



Identification and characterization of novel *ERBB4* variant associated with sporadic amyotrophic lateral sclerosis (ALS)

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is the most common type of motor neuron disease characterized by progressive motor neuron degeneration in brain and spinal cord. Most cases are sporadic in ALS and 5–10% of cases are familial. >50 genes are known to be associated with ALS and one of them is *ERBB4*. In this paper, we report the case of a 53-year-old ALS patient with progressive muscle weakness and fasciculation, but he had no cognitive decline. We performed the next generation sequencing (NGS) and *in silico* analysis, it predicted a highly pathogenic variant, c.2116 A > G, p.Asn706Asp (N706D) in the *ERBB4* gene. The amino acid residue is highly conserved among species. *ERBB4* is a member of the ERBB family of receptor tyrosine kinases. *ERBB4* has multiple tyrosine phosphorylation sites, including an autophosphorylation site at tyrosine 1284 residue. Autophosphorylation of *ERBB4* promotes biological activity and it associated with NRG-1/*ERBB4* pathway. It is already known that tyrosine 128 phosphorylation of *ERBB4* is decreased in patients who have ALS-associated *ERBB4* mutations. We generated *ERBB4* N706D construct using site-directed mutagenesis and checked the phosphorylation level of *ERBB4* N706D in NSC-34 cells. We found that the phosphorylation of *ERBB4* N706D was decreased compared to *ERBB4* wild-type, indicating a loss of function mutation in *ERBB4*. We report a novel variant in *ERBB4* gene leading to ALS through dysfunction of *ERBB4*.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal neurodegenerative disease characterized by the loss of motor neurons in the brain and spinal cord. As the disease progresses, patients experience twitching, and paralysis. In ALS, most of patients are sporadic cases with only 5–10% of patients having familial (inherited or genetic) cases [1,2]. Patients with ALS often experience cognitive deficit and behavior impairment [3–5] some patients overlap clinical features with frontotemporal dementia (FTD) [6,7]. Riluzole (which inhibits glutamate release) [8,9], Edaravone (a ROS scavenger) [10,11], and Relyvrio (sodium phenylbutyrate and taurursodiol) [12,13] are representative drugs for ALS treatment. Riluzole was firstly approved in 1995 as a treatment for ALS, several clinical trials showed riluzole improve survival in ALS patients [14–16]. Edaravone clinical trial including a specific group of patients indicated a slower functional decay over 6 months [17]. Relyvrio (AMX-0035) was approved in 2022 for ALS treatment [18], supported by a phase-2 clinical trial showing a slower functional decay, currently phase-3 trial is close to end [12]. To date, >50 genes have been identified as ALS-associated genes. The most common pathogenic mutations in patients with ALS are found in the *SOD1*, *C9ORF72*, *FUS*, and *TARDBP* genes [19]. Besides, *OPTN*, *TBK1*, *SQSTM1*, *UBQLN2*, *VCP*, and *ATXN2* are also linked to ALS pathogenesis [20,21]. In 2013, *ERBB4* mutations (R927Q and R1275W) were first identified in ALS patients using whole-genome sequencing [22]. These

amino acids are highly conserved among species. Functional analysis suggested that these mutations decreased autophosphorylation compared to *ERBB4* wild-type upon NRG-1 stimulation.

ERBB4 is a member of the epidermal growth factor receptor (EGFR) family, which has four subfamilies: *ERBB1*, *ERBB2*, *ERBB3*, and *ERBB4* [23]. These proteins are receptor tyrosine kinases that form homo- and heterodimers to activate cellular functions [23]. To activate the ERBB signaling pathway, ligands such as neuregulins (NRG-1, NRG-2, NRG-3, and NRG-4) and EGF-like growth factors (TGF- α , Amphiregulin, Ephiregulin, and Betacellulin) must bind to the ERBB receptor [24]. ERBB proteins are in the cell membrane and transmit signals into the cell. The ERBB pathway regulates cell proliferation, migration, differentiation, apoptosis, cell motility, and neuronal development [25,26]. The ERBB pathway is also related to neuropsychiatric diseases such as schizophrenia [27,28] and several neurodegenerative diseases including Alzheimer's disease (AD) [29–31], Parkinson's disease (PD) [27], Multiple sclerosis (MS) [32], and ALS [22]. Several papers have reported that *ERBB4* mutations are related to ALS pathogenesis [22,33–38]. When *ERBB4* is activated by ligands, it undergoes proteolytic cleavage, leading to the release of the intracellular domain (ICD) from the cell membrane. This domain can translocate into the nucleus and act as a transcription factor [39–41].

In this study, we report a novel *ERBB4* variant in Korean ALS patient. We found that the *ERBB4* mutation showed a decreased phosphorylation level compared to *ERBB4* wild-type. Furthermore, *ERBB4* is known as a

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; AD, Alzheimer's disease; NGS, next generation sequencing; FTD, frontotemporal dementia; PD, Parkinson's disease; MS, Multiple sclerosis; MMSE, mini-mental status examination; ALSFRS-R, amyotrophic lateral sclerosis functional rating scale; MRC, medical research council; EGFR, epidermal growth factor receptor; A β , Amyloid beta.

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membrane-localized protein. The *ERBB4* mutation showed reduced phosphorylation in the membrane fraction. This means that the *ERBB4* mutation causes loss of function. Additionally, knock-down of *ERBB4* causes cellular toxicity in primary cortical neurons. Our findings suggest that mutations in *ERBB4* lead to loss of *ERBB4* function and motor neuron death in ALS.

2. Material & method

2.1. Reagents and antibodies

The following antibodies were used for immunoblotting: rabbit anti-phospho-HER4/*ERBB4* (Tyr1284) (Cell Signaling Technology, 4757), rabbit anti-*ERBB4*/*HER4* (Abcam, ab32375), rabbit anti-Pan-cadherin (Cell Signaling Technology, 4068), anti- α tubulin-HRP (Cell Signaling Technology, 9099), anti-rabbit IgG-HRP (Cell Signaling Technology, 7074), and anti-mouse IgG-HRP (Cell Signaling Technology, 7076).

2.2. Next generation sequencing

The DNA extraction was performed from the patient's EDTA blood sample after written consent. A total of 4813 genes with approximately 62,000 target exons were captured using the TruSight One Panel from Illumina and the captured DNA was then sequenced using the NextSeq from Illumina. The sequencing data was mapped to the UCSC hg19 reference genome for comparative analysis. The possible pathogenic variant detected was further validated using Sanger sequencing for confirmation.

2.3. Cell line

The mouse motor neuron-like (NSC-34) cell line (Cat.No. CLU140; Cedarlane) are well-characterized lower motor neuron-like cells generated by the fusion of embryonic mouse spinal cord cells and mouse neuroblastoma cells [42]. NSC-34 cells show morphological and physiological similarities to mature primary motor neurons. NSC-34 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, 11,995–065) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, 16,000–044) and 50 μ g/ml penicillin-streptomycin (P/S; Gibco, 15,140–122).

2.4. Transfection

NSC-34 cells in 6-well plates (30×10^4 cells/ml) were transfected with 4 μ g of *GFP* (pCMV6-AC-*GFP*, Origene Technologies, PS100010), human *ERBB4* (pCMV6-AC-*ERBB4*-*GFP*, Origene Technologies, RG220449, JM-a/CVT-1), *ERBB4* N706D-*GFP*, or *ERBB4* R937Q-*GFP* using ViaFect Transfection Reagent (Promega, E4982). *ERBB4* N706D generated by QuikChange Multi Site-Directed Mutagenesis kit (Agilent, 200,514) followed protocol [43] using oligo nucleotide primer (NM_005235.2, 5'-CACCCAATCAAGCTCAACTTCG-3' to 5'-CACCCGATCAAGCTCAACTTCG).

2.5. Cytotoxicity test

NSC-34 cells (30×10^4 cells/ml) were grown in 24-well plates and transfected *GFP*, *ERBB4*-*GFP*, *ERBB4* N706D-*GFP* or *ERBB4* R927Q-*GFP* for 48 h. To measure cytotoxicity, Cell Counting Kit-8 (CCK-8; Enzo Life Science, AXL-850-039-KI02) was used in accordance with the manufacturer's instructions. Briefly, 50 μ L of CCK-8 reagent was added to each well, and the plate was incubated at 37 °C for 2 h. Absorbance was measured at 450 nm using microplate reader (Tecan). Cell viability was expressed as a percentage of control (GFP-transfected) cell viability. All experiments were performed in triplicate.

2.6. Immunoblot analysis

NSC-34 cells were homogenized in Cell Lysis Buffer (Cell Signaling Technology, 9803) containing protease (Roche, 11,836,153,001) and phosphatase (Roche, 4,906,837,001) inhibitor cocktails. Protein concentrations of cell lysates were determined by BCA protein assay (Thermo Fisher Scientific, 23,225). Next, the protein extracts were mixed with 4 $\times$ Bolt LDS sample buffer (Invitrogen, B0007) and 10 $\times$ Bolt Sample Reducing Agent buffer (Invitrogen, B0009), and then they were boiled at 95 °C for 5 min. An equal amount of protein from each sample was separated NuPAGE 3–8% Tris-Acetate gels (Invitrogen, EA0378BOX), and then it was transferred to polyvinylidene difluoride (PVDF) membrane. After blocking the membrane with 2% BSA or 5% skim milk in TBS with 0.025% Tween 20, blots were probed with antibodies as indicated and detected with an ECL prime kit (GE healthcare, RPN2232). Samples from three independent experiments were used, and the relative expression levels were determined using Fusion-FX imaging system (Viber Lourmat).

2.7. Cytosol/membrane fraction

Cells were fractionated using Mem-PER Membrane Protein Extraction Kit (Thermo Fisher Scientific, 89,842). Cytoplasmic fractions were obtained Permeabilization buffer centrifugation at 16,000 $\times$ g for 15 min at 4 °C. Membrane fractions were obtained Solubilization buffer centrifugation at 16,000 $\times$ g for 15 min at 4 °C. The extracts were mixed with 4 $\times$ Bolt LDS sample buffer and 10 $\times$ Bolt sample Reducing Agent buffer, and then they were boiled at 95 °C for 5 min.

2.8. Statistical analyses

Data were analyzed by Student's *t*-test (Vassar Stats, www.vassarstats.net) or one-way ANOVA test depending on comparison variables with post-hoc analysis as indicated (GraphPad Prism Software). Differences were considered significant when $p < 0.05$ and are indicated as follows: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; n.s., not significant.

3. Result

3.1. Case presentation

A 53-year-old male presented with progressive right limb weakness and fasciculation. He complained of right-hand weakness 3 months before and progressed to have right dominant leg weakness a month before the initial visit to our outpatient clinic. He had no dysarthria or dysphagia at the initial presentation. The patient had no history of systemic disease and no family history of any genetic disease. On the initial neurological examination, he did not have dysarthria or tongue atrophy and he had no abnormalities in the cranial nerves. However, the motor examination showed motor weakness with a medical research council (MRC) grade of 4 right arm flexion with a MRC grade of 3 in the finger fanning of the right hand along with muscle atrophy in the first dorsal interosseous muscles. The patient also had a weakness of MRC grade of 4 in thigh flexion in the right side and there was no gross motor weakness observed in his thigh extension, knee flexion, extension, ankle dorsiflexion and plantarflexion. The patient also demonstrated hyper-reflexia in the right biceps, brachioradialis, knee and ankle jerks. The pathologic reflexes, including Hoffmann signs and Babinski signs were present. Routine laboratory tests, including complete blood count, electrolytes, liver, and renal function tests, were normal. Autoimmune, immunofixation electrophoresis, and paraneoplastic antibodies were unremarkable. Viral markers in the serum and cerebrospinal fluid study were negative. The CSF study showed normal protein and glucose levels, with a level of 72 mg/dL and 30 mg/dL, respectively. The cervical spine magnetic resonance imaging was unremarkable without significant radiological compression. The nerve conduction study was performed in

bilateral median, ulnar and radial nerves in the upper extremities and tibial, peroneal, and superficial peroneal nerves. The nerve conduction study showed a motor dominant neuropathy without sensory involvements. The needle electromyography (EMG) showed active denervation in the cervical, thoracic paraspinal, and lumbosacral innervated muscles such as first dorsal interosseous, extensor digitorum communis, biceps brachii, thoracic paraspinal muscles, tibialis anterior, gastrocnemius, and vastus lateralis muscles but showed normal needle EMG study in the bulbar muscles. The mini-mental status examination (MMSE) was 30 with no cognitive decline. Based on neurological and electrophysiological studies, the patient manifested both upper and lower motor neuron signs in the cervical and lumbosacral regions and he was diagnosed with clinically probable ALS *via* revised El Escorial criteria. The baseline revised ALSFRS-R was 44. Pulmonary function studies showed a forced vital capacity of 96% and video fluoroscopy was unremarkable. Next-generation sequencing revealed a likely pathogenic variant in c.2116 A > G, p.Asn706Asp in the *ERBB4* gene (Fig. 1A-C). We performed *in silico* analysis using SIFT (<http://sift.jicvi.org>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>), and Mutation Taster ([http://](http://mutationtaster.org)

mutationtaster.org). The prediction result showed this variant is rare and highly deleterious. We also ruled out *C9ORF72* mutation *via* separate sequencing. The patient was treated with edaravone when the patient's ALSFRS-R was 30, but there was a significant rapid decline of ALSFRS-R to 22 along with significant neurological deterioration after six months, and he was intubated due to respiratory difficulty (Fig. 1D).

3.2. Characterization of *ERBB4* N706D mutant in neuronal cells

The tyrosine phosphorylation of *ERBB4* (Tyr1284) is a significant indicator of its biological activity, as previously reported [44]. To assess the impact of the novel *ERBB4* variants identified in the ALS patient on *ERBB4* functions, we examined the Tyr1284 phosphorylation level of *ERBB4* in NSC-34 cells expressing either wild-type or mutant *ERBB4* proteins. The *ERBB4* mutations (N706D and R927Q) were introduced into the pCMV6-AC-*ERBB4*-GFP plasmid encoding GFP-tagged human *ERBB4* by site-directed mutagenesis according to the protocol described in the QuikChange Multi Site-Directed Mutagenesis Kit. Then, we transfected DNA vectors expressing GFP-tagged *ERBB4*, *ERBB4* N706D,

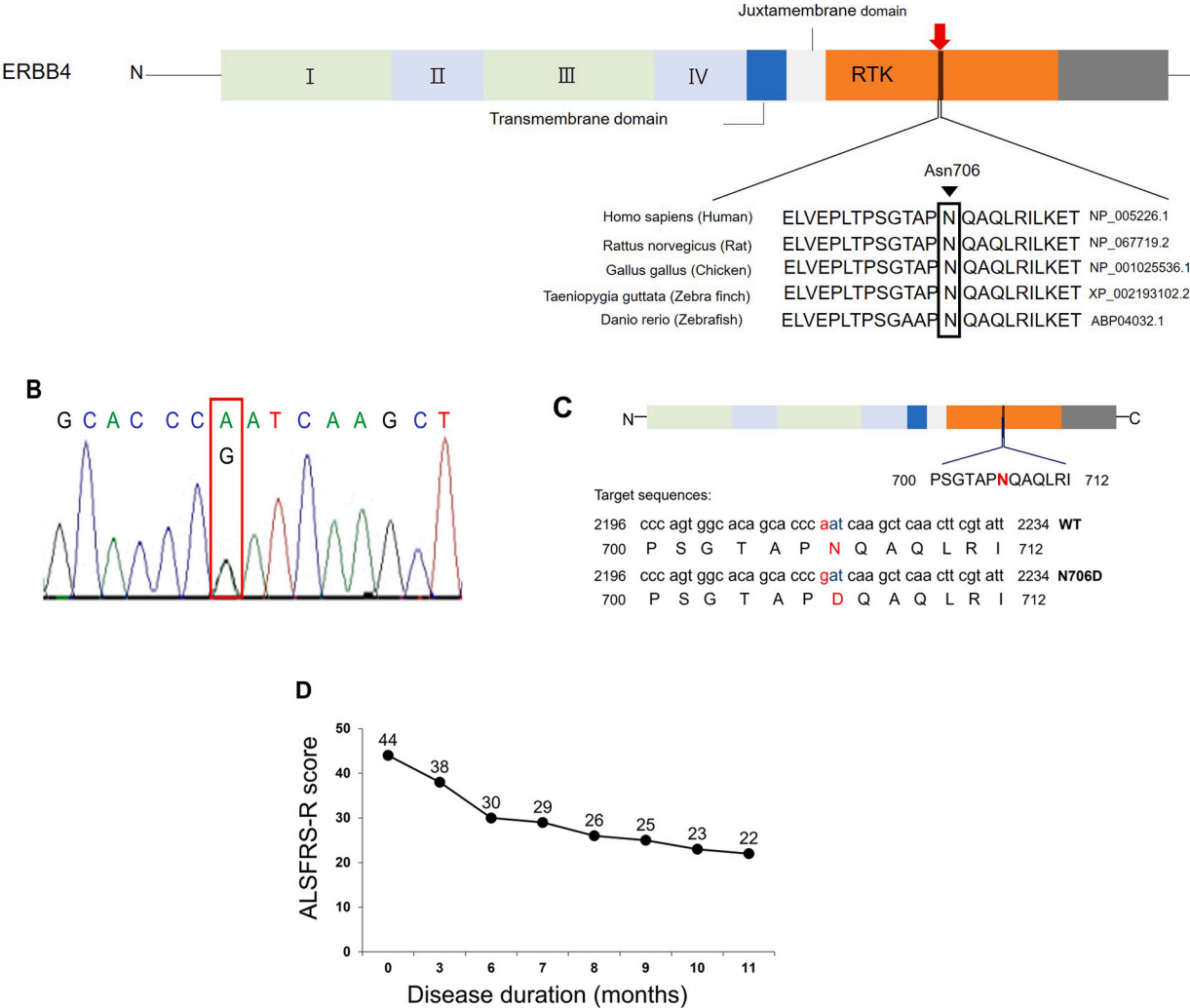


Fig. 1. A new *ERBB4* mutation conserved among species. (A) Schematic representation of *ERBB4* protein. *ERBB4* contains ectodomain (I-VI), transmembrane domain (TM), Juxtamembrane domain, and receptor tyrosine kinase (RTK) domain. New *ERBB4* mutation (N706D) locate in tyrosine kinase domain. Amino acid sequence alignment across multiple species shows conservation of the *ERBB4* mutated amino acid. Asparagine 706 is preserved among species. (B) Validation of mutation by Sanger sequencing. c.2116 A > G mutation detected in ALS patient. Verified amino acid is highlighted by red. (C) Illustration of target sequence for N706D in *ERBB4*. (D) Serial changes in ALSFRS-R total score during 11 months. Edaravone treatment started at 6 months. Edaravone was initially administered for 14 consecutive days, followed by a 2-week drug-free period, and subsequently administered for 10 days within a 2-week period. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or *ERBB4* R927Q (an ALS-associated mutation previously reported) into NSC-34 cells. We observed that ERBB4 N706D resulted in a significant decrease in the phosphorylation level when compared to ERBB4. However, we did not observe any changes in the phosphorylation level with ERBB4 R927Q mutation (Fig. 2A). When ERBB4 activated, ERBB4 is cleaved by γ -secretase and released from membrane. ERBB4-ICD translocate into nucleus and acts as transcription factor [45,46]. In our experiment, expression levels of ERBB4-ICD are not change by *ERBB* or *ERBB* N706D or R927Q overexpression (Fig. 2A). ERBB4 is transmembrane protein containing ligand binding site, it cleaved by γ -secretase and releases intracellular domain from cell membrane and translocate into nucleus [45]. Therefore, it is crucial for ERBB4 to be localized at the cell membrane to function appropriately. We fractionated NSC-34 cells expressing GFP-labeled *ERBB4*, *ERBB4* N706D, and *ERBB4* R927Q into cytosol and membrane components. We used Western blotting to assess the level of ERBB4 in the membrane fraction. Our observations revealed that only ERBB4 N706D exhibited lower membrane localization compared to the wild-type protein (Fig. 2B). The ratio of ERBB4 to p-ERBB4 in the membrane fraction was lower in both the N706D and R927Q mutants compared to the wild-type protein (Fig. 2B).

These results suggest that the novel *ERBB4* variant is a loss of function mutation similar to previously known ALS-associated ERBB4 mutations. Next, we assessed cell viability by overexpressing GFP-labeled *ERBB4*, *ERBB4* N706D, or *ERBB4* R927Q in NSC-34 cells using the Viafect Transfection Kit. Our findings demonstrated that overexpression of ERBB4 significantly decreased cell viability compared to the control group. Furthermore, ERBB4 N706D exhibited stronger cytotoxicity than the wild-type protein. These results suggest that the ERBB4 N706D mutation leads to enhanced cytotoxicity compared to the wild-type (Fig. 2C). All these results suggest that reduced *ERBB4* function by novel mutation may be a pathogenic mechanism in ALS.

4. Discussion

The advancement of genetic technology, including whole-genome sequencing and gene-editing, has provided new opportunity to understand about ALS-associated genetic mutations [47]. Using targeted-next generation sequencing, we found a novel variant of *ERBB4* (c.2116 A > G) in ALS patient. The variant was a mis-sense mutation that replaced the 706th arginine in the ERBB4 protein with an aspartic acid, and the

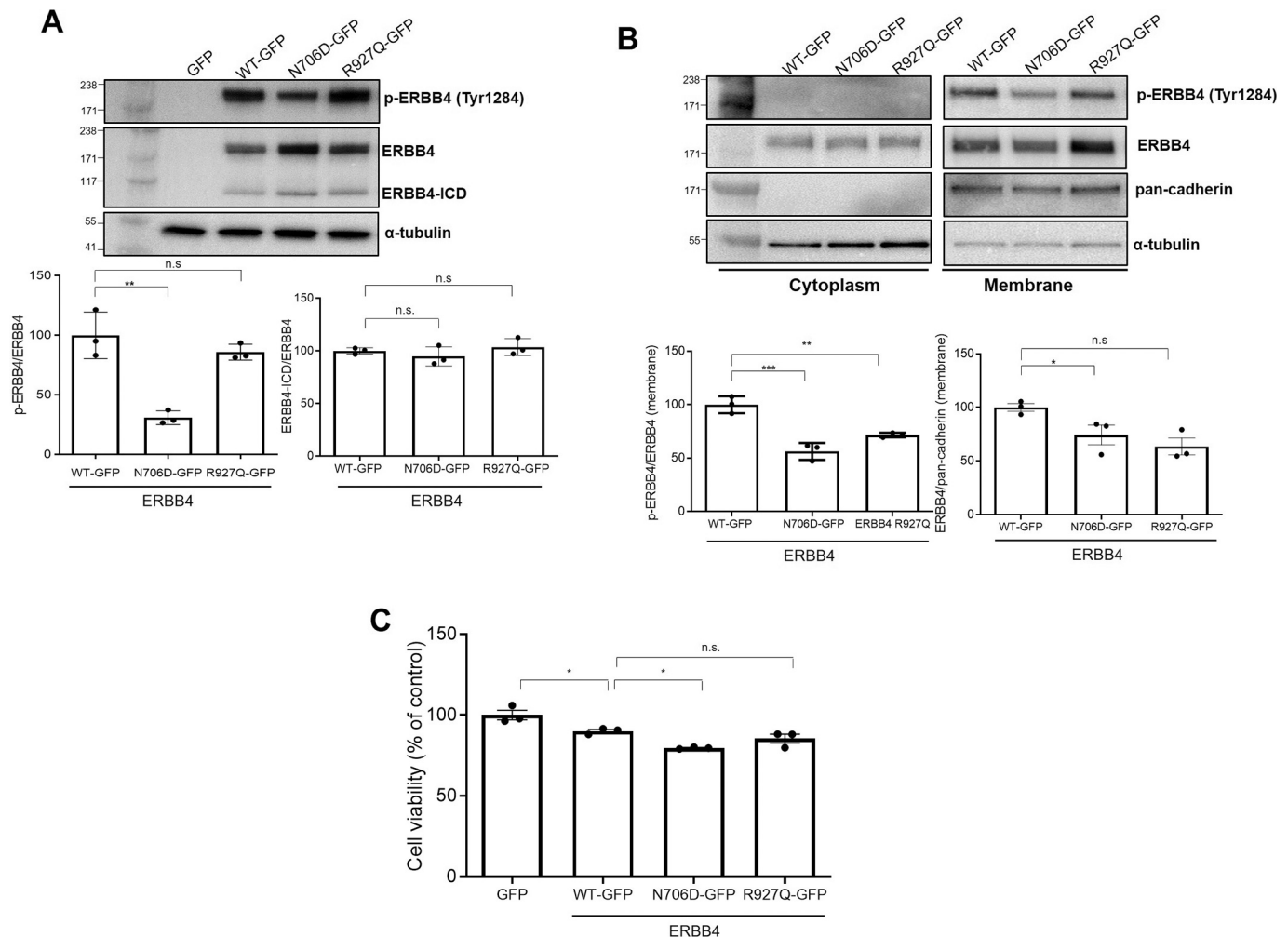


Fig. 2. ERBB4 N706D mutation decrease biological activity.

Overexpression of GFP, *ERBB4* or *ERBB4* mutants (N706D and R927Q) were transfected in NSC-34 cells for 48 h. (A) Cell lysates were immunoblotted with ERBB4 and phospho-ERBB4. Phosphorylation levels of ERBB4 were significantly decreased in ERBB4 N706D mutation. Quantitative data shows phospho-ERBB4 to ERBB4 and ERBB4-ICD to ERBB4. α -tubulin used for internal control. (B) Cells were fractionated to cytosol and membrane. Cell lysates were immunoblotted with ERBB4 and phospho-ERBB4. α -tubulin (cytosol) and pan-cadherin (membrane) were used for internal control. Expression of ERBB4 levels were significantly decreased in ERBB4 N706D mutation in membrane fractions. Quantitative data shows phospho-ERBB4 to ERBB4 and ERBB4 to pan-cadherin in membrane fractions. (C) Cell viability was measured using a CCK-8 assay. Quantification was performed on mean $\pm$ standard error (SEM) from 3 or 4 independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. not significant (One-way ANOVA with Tukey's multiple comparisons test).

mutation was predicted to be pathogenic by multiple *in silico* assays. To characterize the new mutant, we expressed it in NSC-34 cells and determined its phosphorylation level and membrane localization. Similar to the previously discovered ALS-associated *ERBB4* mutation, the new *ERBB4* mutation (N706D) caused reduced phosphorylation of *ERBB4* and membrane localization compared with *ERBB4* wild-type. Moreover, knock-down of *ERBB4* in primary neurons reduces neuronal survival.

ERBB4 is one of the members of *ERBB* family, which also includes *ERBB1*, *ERBB2* and *ERBB3*. *ERBB4* generated four isoforms (JM-a, JM-b, CYT-1, and CYT-2) via alternative splicing [48,49]. When NRG-1 binds to *ERBB4*, *ERBB4* undergoes hetero- (with *ERBB2*), or homo- dimerization and autophosphorylation to plays role in cellular functions. NRG-1 and *ERBB4* expressed in various central nervous system including motor neuron of spinal cord [34,50,51]. Impairment of NRG-1/*ERBB4* pathway induce impairment of neuronal migration, neurite outgrowth, and synapse formation [52]. NRG-1/*ERBB4* pathway also regulates both excitatory (glutamatergic) and inhibitory (GABAergic) transmission of neurons [53–55]. It is already known that *ERBB4* also implicated in neurodegenerative diseases such as ALS, PD, and AD. *ERBB4* express dopaminergic neuron of substantia nigra [56], *ERBB4* expression increased in dopaminergic neuron in PD patients compare with controls [57]. Moreover, *ERBB4* expression significantly upregulated in hippocampal neurons in AD patients [58]. Amyloid beta (A β)-induced tau phosphorylation is one of the factors in AD, inhibition of *ERBB4* restore A β -induced toxicity through reduction of tau phosphorylation [59].

ERBB4 expression are reduced in SOD1^{G93A} mouse and sporadic ALS patient's motor neurons. Furthermore, motor neurons with reduced *ERBB4* expression had higher rates of cytoplasmic accumulation of TDP-43, a typical ALS pathology, in the spinal cord of ALS patients [34,51]. Previous study has reported that *ErbB4* mRNA is a binding partner of TDP-43, and mislocalization of TDP-43 influences transcription of *ErbB4* in mouse primary neurons [60]. Moreover, knock-down of *TDP-43* in primary neurons impaired NRG-1/*ERBB4* pathway leads to dendritic loss and neuronal toxicity [60].

ERBB4 related ALS manifested autosomal dominantly inherited adult onset or sporadic cases demonstrating typical upper and lower motor neuron involvement, slow progression, without cognitive impairment [22]. The first reported *ERBB4*-related familial ALS manifested as late onset, incomplete penetrance within families, and the causal variant was found in the tyrosine kinase domain (R927Q) and C-terminal domain (R1275W) of the *ERBB4* gene. In this report, patients slowly progressed for more than five years after symptom onset, clinically reflecting a slow progression. Our case is consistent with the previous study as our patient also presented with the classical form of ALS without cognitive impairment. However, our patient showed rapid progression leading to a bedridden state with the usage of mechanical ventilator support within 18 months from symptom onset, which differs from the previous report. In line with previous studies, *ERBB4* plays a role in neurodegenerative diseases, including ALS. Our results provide new insight into *ERBB4*-mediated ALS pathogenesis.

CRediT authorship contribution statement

Younghwi Kwon: Writing – review & editing, Writing – original draft, Data curation. **Minsung Kang:** Data curation. **Yu-Mi Jeon:** Writing – review & editing. **Shinrye Lee:** Writing – review & editing, Visualization, Funding acquisition. **Ho-Won Lee:** Writing – review & editing, Writing – original draft. **Jin-Sung Park:** Writing – review & editing, Writing – original draft, Funding acquisition. **Hyung-Jun Kim:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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