奈医誌. (J. Nara Med. Ass.) **47**, 354～360, 1996

FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS : MUTANT CU/ZN SUPEROXIDE DISMUTASE

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Received October 11, 1996

Abstract : Six Japanese families with familial amyotrophic lateral sclerosis (FALS) were subjected to genetic analysis of the Cu/Zn superoxide dismutase gene (SOD 1). Sequence analysis revealed two novel mutations in two independent families. A T-to-A transversion causes a Val 17 Glu substitution in one family. A T-to-G mutation leads to a Leu 8 Val substitution in another family. Other four families had unique clinical symptoms, and normal coding sequences of the SOD 1 gene, suggesting that FALS is a complex of heterogeneous motor neuron diseases.

Index Terms

familial amyotrophic lateral sclerosis, superoxide dismutase, gene mutation

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a degenerative disorder in which the motor neurons in the motor cortex, brain stem and spinal cord degenerate leading to muscular atrophy and weakness. Death from respiratory failure occurs commonly within five years after the disease onset. Although the pathophysiology of ALS remains unknown, the linkage of familial form of ALS (FALS) to mutation in the Cu/Zn superoxide dismutase (SOD 1) gene has been identified in some patients[1-3]. Recent reports of SOD 1 mutations in sporadic ALS patients and of motor neuron death in transgenic mice expressing a human SOD 1 mutation enforce the critical role that mutant SOD 1 plays in the pathogenic process of ALS[4,5]. In this paper, we report an investigation of the coding sequences of the SOD 1 gene in four Japanese families with FALS, in addition to previous cases[2,3].

PATIENTS

Six families with FALS were studied. All the families were Japanese, and have no marital relation to Caucasian or other races. Family trees are shown in Figure 1.

Family 1

A 42-year-old woman died of general wasting, who had an eight years' history of progressive muscular atrophy and required assisted ventilation for the last three years of her life. She first noted weakness of the hands and left leg, and then presented with respiratory failure at age 36. There were no ocular symptoms. Atrophic change and fasciculation were obvious in the tongue. Pathological reflexes were definitely positive in the extremities.

The proband's father, who was 66 years old, noted progressive weakness and atrophy of the leg muscles, but no sensory symptoms at age 63. There were no abnormal findings in the tongue and upper limbs. During the following six months, the upper limb weakness, increased deep

tendon reflexes in the lower limbs and Babinski's reflex became evident. Two years after the onset of the disease, he was confined to a wheel chair.

Family 2

The proband was a 42-year-old woman who first noted muscular weakness and atrophy in the legs at age 41. Cranial nerves were normal. There were no pathological reflexes, but obvious fasciculations in the thigh and legs. Sensations were normal. EMG studies revealed denervation in the affected muscles, and nerve conduction study of the tibial nerves showed a normal result. In three successive generations, six individuals were affected by the progressive muscular atrophy and weakness.

Family 3

A 45-year-old man noted progressive weakness of the legs at age 30, over the next three years, the weakness appeared in the upper limbs. He showed urinary incontinence and sensory symptoms two years after the disease onset. In three consecutive generations, eight individuals were affected with FALS.

Family 4

A 54-year-old man developed pill-rolling posture of the hands. At age 55, he noted progressive weakness of his legs, bulber symptoms and urinary incontinence. He had a limitation in vertical gaze, aphonia and bradykinesia at age 57. Muscular atrophy was evident in all limbs. There was no ataxic movement. Sensations of pain, touch, temperature and vibration were normal. The patient's sib affected with FALS died at age 30.

Family 5

A 64-year-old woman noted bulbar symptoms at age 54. She exhibited gait difficulty, and the hands became clumsy. Dystonia-like movement appeared in the shoulder and arms. At age 59, she had no ocular symptoms, but presented with atrophy and fasciculation in the tongue, and increased deep tendon reflexes in all the limbs. There were choreatic movement and torticollis. Touch, pain and pressure sensations were normal, but vibratory sensation was decreased. Co-ordination was normal. Autonomic disturbances were normal. The patient's sister (70-year-old), affected by FALS, is mechanically ventilated since age 67.

Family 6

A 52-year-old man noted weakness of the leg muscles. The walking difficulty was progressive, and a shoulder dullness appeared. His brother was presumably affected by FALS.

METHODS

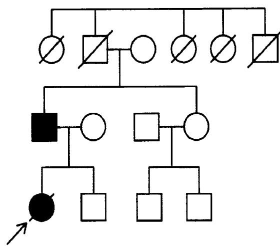
PCR amplification and sequencing of SOD 1 gene and cDNA fragments

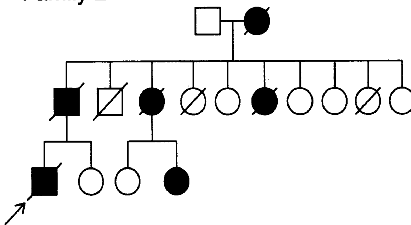
Genomic DNAs were extracted from peripheral blood leukocytes of patients and controls. Based on the published sequences, oligonucleotide primers flanking each exon of the SOD 1 gene were prepared[6]. The five exons were amplified from genomic DNA using Gene Ampkit (Perkin Elmer Cetus). Total RNAs were extracted from lymphocytes by the method described by Chomczynski and Sacchi[7]. Total RNAs from lymphocytes were reverse transcribed into first-strand cDNA using oligo dT primer and M-MLV reverse transcriptase as suggested by supplier (Pharmacia LKB). cDNA reaction was used as a template for SOD 1 cDNA amplification. The primer sequences flanking the entire coding region were: 5'-TTCCGTTGCAGTC-CTCGGAAC-3' and 5'-TTTCTACAGCTAGCAGGATAAC-3'. PCR run consisted of 40 cycles

of denaturation at 94℃, annealing at 55℃ and extension at 72℃ for 60 sec each step. PCR products were electrophoretically separated on 3 % NuSieve GTG agarose (FMC Corp.), and directly sequenced by automated fluorescence-sequencer (Applied Biosystems model 373 A).

Restriction site analysis of PCR-products

The SOD 1 gene fragments containing predicted mutation sites were amplified from genomic DNA of the patients and controls. The fragments were digested with an appropriate restriction enzyme and electrophoresed on polyacrylamide gel, stained with ethidium bromide and photo-





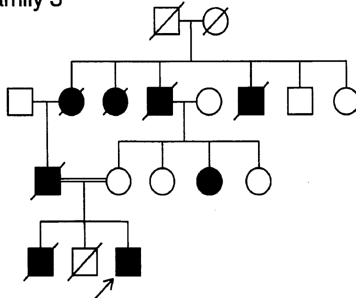


Fig. 1. Pedigree of the FALS (Families 1, 2, 3,). Male family members are represented by squares, female by circles, deceased by diagonals, and affected by solid symbols. The proband of each pedigree is indicated by an arrow.

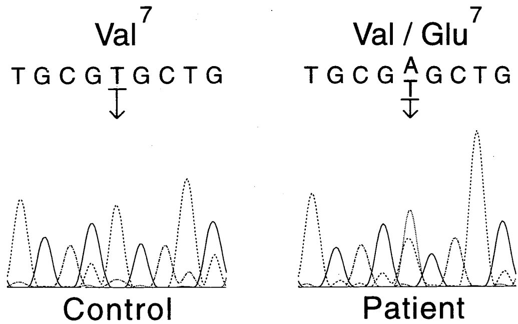


Fig. 2. Direct sequence analysis of RT-PCR products from control and FALS patient. FALS patient is heterozygous for a T-to-A trasversion

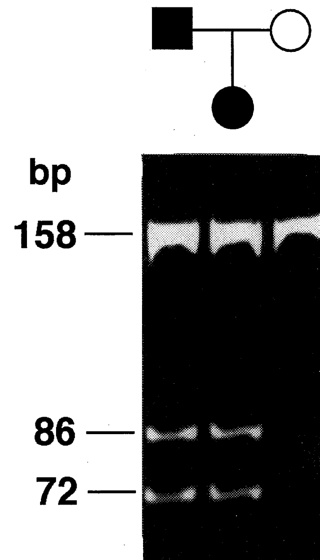


Fig. 3. RFLP analysis of exon 1 of the SOD 1 gene. PCR products of exon 1 was digested with *Alu* 1 and electrophoresed on 10% polyacrylamide gel. Two extra fragments (72 and 86 bp) were observed in the affected father and daughter.

graphed over UV lights.

Enzyme activity and Immunoblotting of red blood cell SOD 1

Red blood cells were isolated from heparinized venous blood obtained from patients with FALS and controls. SOD 1 protein was partially purified by the method described by Deng et al.[8]. Enzyme activity was measured by the method of the xanthine-xanthin oxidase/nitro blue tetrazolium system described by Beauchamp and Fridovich[9]. Partially purified SOD 1 protein was reduced and electrophoresed in a 15 % SDS-PAGE accoring to Laemmli[10], followed by staining with Coomassie blue R 250. For immunoblotting, red blood cell proteins were transferred to nitrocellulose membranes[11]. The membranes were blocked with 4 % skim milk for 2 h, and incubated overnight at 4℃ with goat anti-human SOD 1[12]. After washing the membranes, the blot was incubated with diluted, affinity-purified, peroxidase-conjugated antigoat IgG (Dako). The immunoblot was again washed three times in PBS and then developed in PBS using 4-chloro-1-naphthol as substrate.

RESULTS AND DISCUSSDON

In Family 1, the patients were a heterozygosity indicative of one normal allele and one variant allele with a T-to-A transversion (Figure 2). This mutation causes the codon change

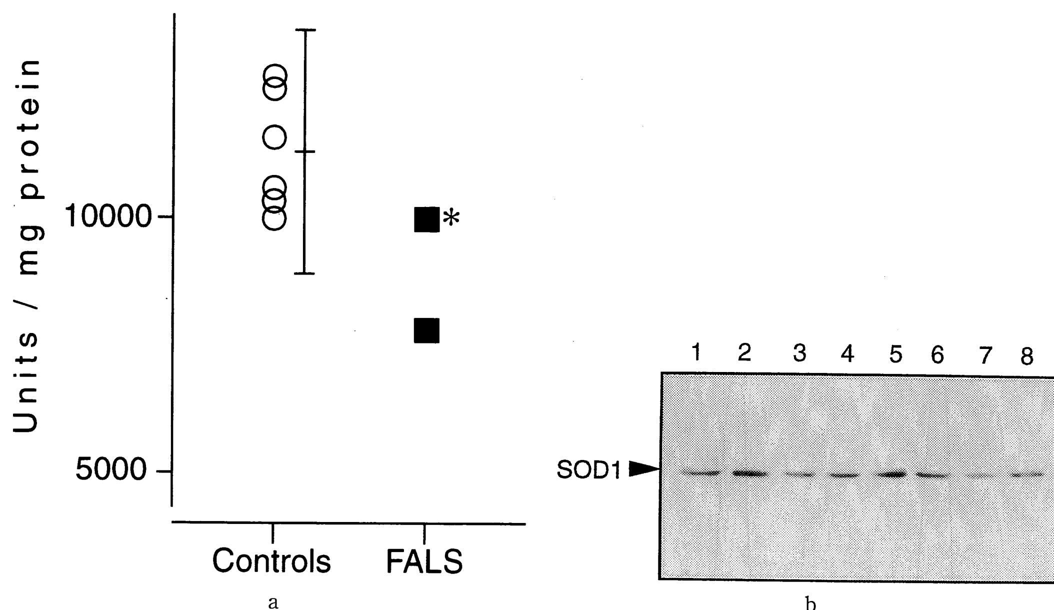


Fig. 4. (a) SOD 1 activities of partially purified enzyme from red blood cells of controls (open circles) and FALS patients (closed squares).
Affected daughter (*) had multiple transfusions of red blood cells for her gastrointestinal bleeding. For the controls, the mean ±2 SD as estimation of 95% confidence interval of the population is shown.
(b) Immunoblot analysis of SDO 1 in red blood cells.
40 μg of red blood cell proteins were analyzed by immunoblotting using anti-SOD 1 IgG. Lane 1, 4-6 and 8, controls; lane 2, FALS-affected daughter; lane 7, FALS-affected father.

from GTG coding for valine to GAG coding for glutamic acid at position 7 of the 153-residue SOD 1 molecule. As the mutation predicted the generation of an *Alu* I restriction site, PCR products containing exon 1 were digested with the restriction enzyme to confirm the linkage between this mutation and FALS. This *Alu* I site assay indicated that the causative gene for this type of FALS is the mutant SOD 1 (Val 17 Glu) gene presented here (Figure 3). The enzymatic activity and protein of SOD 1 were reduced in the patient (Figure 4). The most frequent mutation (Ala 4 Val) and another (Ala 4 Thr) are present at position 4, close to the mutation site as presented here, all the three locate at the middle of the first β-stand, one of the major conserved regions of SOD 1[8,13]. Since, unlike valine having a nonpolar may alter the three-dimensional structure, destabilizing dimer contact or subunit fold in the SOD 1 molecule[8].

In Family 2, a T-to-G mutation in exon 4 of the SOD 1 gene, caused a Leu 84 Val substitution, was identified (Figure 5). Other coding regions contained normal sequence. The Leu 84 Val mutation forms a novel structure, as it occurs outside the β strands and loops forming the β barrel. This mutation is located at the end of the Zn-binding loop where it joints β strand (residues at 85-91), and a hydrophobic anchor for the end of Zn loop. There is an adjacent known ALS mutation site at Gly 85[8], a conserved left-handed glycine that is important for the junction of the Zn-binding loop with the β barrel. The mutation Leu 84 Val may result in defective packing and β barrel stability in the region underlying the Cu and Zn-binding sites. It is notable that the duration of clinical course was very variable in individuals of this famiy. The clinical courses for the five affected members were, 7, 21, 36, 36 months and 15 years. Contrary to the previously reported family with the same mutation (1.6±0.5 years), the clinical courses in our patients were slowly progressive[14]. These results suggest that other

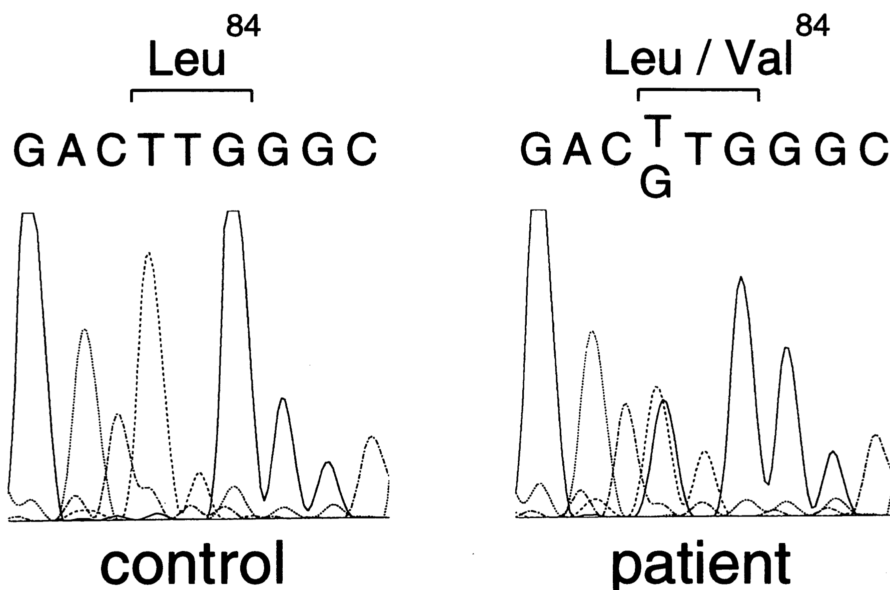


Fig. 5. The direct sequence analysis of PCR products of SOD 1 exon 4 from control and patient. Patient is heterozygous for a T-to-G change, resulting in a Leu 84 Val substitution.

factors in addition to the genetic ones influence the clinical manifestations.

In other four families with FALS, coding sequences were identical to that in the published sequences of the normal SOD 1 cDNA[1]. Unique clinical features included sensory symptoms in Family 3, urinary incontinence in Families 3 and 4, Parkinsonian symptoms and ocular symptoms in Family 4, and choreatic movement in Family 5. Thus the considerably clinical variety of symptoms suggests that other genetic factors may be also involved in the pathogenesis of FLAS.

The mechanism by which SOD 1 mutations cause ALS should be explained. One possibility is that the activity of SOD 1 is reduced, leading to an accumulation of toxic superoxide radicals in FALS patients. This explanation can be supported by the fact that mutations in SOD 1 are uniformly destabilizing, resulting in proteins with shorter half-lives and decrease in enzyme activity[8], thus increasing oxygen free radical stress. Alternatively, the activity of SOD 1 might be increased, leading to excessive levels of hydrogen peroxide and highly toxic hydroxyl radicals. Furthermore, given that the total activity appears to be $<50\%$ of normal, we can not exclude a dominant-negative effect in which the variant subunit protein interferes with the function of the normal subunit protein.

An important quention is why FALS transmits by an autosomal dominant mode, as the loss-of-function usually results in a recessive disorder. As noted above, one possibility is that mutant SOD 1 has a dominant-negative effect. Another possibility is that in addition to reducing SOD 1 activity, this mutation may confer a novel, potentially cytotoxic function to SOD 1 protein. A further question is why decrease or alterations in SOD 1 activity might selectively damage motor neurons, given the ubiquitous expression of the gene. Nonetheless, overexpression of SOD 1 in transgenic mice led to an apparently specific defect in distal motor neuron terminals, indicating that this gene can selectively affect motor neurons[4]. Alternatively, the motor neurons are highly sensitive to a decrease in SOD 1 activity. Furthermore, because SOD 1 enzyme is susceptible to various inhibitors, the possibility that FALS may be caused by intoxication of motor neurons with such an inhibiting agent should be considered. Clinical and genetical investigations of the six families indicate that additional genetic, sexual, or environmental factors influence the expression of FALS.

ACKNOWLEDGMENT

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and a Nakajima Memorial Research Grant, Nara Medical University.

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