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Multiple Transcripts of the Human Cu,Zn Superoxide Dismutase Gene

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We have identified five alternatively spliced transcripts of the gene for human Cu,Zn superoxide dismutase (SOD1), a causative gene for autosomal dominant amyotrophic lateral sclerosis (ALS). The splice variants of wild-type or mutant SOD1 were expressed in a tissue-specific manner; therefore, their expression may be regulated to modify SOD1 function. In addition, the expression in the brain implies that variants may play a role in the nervous system, the region involved in ALS. Immunoblot study of HeLa cells transfected with two variants encoding C-terminal truncated proteins did not show the proteins of expected size. However, this observation is consistent with the previous study of C-terminal truncated mutant proteins that cause ALS, suggesting that both variant and mutant proteins may share certain properties, such as instability or insolubility in the cytosol. These findings suggest that the splice variants may contribute to a physiological function of SOD1 or to a pathological mechanism in ALS.

Key Words: **Cu,Zn superoxide dismutase; amyotrophic lateral sclerosis; alternative splicing; variant.**

Cu,Zn superoxide dismutase (SOD1), a cytosolic enzyme catalyzes the conversion of superoxide anion to oxygen and hydrogen peroxide for cellular defense against the oxidative stress (1). Mutations of the gene encoding human SOD1 have been identified in autosomal dominant and rare recessive or sporadic amyotrophic lateral sclerosis (ALS) cases (1–4). This disease is characterized by fatal, progressive muscle weakness and atrophy due to motor neuron degeneration. The mechanism of the neurodegeneration is thought to be an unknown toxic gain of function of the mutant protein rather than a loss of function of SOD1. This prediction is supported by the transgenic mice overexpressing mutant SOD1 that develop disease similar to that of ALS patients despite harboring more SOD1 activity than the non-transgenic littermates (5). Since most of the mutant proteins have only one amino acid change, certain part of the peptides may be necessary for the toxicity (1). Moreover, several reports of C-terminal truncated SOD1 proteins predicted by nonsense or frameshift mutations demonstrate that the remaining N-terminal part of the protein may be important for the motor neuron degeneration (6). Alternative splicing is a widespread mechanism for regulating gene expression and generating isoform diversity (7). The variant proteins produced by alternative splicing exert their distinct function with various mechanisms, ranging from the inhibition of the wild-type protein to the change in cellular localization (8, 9). In this study, we have identified multiple alternatively spliced transcripts of the gene for SOD1. The splice variant transcripts from normal controls and ALS patients with different mutations were expressed in various tissues.

MATERIALS AND METHODS

Reverse-transcribed polymerase chain reaction study. Total RNA was isolated from lymphoblasts of three normal controls and ALS patients with heterozygous Ala4Val (A4V), His46Arg (H46R), Gly93Ala (G93A), Val148Ile (V148I), and homozygous Asp90Ala (D90A) SOD1 mutations. Two micrograms of RNA was reverse-transcribed (RT) into a first-strand cDNA with oligo dT primers and random hexamer primers. To analyze the distribution of the transcripts in various tissues, we purchased cDNA panels from Clontech Laboratory Inc. (Palo Alto, CA). These cDNAs are derived from poly A^+RNA of non-disease control subjects. PCR was performed with the following primer pairs in the presence of [α-^{32}P]dGTP: P1.0; 5′-TTCCGTTGCAGTCCTCGGAA-3′ and P10; 5′-TTCTACAGCTAGCAGGATAACA-3′ were used for amplifying the fragment containing exons 1 to 5 of SOD1 cDNA and P1.0 and 238R; 5′-ACAGCCTGCTGTATTATCTCCAA-3′ were for exons 1 and 2. PCR runs consisted of 30–35 cycles of 1 min each at 94, 60, and 72°C. PCR fragments were separated on 4% polyacrylamide gels, dried, and detected with a phosphor imager (Storm 860, Molecular Dynamics, Sunnyvale, CA). The detected bands were cut from the gel, reamplified, and sequenced with the same primers. When the sequence

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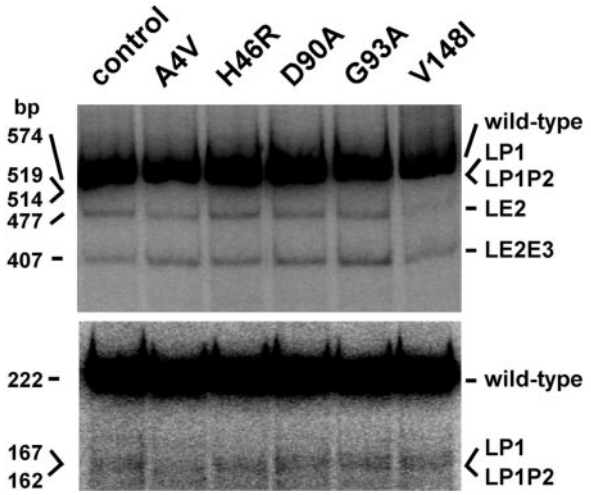

FIG. 1. Reverse-transcribed (RT) and amplified human Cu,Zn superoxide dismutase (SOD1) mRNA from lymphoblasts of normal controls (one representative case was shown) and amyotrophic lateral sclerosis patients with various SOD1 mutations, including heterozygous Ala4Val (A4V), His46Arg (H46R), Gly93Ala (G93A), Val148Ile (V148I), and homozygous Asp90Ala (D90A) mutations. Upper panel shows the RT-PCR products amplified using P1 primer in exon 1 and P10 primer in exon 5. Each lane has two small extra bands in addition to the band corresponding to the wild-type transcript. Lower panel shows PCR fragments amplified using P1 and 238R primer in exon 2. The extra band detected here contains two different variants. The variants' designation is described in the text and in the legend to Fig. 3.

result was not clear, for example, formation of the heteroduplex or mixture of two fragments because of their similar sizes, we cloned these bands into TA cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA) and confirmed the sequences. We also used different primer pairs for amplification of the variants, P1.4; 5′-GTTTGCGTCGTAGTCTCCTGCA-3′ in exon 1 and 416R; 5′-GTGTGCGGCCAATGATGCAAT-3′ in exon 4.

Plasmid construction. The SOD1 cDNAs for variants lacking exon 2 and lacking exons 2 and 3 were amplified from lymphoblast mRNA by RT-PCR method with the primers 5′-GTGAAGCTTGCCACCATGGCGACGAAGGCCGTGTGC-3′ and 5′-ATTTCTAGAGCTAGCAGGATAACA-3′. These primers created new restriction sites, *Hin*dIII and *Xba*I for cloning into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). To eliminate any possible PCR errors, entire coding regions of the cloned cDNAs were sequenced. A D90A mutant cDNA vector was also cloned as a control of expression system, since this mutant can be electrophoretically differentiated from the endogenous human SOD1 in HeLa cells (10).

Transfection study. HeLa cells were maintained in Dulbecco's modified Eagle's medium with 20% fetal bovine serum. A day before transfection, 1.6×10^5 cells are seeded in 6-well plates. The cells were transfected with the expression vectors containing SOD1 variant or mutant cDNA using Superfect (Qiagen, Germany). The cells were harvested 48 h after transfection, and subjected to the immunoblot study. In the transfected cells variant or mutant SOD1 mRNA expression was confirmed by RT-PCR method (data not shown).

Immunoblot study. The transfected cells were suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl, 0.5% Triton X100, and protease inhibitor cocktail (Complete Mini, Boehringer Mannheim, Germany). Eight micrograms protein of the supernatant from each cell sample was electrophoresed on 16% sodium dodecyl sulfate–polyacrylamide gel, transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA), and immunoblotted with rabbit anti-human SOD1 polyclonal antibodies as used previously (6). Immunoreactive proteins were visualized by using the horse-radish-peroxidase-based chemiluminescent method (Pierce, Rockford, IL).

RESULTS

Identification of SOD1 splice variants and distribution of variant transcripts in various tissues. The RT-PCR of the lymphoblasts from controls and the ALS patients showed several bands smaller in size than the wild-type band (Fig. 1). These were also found in various control tissues including the brain (Fig. 2). The band intensity varied between individuals with different SOD1 mutations and in various tissues (Figs. 1 and 2). For example, the bands of LE2 and LE2E3 for V148I lymphoblasts were weaker than those of other mutants and controls. In addition, the band containing the smallest fragment was not seen in lymphoblasts and the lung. Sequence analyses of these bands identified five alternative splice variants. The results were confirmed by PCR using other primer pairs, and subsequent cloning and sequencing. These include the variant transcripts lacking a part of exon 1, designated as LP1; a part of exon 1 and a part of exon 2, as LP1P2; an entire exon 2, as LE2; an entire exon 2 and 3, as LE2E3; and a part of exon 1 and an entire exon 2 and 3, as LP1E2E3 (Fig. 3).

Immunoblot study in transfected HeLa cells. The cells expressing with the D90A mutant showed an extra band in addition to the band of their endogenous human SOD1, while the nontransfected and LE2 or

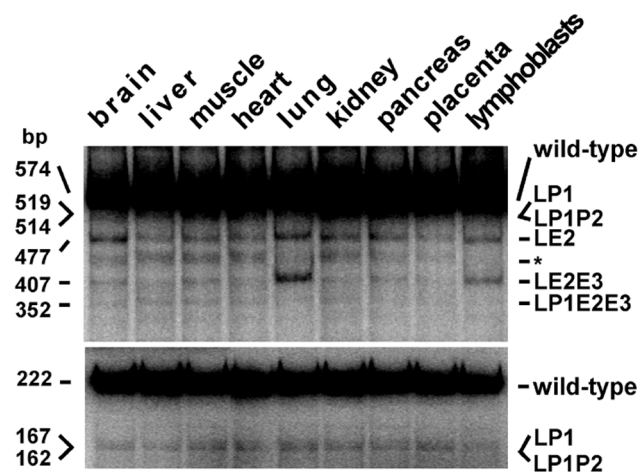

FIG. 2. Tissue distribution of the Cu,Zn superoxide dismutase (SOD1) variant transcripts from control subjects. Band marked with an asterisk (*) is heteroduplex of LE2E3 and the wild-type transcripts. Upper panel shows the RT-PCR products containing exons 1 to 5. Each lane has at least two small extra bands in addition to the band corresponding to the wild-type transcript. Lower panel shows PCR fragments containing exons 1 and 2. The variants' designation is in the text and in the legend to Fig. 3.

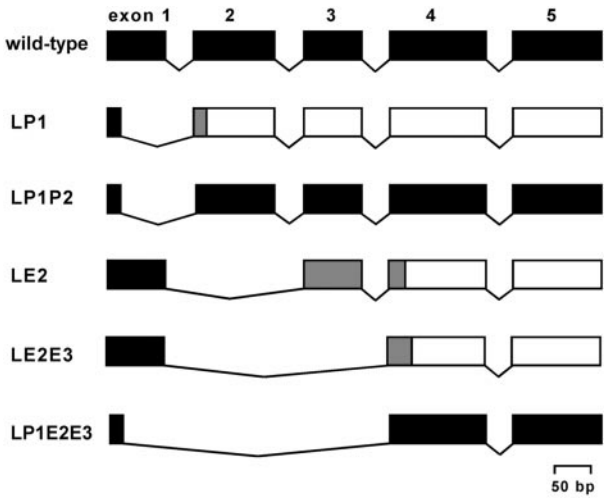

FIG. 3. Schematic representation of wild-type and variant Cu,Zn superoxide dismutase (SOD1) transcripts. LP1, the transcript lacking a part of exon 1; LP1P2, lacking a part of exon 1 and a part of exon 2; LE2, lacking an entire exon 2; LE2E3; lacking an entire exon 2 and 3, LP1E2E3, lacking a part of exon 1 and an entire exon 2 and 3. The variants of LP1, LE2, and LE2E3 lacking 55 (nt 18–72), 97 (nt 73–169), and 167 (nt 73–239) bases, respectively, change the translational reading frame, while LP1 and LP1E2E3 lacking 60 (nt 18–77) and 222 (nt 18–239) bases, respectively, conserve the frame. The nucleotide number (nt) of SOD1 cDNA is based on counting the "A" in the initiation codon of "ATG" as one. Black area is a normal coding region, gray area is an aberrant coding region, and open area is a noncoding region. The predicted variant proteins share five amino acids (a.a.) for LP1 with the wild-type SOD1 (153 a.a.), 23 a.a. for LE2 and LE2E3, 79 a.a. for LP1E2E3, and 133 a.a. for LP1P2.

LE2E3 transfected cells did not show any additional bands (Fig. 4).

DISCUSSION

Here we report five alternatively spliced transcripts of the gene coding for human SOD1. Others have found two species of SOD1 mRNA with different poly A sites, but not alternatively spliced variants (11). This is partly because the amount of these variants was too low to be detected with Northern blot methods. The discovery of the variants was possible by using RT-PCR combined with a phosphor imager, which is highly sensitive compared with conventional methods (12). Although the variants were detected with PCR-based method, these are not PCR artifacts because of the following reasons. (i) PCR with different pairs of primers showed the bands corresponding to each variant (data not shown). (ii) The same variant transcripts were identified in different tissue and individual samples. Moreover, these variants may be amplified from intact SOD1 mRNA by using poly A^+ RNA derived or oligo dT primed cDNA as RT-PCR templates. Why does the alternative splicing occur in the gene for SOD1? In search for answers to this question we applied the scoring method of Shapiro and Senapathy to analyze the degrees of match between SOD1 splicing site sequences and the corresponding consensus sequences (13). Among the calculated scores of the splice donor sites, 77.2, 85.9, 80.1, and 92.2, the intron 1 donor site showed the lowest degree of match (77.2) that may reduce the splice site usage, leading to alternative splicing. This explanation seems plausible because the splice site selection is highly dependent on the relative strength of the whole sequence in context with individual splicing signals (14). The region 55-bases upstream of the normal splice donor site may thus be selected as the alternative splice site, producing the variants lacking a part of exon 1. The same calculation was applied to the splice acceptor site. Among the splice scores of 84.4, 79.9, 89.6, and 85.9, the lower scores shown in intron 1 and intron 2 (84.4 and 79.9) may reduce the usage of the splice site, leading to the lack of an entire or a sectioned exon 2 and/or an entire exon 3 Although the splice score flanking exon 3 (79.9 and 80.1) is lower than that of exon 2 (84.4 and 85.9), we could not find variant lacking exon 3 alone. Therefore, the splice selection should be related to other factors, such as exonic enhancers (15). The exon 3, in fact, has polypurine sequences, which is a known exonic enhancer that facilitates the exon recognition (15). This theory may account for relatively stronger exon recognition of exon 3 than exon 2, which prevents SOD1 mRNA from lacking exon 3 alone. Alternative splicing may be explained by the structural feature of the gene for SOD1, however, the gene structure is not the only factor that contributes to the generation of splice variants. For example, the pattern of R-PCR bands of the variants was variable in their intensity when these variants were expressed in lymphoblasts harboring different SOD1 mutations. This indicates that variant expres-

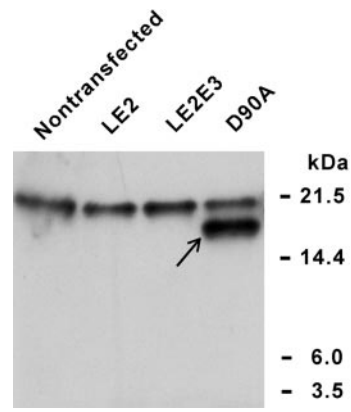

FIG. 4. Immunoblot study for HeLa cells transfected with mutant and variant Cu,Zn superoxide dismutase (SOD1). Nontransfected cells showed endogenous human wild-type SOD1 protein. D90A mutant protein (arrow) was expressed in addition to the endogenous protein, while variant proteins were not detected in their expected size, 6.0 kDa for LE2 and 3.5 kDa for LE2E3.

sion level was different between various SOD1 mutations, although the precise quantitation was not examined. The variation in the intensities was also observed in various tissues from control subjects. These findings suggest that the variant expression is not constant rather it is influenced by SOD1 mutation or tissue specificity. This may indicate that the variants are regulated in their expression to modify SOD1 function. In addition, the expression in the brain implies that variants may play a role in the nervous system, which is the region involved in ALS.

Low amount of variants mentioned above raises questions about whether these variants have physiological or pathological significance. In another case, such as with transcription factor E3, the transcripts were present in submolar amounts, 2–20% of wild-type, which strongly inhibited the wild-type protein (8). What is the function of the variants? Except for LP1P2 variant encoding 133 amino acids (a.a.), other variants encode substantially short proteins, 5–79 a.a. shared with the wild-type protein, while the shortest disease-causing mutant protein has 118 a.a. shared (6). Because all but LP1P2 variant protein lack the active site of the enzyme encoded in exon 2 and 3, these variant proteins probably do not have SOD1 activity. However, all variant proteins conserve at least a part of the dimer contact regions encoded in exon 1, and therefore, can interact with the wild-type proteins. The variants may thus affect the wild-type proteins as some mutant SOD1 or other gene products do (2, 8, 16). Moreover, even small proteins can exert significant biological effect on cells, as in the case of insulin-like growth factor receptor where only a 17-amino-acid peptide has a function contradictory to the wild-type protein activity, leading to cell death (17). To determine the variant function, we constructed the expression vectors with the LE2 and LE2E3 variant transcripts, which encode C-terminal truncated SOD1 proteins. We chose these two variants, because the disease causing truncated mutant mRNAs also encode C-terminal truncated proteins. The immunoblotting of the transfected HeLa cells failed to show the expected-size band of the truncated proteins, but showed the D90A mutant band (Fig. 4). One of the possible reasons is that the polyclonal antibody against whole SOD1 protein may not recognize the variant proteins. To eliminate this possibility, we used the human SOD1 specific antibody raised against the peptide at position 17–36 of human SOD1, which can show human wild-type SOD1, but could not detect the variant proteins (data not shown). Therefore, it is plausible that the expression level of the truncated proteins was too low to be detected in the cytosol. This finding is also consistent with the previous study of the C-terminal truncated mutant proteins that causes ALS (6), suggesting that both variant and mutant proteins have the same kind of instability or insolubility in the cytosol. In another study for glutamate transporter EAAT2, alternative spliced form was not detected at the protein level in the cytosol, but exerted significant biological function including inhibition of the wild-type transporter activity (16).

An important question is whether these variants are associated with the disease. These variants were found in both ALS patient and controls, therefore, if the variant protein is toxic, the toxicity is not obvious under normal condition. However, it is possible that cellular stress may increase the variant protein, leading to the motor neuron degeneration. In other diseases such as Alzheimer disease, the presenilin-2 splice variant is increased in hypoxic condition, which may contribute to the disease state (18). Alternatively, the truncated protein may reduce the cellular toxicity as shown in the BRCA1 splice variant product (19). At present, it is difficult to determine the fundamental role played by these variants. However, the tissue specific expression suggests that these variants may be regulated in their expression to modify SOD1 function. In addition, the variants may contribute to cellular viability, because the truncated mutant SOD1, sharing certain protein property with the variant protein, contributes to the motor neuron degeneration. Although further functional analysis is needed, the alternative spliced variants may provide a new insight into the physiological function of SOD1 or the pathological mechanism in ALS.

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