Identification of a Missense Mutation (LDH-H : R171P) in a Lactate Dehydrogenase Deficiency Patient Using the Reverse Transcription-Polymerase Chain Reaction

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Accepted for publication on May 27, 1999

ABSTRACT. A complete deficiency of lactate dehydrogenase subunit H was identified in a 41-year-old female with paralysis of her left lower limb. The propositus had extremely low LDH activity and five of her family members had from lower to normal level of LDH activity. DNA sequence analysis of the propositus identified a G→C transversion mutation at codon 171 from CGC (Arg) to CCC (Pro). PCR-primer introduced restriction analysis (PCR-PIRA) using a specific mismatched primer was employed to introduce a new *Nru*I restriction site into the PCR product. A family analysis by digestion with *Nru*I disclosed that her parents, younger sister and two daughters had the same mutation. This mutation, R171P, was newly discovered in Japan.

Key words: lactate dehydrogenase — missense mutation — RT-PCR

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the oxidation of pyruvate to lactate in the final step of the glycolytic pathway. This enzyme is formed as a tetramer by combination of two different subunits, M (muscle, A) and H (heart, B).

These subunits give rise to five different isozymes of the tetrameric molecule, $LDH_1(H_4)$, $LDH_2(H_3M)$, $LDH_3(H_2M_2)$, $LDH_4(HM_3)$ and $LDH_5(M_4)$. The first case of a complete deficiency of LDH-H in a male with slight diabetes was reported as a phenotype by Kitamura *et al.*[1] Thereafter several similar cases of LDH-H subunit deficiency were found out.[2,3] Since the entire coding region of the LDH-H gene was reported by Takano *et al*,[4] nine genetic variants of LDH-H deficiency have been found in the Japanese population, LDH-H : A35E,[5] LDH-H : 103 (8 bp, dup),[6] LDH-H : S131R,[7] LDH-H : 139 (2 bp, dup),[8] LDH-H : 145 (4 bp, dup),[6] LDH-H : Y147Term,[8] LDH-H : F172V,[5] LDH-H : R173H[9] and, LDH-H : M176L.[5] All these variants are characterized by a deficient or unstable type of LDH-H. In this paper, we report a new case of a missense mutation of the deficient type of LDH-H.

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MATERIALS AND METHODS

Case report

The propositus was a 41-year-old female who visited Yamaguchi Prefectural Central Hospital because of paralysis of her left lower limb. A physical examination was normal, and a routine laboratory examination showed no abnormalities except for extremely low serum LDH activity, 40 IU (normal range, 200-400 IU). Her parents had a consanguineuos marriage. LDH studies carried out on her family members led to the suspicion that her parents, younger sister and two daughters might have the LDH deficiency.

LDH activity and isozyme analysis

LDH activity was measured by the modified spectrophotometric monitoring method of Wroblewski *et al*.[10]

Erythrocytes, leucocytes and platelets were prepared by the method described by Kamada *et al*.[3] The LDH isozymes were electrophoretically identified by the modified method of Shioya *et al*,[11] using a cellogel membrane and an LDH reagent kit (LDH Isozyme Test, Wako Chemical, Tokyo, Japan) according to the manufacturer's instructions.

Total RNA extraction and reverse-transcription-PCR amplification (RT-PCR)

Total RNA from whole blood was prepared using catrimox-14™ surfactant (Takara, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was generated from total RNA with a 1st-strand™-cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) with Molony murine leukemia virus reverse transcriptase (RT) and a random primer. Thirty cycles of polymerase chain reaction (PCR) amplification of cDNA consisting of denaturation at 90°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1.5 min with a sense primer 5'-CCGCACGACTGTTACAGA-3', and an antisense primer, 5'-TGTTCAAGAGCTCAGATTGC-3' were performed. One-tenth of the first PCR product was subjected to nested PCR amplification under the same conditions as those of the first PCR with a different primer set; that is, a sense primer of 5'-CGACTGTTACAGAGG-3' and an antisense primer of 5'-AAGCATTAAACCAAG-3'. The purified PCR product was directly sequenced by a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and a DNA sequencer (model 373; Applied Biosystems, Foster City, CA, USA).

Genomic DNA analysis

Genomic DNA extraction from white blood cells was performed according to the standard procedures.[12] PCR amplification of exon4 in the LDH gene was carried out according to the method of McGuire *et al*,[13] using a primer set consisting of a sense 5'-AGTGGACATTCTTACGTATG-3' and an antisense 5'-CATCCATGGCA-GCTG-3'.

PCR-primer introduced restriction analysis (PCR-PIRA)

Genomic DNA was amplified by the PCR method with a specific mismatched primer under the same conditions as those used with the DNA

amplification method except for the primers. The two oligonucleotide primers used were : sense 5'- AGTGGACATTCTTACGTATG - 3', which binds to the first position in exon 4 (nucleotide 420 to 439), and antisense 5'- TGAATGCCAAGTTTTTCAGCCATAAGGTCG - 3', which binds to the coding base in exon 4 (nucleotide 545 to 516) and introduces a mismatch single base (A→C) at its penultimate position (asterisk) from the 3' end.

RESULTS

LDH activity

The LDH activity of the family members is shown in Table 1. The propositus showed extremely low LDH activity. The LDH activity of five family members ranged from lower to normal levels. The family tree is shown in Fig 1.

LDH isozyme analysis

The zymogram of LDH isozymes from the propositus samples (serum,

TABLE 1. Lactate dehydrogenase activity and H/M ratio in sera and erythrocytes of the propositus and her family members.

	Serum		Erythrocytes	
Subject	LDH Activity	H/M	LDH Activity	H/M
I-1 Father	202	1.0	__a	—
I-2 Mother	199	1.0	__a	—
II-1 Propositus	40	—	11	—
II-2 Sister	236	1.0	160	1.9
III-1 Daughter-1	143	1.1	120	2.1
III-2 Daughter-2	210	1.0	__a	—
Normal range	200-400 IU/I	1.5-2.5	166-203 U/gHb	2.8-4.4

a : not examined

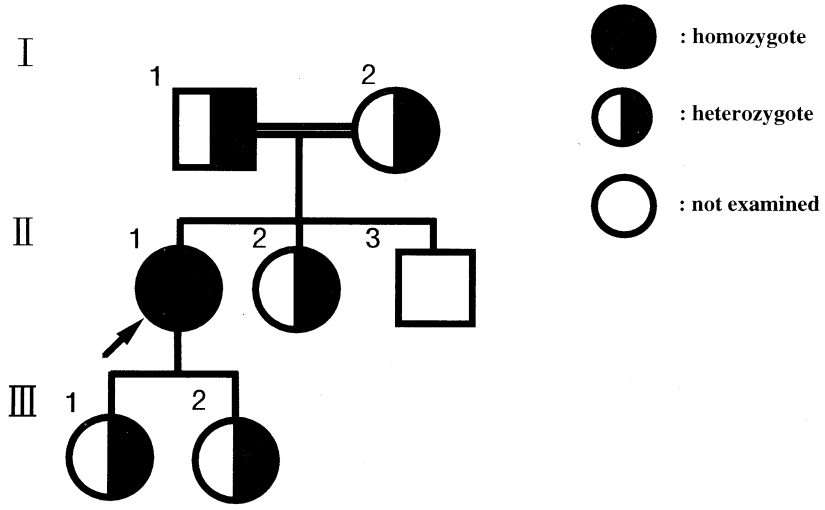

Fig 1. Family tree of the propositus. The arrow indicates the propositus.

leucocytes, erythrocytes, platelets and saliva) showed only one band of LDH_5 (M_4), as shown in Fig 2. These results indicated that the propositus is a homozygote with complete deficiency of LDH subunit H. Although five isozymes could be found in sera of her five family members, the distribution of the LDH activity was different from a normal subject (data not shown).

DNA sequence of a mutant cDNA

The entire coding region of the cDNA of the LDH-H from the propositus was amplified and sequenced. Sequence analysis verified that the LDH-H gene of the propositus (II-1) had a G→C transversion mutation in nucleotide 512, which converted codon 171 from CGC (Arg) to CCC (Pro), resulting in the homozygous state of this mutation, as shown in Fig 3. The propositus's mother (I-2) had two bases, G and C, at nucleotide 512, indicating she is heterozygous. The control subject showed one base, G only, at nucleotide 512, which is seen in the normal LDH-H DNA sequence. This mutation was

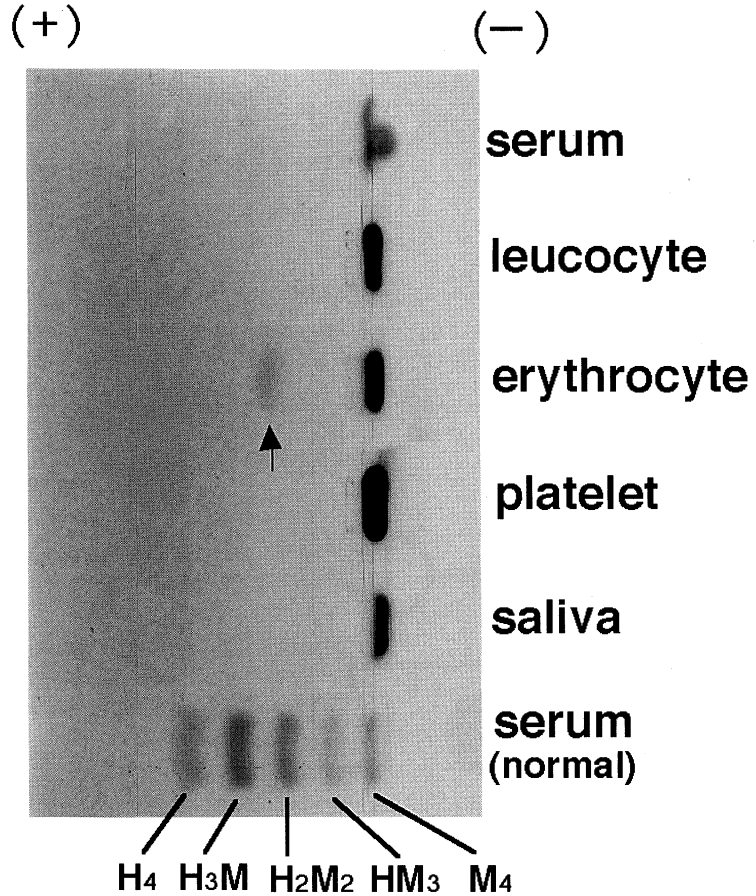

Fig 2. Zymograms of the LDH isozyme obtained from serum, reticulocytes, erythrocytes, platelets and saliva of the propositus and from the serum of a normal subject. Arrow showes the position of hemoglobin in the hemolysate.

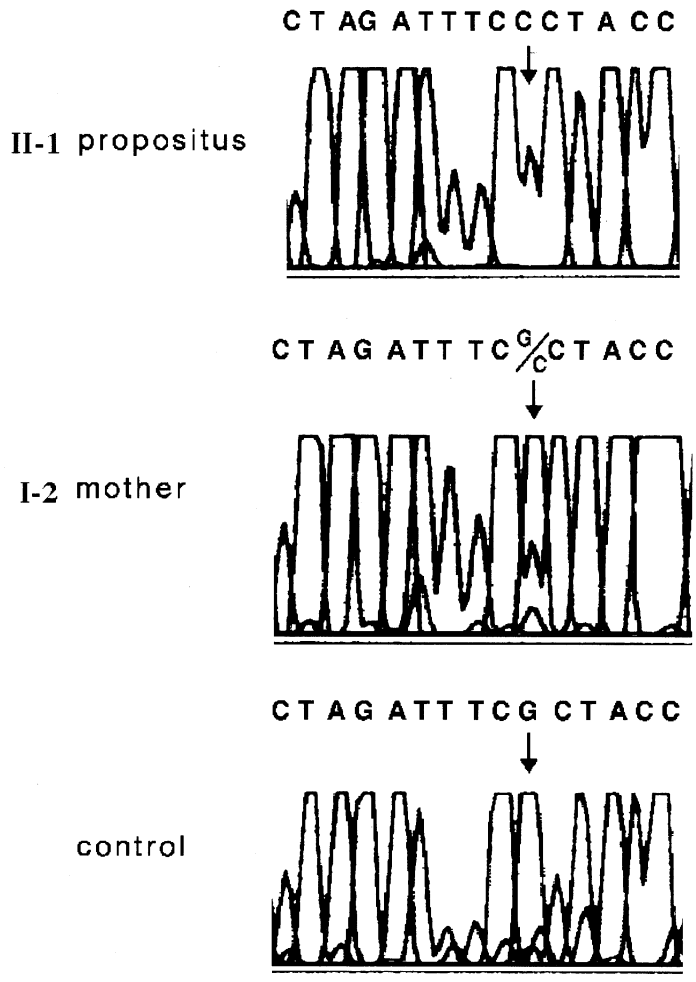

Fig 3. Sequence analysis of amplified cDNA.
The propositus (II-1) is homozygous for this substitution CGC→CCC at codon 171 (Arg→Pro). Her mother (I-2) is heterozygous for the same mutation (G and C). The normal control is shown at the bottom.

confirmed by direct sequencing analysis from the PCR product of the genomic DNA of the propositus (data not shown).

PCR-PIRA

A PCR product of 126 bp derived by the mismatched PCR method from the normal subject was completely digested into two fragments of 96 bp and 30 bp with *Nru*I. On the other hand, the PCR product of this mutation from the homozygote (II-1) was found to be resistant to *Nru*I digestion and that of the propositus's mother (I-2), showed two fragments of 126 bp and 96 bp on agarose gel, as shown in Fig 4.

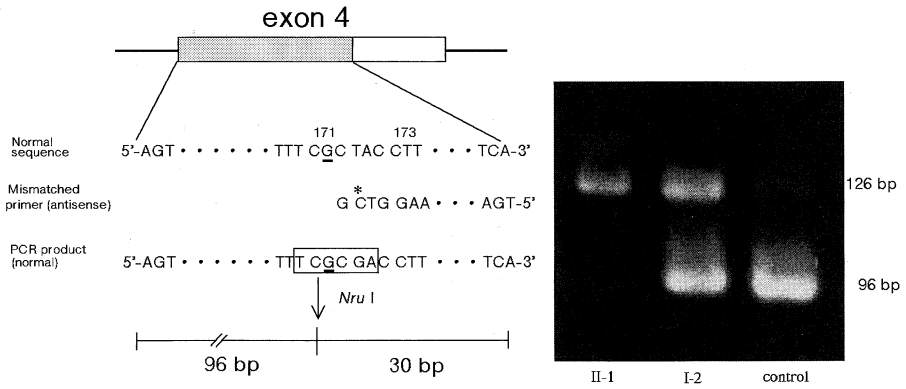

Fig 4. Scheme for detection of Arg 171 Pro mutation of the LDH-H variant. The specific primer is composed of 30 mer and contains a mismatched single base (A →C) at the second position from the 3' end (asterisk). A normal PCR product of 126 bp derived from the normal subject at codon 171 (under bar) has a new *Nru*I restriction site (square box) and has been digested into two fragments of 30 bp and 96 bp with *Nru*I.
Left: separation of *Nru*I restriction fragments of two individuals (I-2 and II-1) on 4% agarose gel.

DISCUSSION

The zymogram of the propositus with complete deficiency of LDH subunit H showed only one band of M_4 isozyme and zymograms of her family members indicated patterns apparently different from that of the normal subject. The H/M ratio in serum LDH determined by calculation of the zymograms of the family members was found to be 1.0~1.1, approxymately half that in the normal subject (1.5~2.5). The H/M ratio for erythrocyte LDH showed the same result as that for the serum LDH, as shown in table 1. These results indicate that the propositus is homozygous for the mutant allele, while her family members are heterozygous, as shown in Fig 1.

The importance of the motif (^{168}DSARFRYL^{175}) containing Arg at residue 171 in LDH-H is shown by its almost absolute conservation among six vertebrates,[4,14,15,16,17,18] as shown in Fig 5.

				171				
human H	D	S	A	R	F	R	Y	L
mouse H	D	S	A	R	F	R	Y	L
pig H	D	S	A	R	F	R	Y	L
human M	D	S	A	R	F	R	Y	L
mouse M	D	S	A	R	F	R	Y	L
dogfish M	D	S	A	R	F	R	Y	L

The underlined residue represents the affected position

Fig 5. Amino acid sequences of LDH-heart (H) and LDH-muscle (M) subunits of LDH in the vicinity of amino acid position 171.

These results suggest that the amino acid Arg at residue 171 is essential to the function of the LDH molecule.

According to Grau *et al*[19] and Clarke *et al*,[20] when pyruvate converts to

lactate in the active center pocket of LDH-H, an enzyme-pyruvate-reduced nicotinamide adenine dinucleotide (NADH) complex is created ; that is, the carbonyl oxygen of pyruvate interacts with the guanidinium group of Arg 109 and with the imidazole group of His 195, and the carbonyl carbon (C-2) of the pyruvate is linked to the hydrogen at C-4 of the NADH nicotinamide ring. Simultaneously, the carboxyl group of pyruvate forms an important salt bond with the guanidinium group of Arg 171 to stabilize the pyruvate in the active center. Therefore, the substitution of Pro for Arg at position 171 may prevent the formation of the salt bridge between pyruvate and amino acid 171 and lessen the stability of the pyruvate. Consequently, the zymogram for the propositus's LDH isozyme showed only one band of M_4 protein. This is the first case of hereditary serum LDH-H deficiency with a point mutation at codon 171.

This work was supported in part by a Research Project Grant (No. 9-114, No. 10-103) from Kawasaki Medical School. We thank Dr. Shiro Miwa (Okinaka Memorial Institute for Medical Research, Tokyo) for the measurment of enzyme activity in blood cells.

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