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Genotypic analysis of families with lactate dehydrogenase A(M) deficiency by selective DNA amplification

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Summary. Genomic DNA prepared from LDH-A-deficient whole blood was amplified by the polymerase chain reaction technique using two primers specific for the active human LDH-A gene. The amplified fragment was examined by direct agarose gel electrophoresis, and a deletion of 20 base pairs (bp) in exon 6 of the LDH-A gene was found. The results permitted a clear distinction between the homozygous mutant, the heterozygous mutant, and wild-type genotypes. Moreover, *Hin*fI digestion and direct sequencing of the amplified product confirmed the results from direct agarose gel electrophoresis. Four families, including 18 individuals, were shown to contain the same mutation, that is a 20-bp deletion in exon 6. All genotypes were consistent with their biochemical phenotypes as evaluated by the ratio of LDH-B to LDH-A subunits in erythrocytes. Thus, all four known affected families in Japan have been shown to carry the same mutant gene, which may have been derived from a single mutational event.

Introduction

In human somatic tissues, five isozymes of tetrameric lactate dehydrogenase (LDH; E.C.1.1.1.27) are formed in vivo by the combination of the LDH-A (muscle) and LDH-B (heart) subunits. The expression of mammalian LDH-A and LDH-B genes is developmentally regulated and tissue-specific (Markert et al. 1975; Li 1989). Analysis of both human LDH-A and LDH-B genes shows that their protein-coding sequences are interrupted by six introns at homologous positions (Chung et al. 1985; Takano and Li 1989).

In humans, four patients with LDH-A deficiency have been reported (Kanno et al. 1980; Maekawa et al. 1984; Kanno et al. 1988; Kumasaka et al. 1989). The first patient was an 18-year-old Japanese man with exertional myoglobinuria and excessive fatigue (Kanno et al. 1980). The second patient was a 26-year-old woman, who was discovered by mass screening for an estimation of gene frequency (Maekawa et al. 1984), and her major complaint was uterine stiffness during pregnancy (Kanno et al. 1988). The third patient was a 23-year-old man who complained of fatiguability and who later had renal failure (Kanno et al. 1988). The fourth patient was a 61-year-old man who was admitted for general fatigue (Kumasaka et al. 1989). Recently, we attempted a molecular characterization of the genetic mutation in the second patient, and a deletion of 20 base pairs (bp) in exon 6 of the LDH-A gene was found (Maekawa et al. 1990). Here we report the use of the polymerase chain reaction (PCR) technique both to identify the 20-bp deletion in the LDH-A gene of 18 members from four families with LDH-A deficiency and to analyze the relationship between genotypes and phenotypes.

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Materials and methods

Subjects

Homozygous and heterozygous individuals were detected according to our described criteria (ratio of LDH-B to LDH-A subunits in erythrocytes; Maekawa et al. 1984). Genomic DNA was extracted, according to a modification of the procedure of Kunkel et al. (1977), from whole blood of family members comprising homozygotes and heterozygotes with LDH-A deficiency as well as those with a normal phenotype.

Oligonucleotide primers

To enzymatically amplify exon 6 of the LDH-A gene, two oligonucleotide primers were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, CA) and were purified using high-performance liquid chromatography. One of them was 5′-CTAGC

ATACATGTGTGCAAGGG-3', which is located 150 nucleotides 5' of exon 6 (primer 1), and the other was 5'-ATGCAGCGTATC ACTACATAGACC-3', which is located 27 nucleotides 3' of exon 6 (primer 2).

Amplification of the LDH-A gene DNA

We amplified the DNA fragment containing exon 6 of the LDH-A gene using a protocol based on a procedure described elsewhere (Saiki et al. 1988). Briefly, 100 µl of reaction mixture containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 10 mg/l gelatin, 200 µmol/l each of dNTPs, oligonucleotide primers 1 and 2 (50 pmol), 2.5 units *Taq* polymerase and 1 µl of DNA extracted from peripheral blood were maintained at 94°C (0.5 min), 58°C (0.8 min), and 72°C (1 min) for 30 cycles.

Identification of the mutant gene

The amplified products were analyzed by direct agarose gel electrophoresis using 2% NuSieve GTG agarose + 1% regular agarose (FMC, Rockland, Me.). The amplified products were confirmed by restriction enzyme digestion with *Hin*fI.

Sequencing of the amplified products

Single-stranded DNA template for direct sequencing was prepared from 1 µl of the amplified double-stranded DNA by the second PCR using primer 2. The oligonucleotides and excess dNTPs were removed by spin dialysis (Centricon 100 microconcentrators; Amicon, Danvers, Mass.). The nucleotide sequences of the DNA templates were determined by the dideoxy chain-termination method with a sequencing protocol using T7 DNA polymerase and deoxyadenosine 5'-(α-^{35}S thio) triphosphate (Gyllenstein and Erlich 1988). The dideoxy sequencing was performed with pimer 1 by using the Sequenase DNA sequencing kit (United States Biochemical Co., Cleveland, Ohio)

Results

Detection of the mutant gene

Figure 1 shows the theoretical scheme of the present study. With the amplification primers used, specific bands of 301 bp and 281 bp were expected from the exon 6 regions of wild and mutant genotypes, respectively. When the amplified DNA fragments were electrophoresed on 2% NuSieve GTG agarose plus 1% regular agarose gels, two bands of 301 bp and 281 bp were easily distinguishable. Moreover, 198 bp and 215 bp fragments were distinguishable when the amplified DNA fragments were digested by *Hin*fI and electrophoresed on the same gel to confirm the absence of one of the two *Hin*fI cleavage sites.

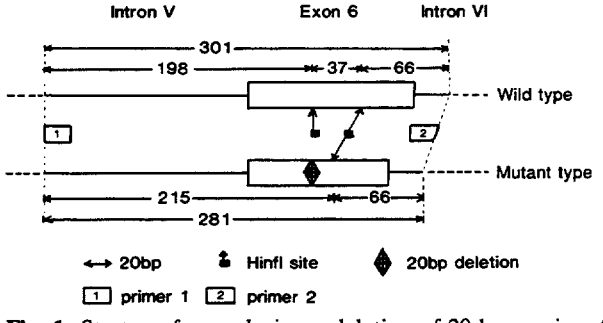

Fig. 1. Strategy for analyzing a deletion of 20 base pairs. A segment of the gene that includes the deletion of 20 bp was amplified by PCR using one pair of allele-specific primers (1, 2). For the wild-type allele, two *Hin*fI recognition sites are present, while the 20-bp deletion in the mutant allele eliminates one of these sites. Thus, a difference in the fragment length between the wild-type allele and the mutant-type allele are observed in both the amplified products and the *Hin*fI-digested fragments

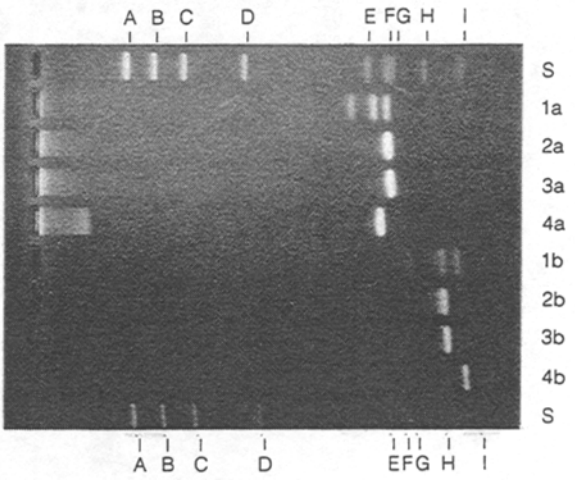

Fig. 2. *Typical electrophoretic pattern of LDH-A deficiency. Genomic DNA was amplified and digested by Hin*fI, as described in the text, and the amplified products were electrophoresed on 2% NuSieve GTG agarose + 1% regular agarose for 2 h at 140 V. *Lane S, Hae*III-digested X174 DNA size marker (*A,* 1353 bp; *B,* 1078 bp; *C,* 872 bp; *D,* 603 bp; *E,* 310 bp; *F,* 271 bp; *G,* 281 bp; *H,* 234 bp; *I,* 194 bp). *Lane 1,* heterozygous individual (Maekawa et al. 1984) for 20-bp deletion. *Lanes 2, 3,* homozygous individual (Maekawa et al. 1984) for 20-bp deletion. *Lane 4,* normal individual; *a* denotes non-digested sample, and *b* denotes *Hin*fI-digested samples

By using this procedure, we examined the genotype of the LDH-A deficient patient described by Maekawa et al. (1984). Figure 2 shows the ethidium bromide-stained gel following electrophoresis. When wild-type DNA was amplified, a predicted 301 bp band, smaller than band E of the molecular size markers, was visualized on an agarose gel by ethidium bromide staining (lane 4a). On the other hand, in mutant-type DNA, a predicted 281-bp band, which is smaller than the one in lane 4a and has a similar length to bands F and G of the molecular size marker, is visualized (lanes 2a, 3a). The difference in DNA fragment length, between 301 bp and 281 bp, is easily detected. In heterozygotes, both wild-type and mutant-type DNA are equally amplified by PCR (lane 1a). The fragment lengths of 301 bp and 281 bp in the agarose gel are compatible with the expectations outlined in Fig. 1. Because the extra band, which migrates more slowly than the 301 bp band, was suspected to be heteroduplex DNA (Nagamine et al. 1989), it was isolated from the gel, denatured at 94°C, renatured at 58°C, and then re-run on agarose gel electrophoresis. The predicted 301-bp and 281-bp bands were regenerated, and the extra band was identified to be a hetero-

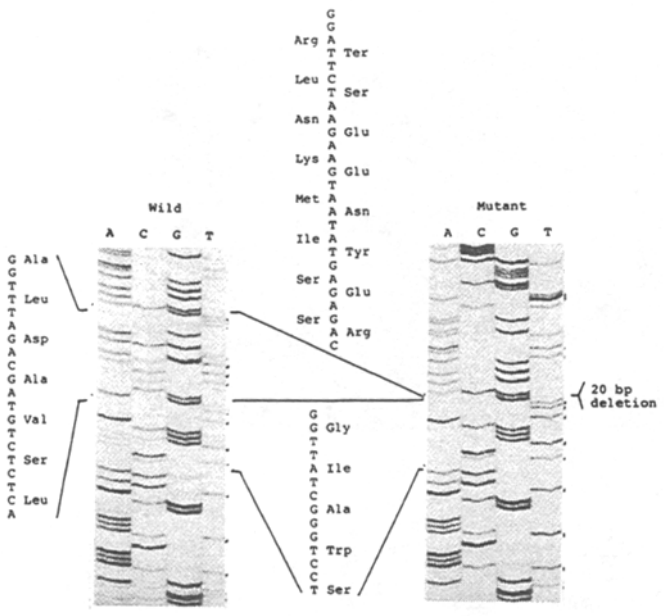

Fig. 3. Representation of the characteristic sequence differences between the wild-type allele and the mutant-type allele. Single-stranded DNA from the second LDH-A deficient patient was amplified and sequenced. The wild-type sequence is shown on the left, and the mutant-type sequence on the right. In the mutant-type gene, the deletion of 20 bp results in a frameshift translation and premature termination *(Ter)* as indicated. Moreover, this individual is homozygous for the mutant allele

duplex of both the 301-bp and the 281-bp fragments (data not shown).

The disappearance of one of the two *Hin*fI cleavage sites in mutant-type DNA was confirmed by digestion of the amplified DNA with *Hin*fI. The disappearance of a *Hin*fI cleavage site results in a 215-bp fragment. As shown in Fig. 2, the smaller 198-bp fragment is visible in the normal control (lane 4b), while the larger 215-bp fragment is observed in the homozygous LDH-A deficiency (lanes 2b, 3b). In the heterozygote both fragments are present (lane 1b).

Confirmation by direct sequencing

We determined the nucleotide sequences of the amplified exon 6 by direct sequencing. The 20-bp deletion in exon 6 of LDH-A gene was observed previously (Maekawa et al. 1990) in the LDH-A-deficient patient reported by Maekawa et al. (1984); this patient was also confirmed to be a homozygote for identical mutant alleles by direct sequencing of PCR-amplified genomic DNA (Fig. 3). Thus, the deletion of 20 bp in exon 6 most certainly exists in this patient.

Correlation between genotypes and phenotypes

Genomic DNA samples from the propositus and family members were independently amplified by PCR and analyzed by direct agarose gel electrophoresis. Figure 4 shows the agarose gel stained with ethidium bromide.

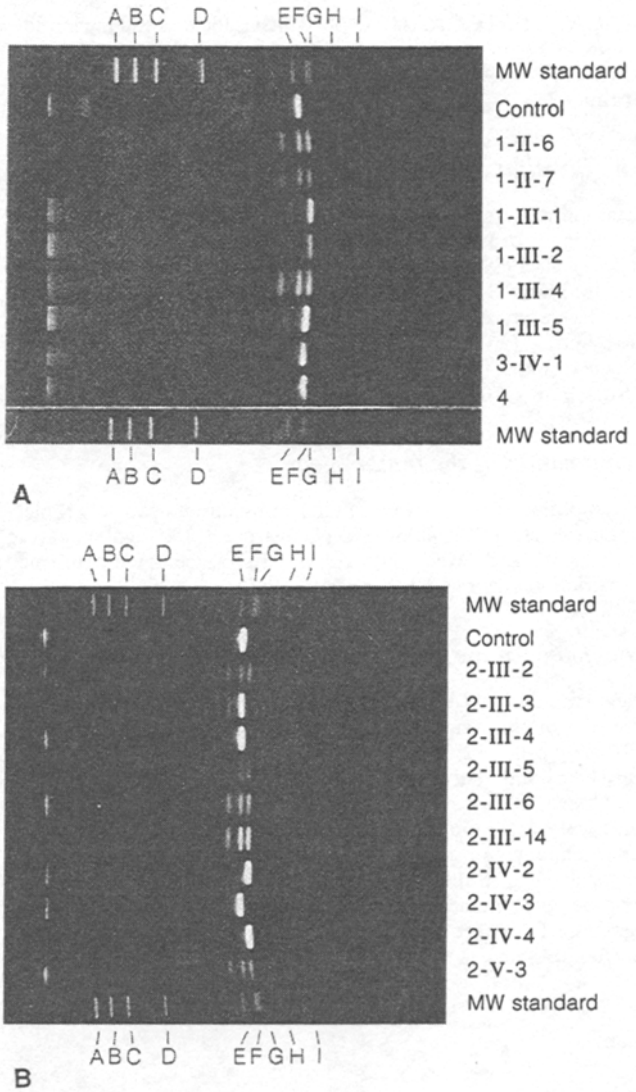

Fig. 4A, B. Use of PCR and agarose gel electrophoresis to detect a deletion of 20 bp in an LDH-A deficiency. DNA was amplified as described in the text, and the amplified products were electrophoresed on 2% NuSieve GTG agarose +1% regular agarose for 2 h at 140 V. The molecular weight *(MW)* standard was a *Hae*III-digested X174 DNA size marker (sizes given in legend to Fig. 2). Control DNA was derived from an individual unrelated to the LDH-A deficiency families. Each individual is indicated by Arabic number – roman – arabic numeral, specifying the family number, the generation in each pedigree, and the individual in the family, respectively (see Fig. 5)

Propositi of four families with LDH-A deficiency all had an identical 281-bp band (Fig. 4A, lanes 1-III-5, 3-IV-1, 4; Fig. 4B, lane 2-IV-2). Thus, all of them are identified as homozygotes of the same mutant allele which has a deletion of 20 bp in exon 6. Moreover, the DNA of both parents and some of the siblings of the first and second families were heterozygotes having both a 301-bp band and a 281-bp band (Fig. 4A, lanes 1-II-6, 1-II-7, 1-III-4; Fig. 4B, lanes 2-III-2, 2-III-5, 2-III-6, 2-III-14, 2-V-3). Some of the homozygotes and heterozygotes have been confirmed by *Hin*fI digestion, while homozygous propositi of LDH-A deficient families have been confirmed

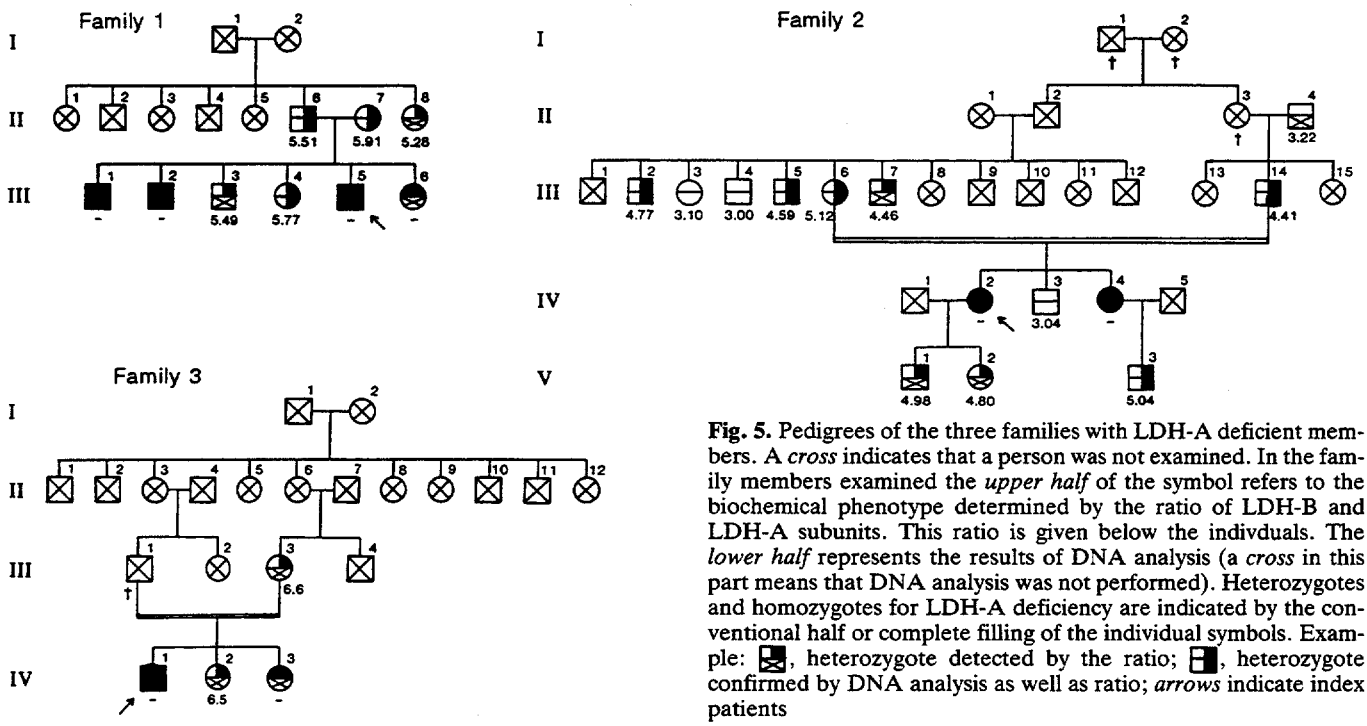

Fig. 5. Pedigrees of the three families with LDH-A deficient members. A *cross* indicates that a person was not examined. In the family members examined the *upper half* of the symbol refers to the biochemical phenotype determined by the ratio of LDH-B and LDH-A subunits. This ratio is given below the indivduals. The *lower half* represents the results of DNA analysis (a *cross* in this part means that DNA analysis was not performed). Heterozygotes and homozygotes for LDH-A deficiency are indicated by the conventional half or complete filling of the individual symbols. Example: ◪, heterozygote detected by the ratio; ◨, heterozygote confirmed by DNA analysis as well as ratio; *arrows* indicate index patients

by direct sequencing (data not shown). On the other hand, some of the sibs of the second case had only the 301-bp band and were homozygous for the wild-type allele (Fig. 4B, lanes 2-III-3, 2-III-4, 2-IV-3).

Discussion

We have previously detected a deletion of 20 bp in exon 6 of the LDH-A gene in a female patient (the second LDH-A-deficient patient) detected by M13 subcloning and DNA sequencing (Maekawa et al. 1990). In the present investigation, we used PCR to amplify genomic DNA from the second patient for gel electrophoresis and direct DNA sequencing, and have confirmed that the patient is homozygous for a mutation consisting of a deletion of 20 bp in exon 6. It is very interesting that the other three patients found in Japan are also homozygous for the same mutant allele.

The biochemical phenotypes in three of the families (Fig. 5) were identified by analysis of the ratio of LDH-B to LDH-A subunits in erythrocytes, as described previously (Maekawa et al. 1984). It is evident that the biochemical phenotypes and the respective genotypes of the individuals analyzed here correspond completely. Thus, LDH-A deficiency is closely linked with the 20-bp deletion in exon 6, and the mutant allele is distributed according to Mendelian segregation.

In each of the second and the third families there is a first-cousin marriage. Thus, it is possible that they are homozygous for an identical mutant allele. However, in the first family no evidence for a consanguineous marriage was found among six ancestral generations; in the fourth family no analysis has been performed. It was somewhat surprising that the mutation in each of the four families is identical, since mutations causing heritable disorders are frequently heterogeneous. However, there are disorders that are caused by a single mutation. For example, adenine phosphoribosyl transferase (APRT) deficiency, which is the cause of urolithiasis, is widely distributed in Japan (Japanese-type deficiency) and is derived from only one mutation (Kamatani et al. 1989). LDH-A deficiency has hitherto been reported only in individuals of Japanese origin. Thus, it is possible that the 20-bp deletion in the LDH-A gene is derived from a single ancestral mutation, in a manner similar to that of the Japanese-type of APRT deficiency.

LDH-A (muscle) deficiency has been classified as a type of hereditary myopathy (Kanno et al. 1988). The propositi of the first and third families have a history of pigmenturia (myoglobinuria) following strenuous exercise. However, the propositus in the second family is not good at sports and has no history of pigmenturia. Thus, the four patients show different clinical expression, even though they have the same mutant allele. The diversity in clinical symptoms is possibly the result of environmental differences.

By the present detection procedure, the PCR products of the wild-type and mutant alleles are clearly distinguishable. In addition, heterozygotes are also distinguishable from homozygous individuals with respect to the wild-type or mutant allele. Thus, DNA amplification and agarose gel electrophoresis provides an easy and convenient method for identifying such mutations.

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