normal cDNA fragment was digested to 306 and 120 bp fragments, and the mutated cDNA was not digested.

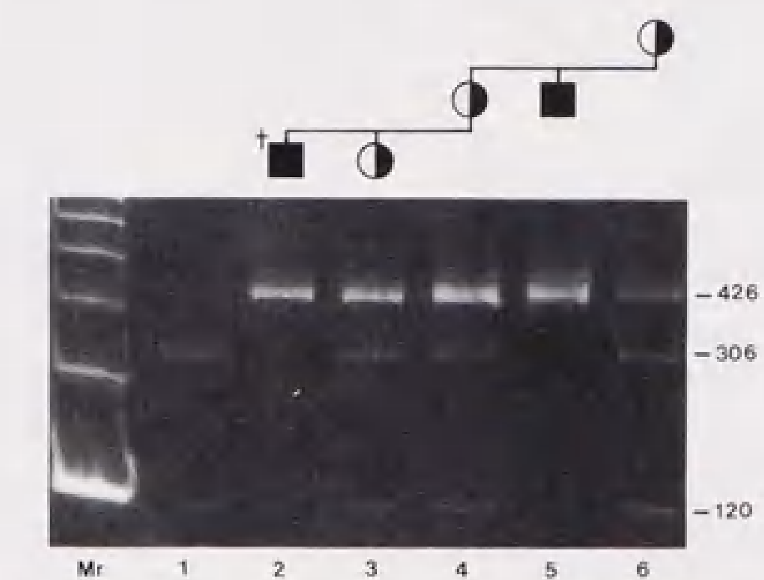

Figure 4. Family study of the mutant allele, for patient 1. Fragment E was amplified using mRNA from white blood cells or autopsied liver, digested with Pst I, and electrophoresed on a 5 % polyacrylamide gel. Lane 1: control, lane 2: nephew (childhood ALD), lane 3: niece, lane 4: sister, lane 5: patient 1, lane 6: mother. It is clear that patient 1 and his nephew are hemizygotes of the mutant allele and the mother, sister and niece are carriers of the mutant allele.

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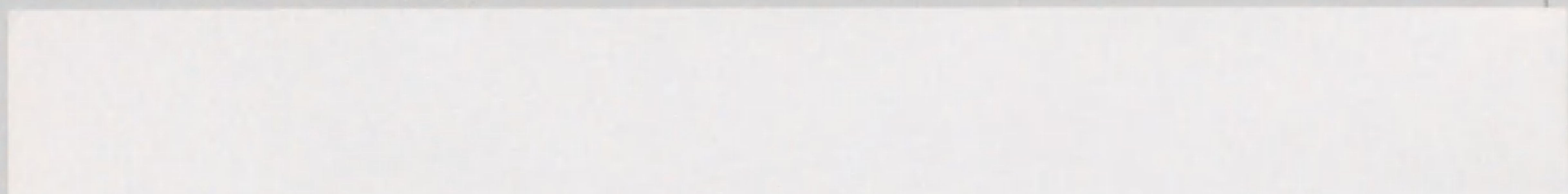

Human Molecular Genetics, 1994, Vol. 3, No. 7 1183–1184

New Gaucher disease mutations in exon 10: a novel L444R mutation produces a new *Nci*I site the same as L444P

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Gaucher disease, a lysosomal storage disease inherited as an autosomal recessive trait, is due to a deficiency of the enzyme glucocerebrosidase, which in turn causes accumulation of glucocerebroside (1). Gaucher disease is characterized by hepatosplenomegaly, bone involvement, and occasionally neurological symptom. According to the clinical findings, this disease is classified into three phenotypes, type 1 (non-neuronopathic form), type 2 (acute neuronopathic form), and type 3 (chronic neuronopathic form). Over 35 kinds of mutations in the coding region of the gene have been reported (2). A single base substitution (1448 T to C) in exon 10 of this gene is the most common mutation in the non-Jewish population (2,3,4), and results in the substitution of proline for leucine in position 444 (L444P). This T to C change produces a new *Nci*I cleavage site. Initially, it was suggested that the L444P mutation was tightly linked to the neuronopathic form (5) and that homozygosity of this mutation occurred exclusively in types 2 and 3. Later we found that homozygosity for the L444P was associated with the non-neuronopathic type in a Japanese teenager by Southern blot analysis of *Nci*I digests (6). Thus genotype/phenotype correlation for the L444P mutation is still unclear.

The patient in our present study was a Japanese female neonate with the most severe type 2 Gaucher disease. She was born at 37 weeks gestation and was delivered by caesarean section due to fetal distress. There was a severe asphyxia and the Apgar score was 1 at 1 minute. Respiration did not become voluntary. Despite intensive care, she died 9 hours after delivery. The diagnosis was made, based on the clinical findings of hepatomegaly, and on evidence of no activity of glucocerebrosidase in the liver at autopsy.

To identify mutations in glucocerebrosidase, genomic DNA was extracted from the white blood cells and autopsied liver by standard techniques (7). DNA across exons 9 and 10 was amplified by 40 cycles of PCR (1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C) using 50 pmol of each primer, 2.5 units of Taq DNA polymerase (Wako), and a DNA thermal cycler (Perkin-Elmer Cetus). The primers sequence were 5′-AA-CTTTGTCGACAGTCCCAT-3′ (forward: 5918–5937) and 5′-GCCCCCAACGCTGTCTTCAGC-3′ (reverse: 6521–6541). To avoid amplification of the pseudogene, the forward primer was designed from sequence specific to the expressed gene (8). Three microliter of the PCR products were digested with *Nci*I (Wako), followed by separation on 2% agarose gel. The size of the intact PCR product was 624 bp; in the presence of the L444P mutation the amplified samples generate fragments of 515 bp and 109 bp after *Nci*I digestion. As shown in Fig.1a, our patient (lane 1) seemed to be heterozygous for the L444P polymorphism as did many patients previously documented, because both the 624 bp and 515 bp fragments were found. The 109 bp fragment could not be visualized clearly on this percentage (2%) of agarose gel.

Mutation Detection Enhancement (MDE) gel (AT biochem) electrophoresis was performed using the same 624 bp PCR products for the detection of other possible mutations in addition to L444P. Five microliter of the amplified samples were denatured at 94°C for 3 minutes and slowly cooled to room temperature for reannealing. Each renatured sample was loaded on a MDE gel at 700 volts for 20 hours, according to the manufacturer's instructions. In this case, our patient (lane 1) showed a clearly different electrophoretic pattern from the L444P patient as shown in Fig.1b, suggesting the presence of different mutations from the L444P. PCR products from the patient and a normal control were subcloned into pT7Blue T-vector (Novagen) and were sequenced by dideoxy chain termination method (9) using Sequenase Version 2.0 DNA Sequencing Kits (USB). The result of sequencing indicated that the patient was a compound heterozygoute with two new mutations, one in each allele. One mutation was a 20 bp deletion with a 2 bp (TG) insertion starting from ^{444}Leu (1447–1466 del 20, ins TG) (Fig. 2). This is the first evidence for deletion/insertion mutation in Gaucher disease. This mutation is not produced from a fusion gene between a true and pseudogene (10), because the pseudogene do not show TG at the same position as the proposed insertions, and the sequences of the 20 bp deletion are absent in the

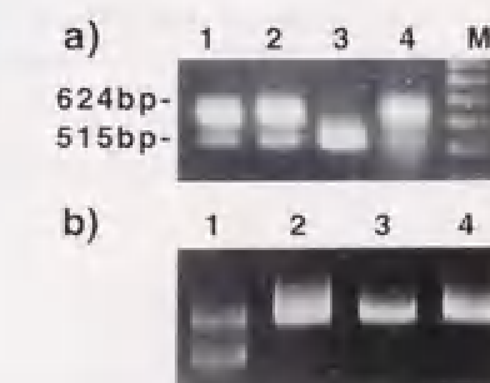

Figure 1. (a). *Nci*I restriction analysis for the detection of the L444P mutation. PCR amplified fragments digested with *Nci*I were separated on 2% agarose gel and were stained by ethidium bromide. The L444P mutation which creates a new *Nci*I site produces a fragments of 515 bp and 109 bp. Without the L444P, a fragment of 624 bp is observed. Lanes 1, 2, 3, 4, and M are as follows; the present case, a patient heterozygous for L444P, a patient homozygous for L444P, a normal control, and 123 bp ladder, respectively. The present case shows the same electrophoretic pattern as the patient in lane 2. **(b).** Mutational screening by MDE gel electrophoresis. Amplified fragments were denatured at 94°C, for 3 minutes, renatured gradually and loaded on MDE gel. Each lane 1 to 4 is the same as shown in Fig. 1a. Only lane 1 (present, case) shows a different electrophoretic pattern.

*To whom correspondence should be addressed

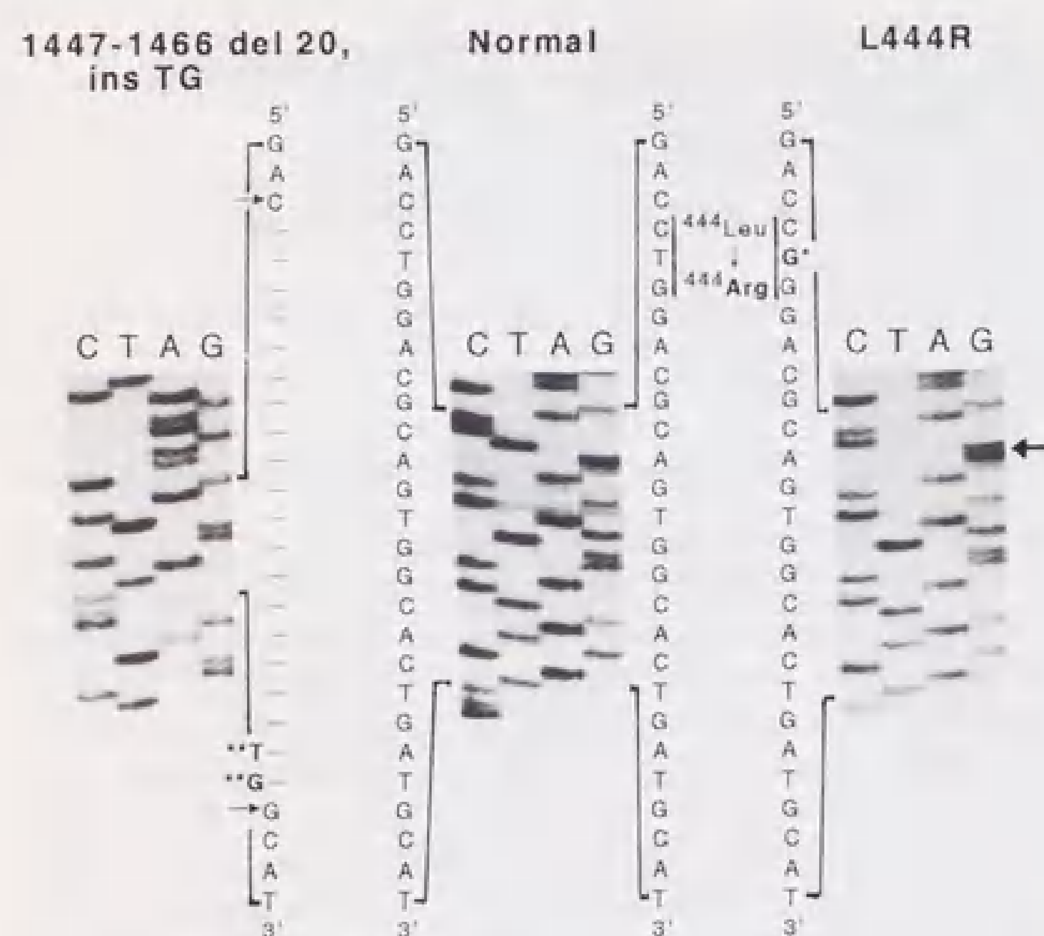

Figure 2. Autoradiograms of sequencing gels showing part of exon 10. The sequence at the center is a normal control. Either side is the two alleles of the present case. The allele designated 1447–1466 del 20, ins TG shows a 20 bp deletion with 2 bp insertion (TG). The allele designated L444R shows an exonic point mutation (1448 T to G) with arginine replacing leucine. '*', '**', '–', '→' denote in order, the mutational nucleotide for the L444R, orphan nucleotides (TG) as an insertion, deleted nucleotides (1447–1466), and the break point of the deletion. The bold arrow indicates a mutational site for the L444R on the autoradiograms.

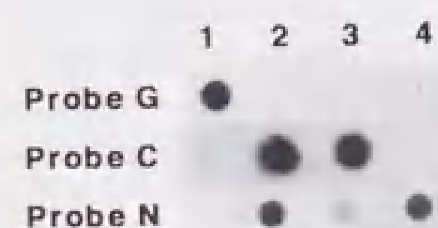

Figure 3. ASO analysis for the detection of L444P and the L444R. Probes G, C, and N were prepared for the detection of the L444R, the L444P, and a normal control, respectively. Lanes 1, 2, 3, and 4 show a present case, a heterozygote for the L444P, a homozygote for the L444P, and a normal control, respectively. The sample from the present case (lane 1) is the only one to hybridize with the probe G for the L444R.

pseudogene (8). This unique mutation must occur by another mechanism. The other new mutation at position 1448 which was not the common L444P but rather a L444R mutation. Namely a single base substitution (1448 T to G: ^{444}Leucine to Arginine) was found at the same position as the common mutational site (1448 T to C: ^{444}Leucine to Proline) (Fig. 2). It is notable that both the L444P and the L444R create a new *Nci*I site at the same position. As a result, the two mutations cannot be distinguished using *Nci*I polymorphism. Thus the utilization of only *Nci*I digestion for the screening of the L444P may result in a misdiagnosis at the genetic level, and some of the patients who have been diagnosed as L444P may have the L444R instead. A test to distinguish these two mutations precisely would provide gene frequencies and may clarify the phenotype/genotype correlation of the L444P mutation, which is poorly understood. In our present study the phenotypic contribution of the L444R cannot be evaluated because the patient is heterozygous.

To distinguish the L444P from the L444R, allele-specific oligonucleotide hybridization analysis was performed. Ten nanogram of each amplified sample was dotted and hybridized separately with the following appropriate mutant (L444R, L444P) and normal oligonucleotide probes; Probe G for the L444R: 5′-G-AACGACCGGGACGCAG-3′ (58°C), Probe C for the L444P: 5′-GAACGACCGGGACGCAG-3′ (58°C), and Probe N for a normal control: 5′-GAACGACCTGGACGCAG-3′ (56°C), respectively. The melting temperatures of each probe are given in parentheses. As shown in Fig. 3, only the sample from the present case (lane 1) hybridizes with the probe G. Samples from individuals heterozygous (lane 2) and homozygous (lane 3) for L444P did not. The sample from the homozygote L444P (lane 3) showed a weak signal with the probe N due to G to T mismatch. ASO was a powerful method to distinct between the two point mutations, but using this method, our patient seemed appears to be homozygote for the L444R. This is because the second allele carrying the deletion mutation, 1447–1466 del 20, ins TG could not hybridize with any of the oligonucleotide probes used in this analysis. However, it is easy to determine that she is a heterozygote for the L444R, if the result of MDE gel electrophoresis is taken into consideration. Therefore we strongly recommend for a precise molecular diagnosis that at least two appropriate mutation detection systems are used, and believe this proposal applies not only in Gaucher disease but in other genetic diseases.

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ABBREVIATIONS

MDE, mutation detection enhancement; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide hybridization.

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