

# p53 and Cell Cycle Proteins Participate in Spinal Motor Neuron Cell Death in ALS

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**Abstract:** Apoptosis has been implicated in many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). We previously demonstrated a role for G1 to S phase cell cycle regulators in ALS with increased levels of hyperphosphorylated retinoblastoma (ppRb) and E2F-1 in ALS spinal cord motor neurons. In this study we examined the levels of the cell cycle checkpoint tumor suppressor protein p53 with concurrent changes in cell death markers during ALS. Expression and subcellular distribution of p53, retinoblastoma, Bax, Fas, and caspases were explored by immunoblot, immunohistochemistry and double-label confocal microscopy in the spinal cord and motor cortex of ALS and control subjects. We identified elevated levels of p53 in ALS spinal cord motor neurons but not neurons in the motor cortex. In addition, there was an increase in Bax, Fas, caspases-8 and -3 proteins in ALS spinal motor neurons. While caspase-3 and TUNEL labeled neurons were positive for ppRb, E2F-1 and p53 in spinal motor neurons, and Fas co-localized with caspase-8 in spinal motor neurons, we failed to observe these results in large neurons in the motor cortex of ALS subjects. We have linked p53 and activation of G1 to S phase cell cycle regulators to an apoptotic mode of cell death ALS spinal cord motor neurons.

**Keywords:** Amyotrophic lateral sclerosis, p53, retinoblastoma, caspase.

## INTRODUCTION

The control of cell proliferation or growth arrest is intimately linked to the control of programmed cell death or apoptosis. Cell cycle components such as ppRb, E2F and p53 function in both cell cycle progression and apoptosis [1-3]. In fact the promoters of p53, p73, human cytochrome c1 and apoptotic protease activating factor 1 (APAF1) contain E2F-1 binding sites suggesting an E2F-1-mediated transcriptional regulation [3-6].  $\beta$ -amyloid-mediated toxicity in PC12 cells was found to be mediated by a E2F-1 - p53 - Bax pathway and the over-expression of E2F-1 in these cells led to DNA fragmentation in addition to the induction of p53 (by E2F-1) and Bax (by p53) [7]. These studies directly link E2F-1 to p53 and apoptosis.

In models of acute and chronic neurological disease, a role for aberrant cell cycle activation and subsequent neuronal apoptosis has been established. These studies include cell death mediated by  $\beta$ -amyloid, presenilin-1, DNA damage, oxidative stress, membrane depolarization, and 6-hydroxydopamine (6-OHDA) [8-12]. DNA damage induced phosphorylation of p53 has been shown to activate cell cycle proteins leading to neuronal cell death [13]. Inhibition of p53 through the use of antisense and dominant negative constructs protects neurons from this wide array of toxic insults [14, 15].

Motor neurons of ALS patients exhibit characteristics of apoptosis in post-mortem tissues [16], and apoptosis is believed to be the mode of cell death in ALS [17]. Increased levels of p53 with concomitant elevation in DNA binding activity has been demonstrated in motor neurons and astroglia of the affected regions of ALS patients but not in controls [18]. This study was corroborated in ALS transgenic mice over-expressing mutant G86R SOD1 protein [19]. Defective DNA repair and enhanced DNA damage are also evident in ALS motor neurons [20-23]. Cell death induced by DNA damage of cultured motor neurons has been linked to Fas and p53 activation [24]. Increased p53 gene expression has been observed in degenerative spinal cords from the wobbler mouse model and in the ventral horns of ALS patients [25].

In the current study we examined the expression and distribution of p53, markers of apoptosis and G1 to S phase cell cycle regulators. We hypothesized that alterations in the cell cycle regulator, p53, will result in concurrent changes in cell cycle proteins and apoptotic death markers during ALS. We present evidence for increased accumulation of p53 in the nucleus of ALS spinal cord motor neurons but not neurons of the motor cortex. Laser scanning confocal microscopy demonstrates that the G1 to S phase cell cycle regulators co-localize with markers of apoptosis (TUNEL and Caspase-3) and p53. Taken together, these results suggest a role for p53 and caspase activation in conjunction with aberrant re-activation of cell cycle regulators during sporadic ALS (SALS).

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## MATERIALS AND METHODOLOGY

### Source of Tissue Samples

The lumbar spinal cord and pre/post-central gyrus (motor/sensory) region from eighteen cases of clinically diagnosed (using El Escorial criteria) and neuropathologically confirmed sporadic ALS (SALS), and ten non-ALS age-matched controls, were utilized to examine protein expression and distribution as previously detailed [26]. All autopsy tissues were obtained from the University of Pittsburgh ALS Tissue Bank. Human tissue samples were obtained at the time of autopsy using University of Pittsburgh Internal Review Board approved consent forms. Approval for use of autopsy human tissues was obtained from the University of Pittsburgh Committee for Oversight of Research Involving the Dead (CORID). Neuropathologic assessment was used to confirm the clinical diagnosis of each subject.

### Antibodies

Monoclonal antibodies were used to detect E2F-1 (KH95; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (DO-1; Santa Cruz), and Fas (B10; Santa Cruz). Polyclonal antibodies were used to detect ppRb (NEN Biolabs, Ipswich, MA), actin (Millipore, Billerica, MA, USA), BAX (BD Biosciences, San Jose, CA, USA), caspase-3 (Cell Signaling Technology, Danvers, MA, USA), caspase-8 (Biomedica Corp, Foster City, CA), p73 (H79; Santa Cruz). The antibody concentrations for immunoblotting were 1:600 (caspase-8), 1:700 (caspase-3), 1:750 (p53, p73, Fas), 1:1000 (BAX) and 1:1500 (Actin). For light microscopy, these antibodies were used at dilutions of 1:100 (caspase-8), 1:150 (p53, caspase-3, Fas), 1:200 (BAX). For confocal laser scanning microscopy, we used primary antibody concentrations at 1:80 (ppRb, p53, caspase-3, caspase-8, Fas), and 1:60 (E2F-1).

### Light and Confocal Microscopy

Paraffin-embedded tissue sections from lumbar or cervical spinal cord and mid-motor cortical regions were used as per published protocols [26]. The dorsal horn of the spinal cord and sensory cortex were the internal controls for immunohistochemistry. The study was conducted in a blinded fashion using eighteen ALS and ten age-matched control cases.

For laser scanning confocal microscopy, tissues were processed as for light microscopy with the following modifications. Primary antibodies were used at a higher concentration (ppRb; and the slides incubated overnight at 4°C. After incubations with biotinylated secondary antibodies (1:1000), the signal was further amplified using the tyramide amplification kit as per manufacturer's protocol. The sections were finally incubated with a 1:500 dilution of streptavidin-Alexa Green (green) or streptavidin-Cy5/Cy3 (red) at a dilution of 1:500. A total of 70-80 and 20-30 motor neurons in the spinal cord and motor cortex, respectively, were counted at 400X magnification. The confocal study was conducted in a blinded fashion. Single labeling and other control experiments were performed to determine optimal concentrations of primary and fluorescently labeled secondary antibodies. Omission of the primary antibody resulted in absence of fluorescent signal.

Crossover control experiments were also performed to determine the specificity of the secondary antibody.

### Protein Extraction

Lumbar spinal cord and motor cortical (mid- and superior-motor cortical regions) frozen tissue from controls and ALS cases were utilized for immunoblotting and DNA binding assays. For total cell lysates, tissue samples were homogenized using polytron homogenizer (PGC Scientific, Gaithersburg, MD) set at 15000 rpm for 45 secs. It was carried out in lysis buffer containing 25mmol/L HEPES (pH 7.4), 50mmol/L NaCl, protease inhibitor cocktail II (SIGMA), and 1% triton-X 100. The homogenized product was spun at 14000 rpm in a cold microfuge and the supernatant saved as the total cell lysate. Nuclear and post-nuclear extracts were extracted as described previously [26]. Briefly, protein extracts were prepared by detergent lysis on ice (0.1% Nonidet P-40, 10 mM Tris (pH 8.0), 10 mM MgCl<sub>2</sub>, 15 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A, and 1 µg/ml leupeptin). The nuclei were collected by low speed centrifugation at 800x g for 5 mins. The supernatant was saved as the post-nuclear supernatant and the pellet containing the nuclei was further extracted with high salt buffer (0.42 mol/l NaCl, 20 mmol/L HEPES (pH 7.9), 20% glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A, and 1 µg/ml leupeptin) on ice for 10 mins. Residual insoluble material was removed by centrifugation at 14,000 x g for 5 mins. The resulting supernatant fraction was collected and termed the "nuclear extract". Protein concentrations were determined by Bio-Rad assay (Bio-Rad, Richmond, CA, USA).

### Immunoblotting

Total cell lysates, nuclear extracts and post-nuclear supernatants were fractionated by electrophoresis on an 8, 10 or 12% sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride nylon membranes (NEN Biolabs) and blocked in 5% nonfat milk/1X PBS or 0.5% BSA / 0.15% glycine in 1X PBS overnight at 4°C. The blots were probed individually with the primary antibodies overnight at 4°C in 0.5% milk/PBS. The blots were washed three times in PBS/0.1% Tween-20 for 15 minutes. Isotype-specific horseradish peroxidase-conjugated secondary antibodies (Chemicon; goat anti-mouse; 1:2000, goat anti-rabbit; 1:2000) were added for 2 hrs at room temperature. The secondary antibodies were washed extensively in PBS / 0.1% Tween-20 (three times for 20 mins). The final reaction products were visualized using enhanced chemiluminescence (ECL; Pierce) and the band intensities were within the linear range of detection. Actin was used to normalize protein levels within each sample. The density of bands was measured using the NIH Image software version 1.58 (National Institutes of Health, USA).

### Electrophoretic Mobility Shift Assay (EMSA)

Gel mobility shift assays were performed as described earlier [26] with the following modifications. The sequences used to probe for p53 specific DNA binding activity were: WT p53 5'-TACAGAACATGTCTAAGCATGCTGGGGA CT -3'; MT p53 5'- TACAGAATCGCTCTAAGCATGCT GGGACT -3'. For competition reactions, unlabeled p53

competitor (3, 30, and 100ng) or unlabeled unrelated competitor (5'-GATCATTCAGGTCATGACCTGA-3'; 100, and 300ng) oligonucleotides were pre-incubated with the protein for 5 mins on ice prior to addition of labeled probe. The positive control for p53 DNA binding activity was camptothecin-treated SHYSY5 neuroblastoma cells.

### Terminal Deoxynucleotidyl Transferase-Mediated Biotinylated UTP Nick End Labeling Staining (TUNEL)

DNA fragmentation was assayed using the fluorescein based apoptosis detection system as per manufacturer's instructions (Promega, USA). Briefly, paraffin embedded sections were processed as for immunohistochemistry and post-blocking were treated with Proteinase K (20µg/ml) for 15 mins. They were then incubated with TdT enzyme/nucleotide mix for 1 hr at 37°C. For double labeling with confocal laser scanning microscopy, the sections were immunolabeled with p53 or ppRb after TUNEL labeling as described earlier. Negative (no enzyme) and positive (DNase I-treated) samples were used. Motor neurons positive for TUNEL and cell cycle proteins (ppRb, p53) were quantified. A total of 30-40 and 20-30 motor neurons in the spinal cord and motor cortex, respectively, were counted at X400 magnification. The study was conducted in a blinded fashion using 11 ALS and 6 age-matched control cases.

### Statistical Analysis

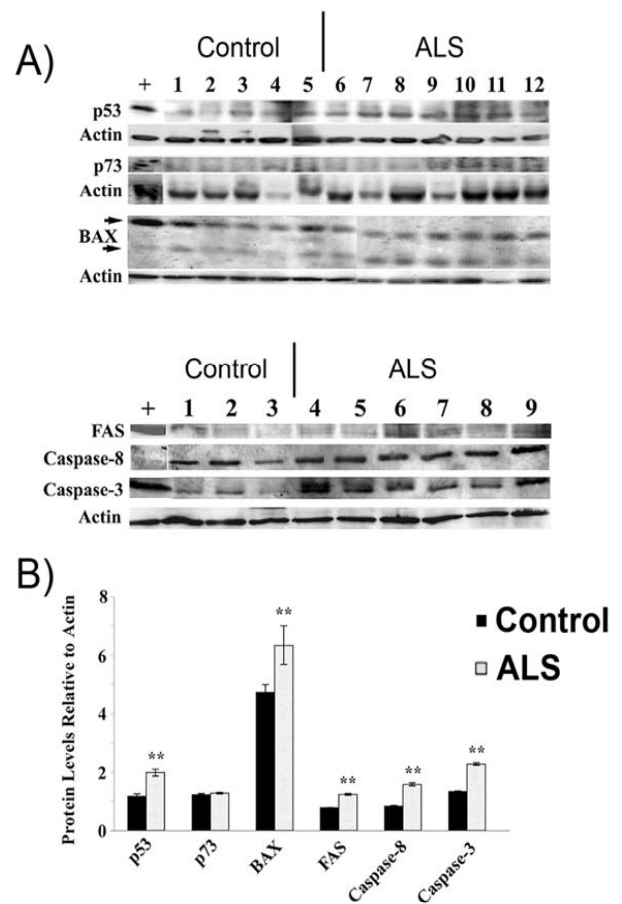
Comparisons between any two groups of data were done using the single-factorial analysis of variance (ANOVA). A *p*-value of  $\leq 0.05$  was considered statistically significant. Numerical data were expressed as means  $\pm$  the standard deviation with "n" signifying the number of experiments or cases.

## RESULTS

### Altered Levels of Cell Death Markers and Cell Cycle Proteins in ALS

We first examined levels of numerous proteins involved in cell death pathways and the cell cycle by immunoblot analysis, using total tissue lysates from lumbar spinal cord and motor cortex of eighteen SALS and ten age-matched control subjects. Representative results are shown in Fig. (1A). The levels of activated p53 were significantly increased in the total lysates of ALS lumbar spinal cord (Fig. 1) and nuclear extracts (data not shown). E2F-1 can also activate other members of the p53 family such p73. Therefore we performed immunoblotting to analyze p73 levels in ALS tissues but found its levels unchanged in ALS spinal cord tissues (Fig. 1). Since p53 can transactivate pro-apoptotic molecules such as Bax, we examined the levels of Bax and other pro-death proteins including Fas, and active caspase-3 and -8. Levels of Bax, Fas, active caspase-3 and -8 were elevated in the total lysates of ALS spinal cord (Fig. 1B). In addition, we observed two immunoreactive bands at 19 and 21 kDa with the Bax antibody. In contrast to results from lumbar spinal cord, p53 was undetectable in total lysates prepared from motor cortex (data not shown).

Fas, caspase-3 and caspase-8 also exhibited no significant changes in protein levels within the motor cortex of ALS and control subjects.

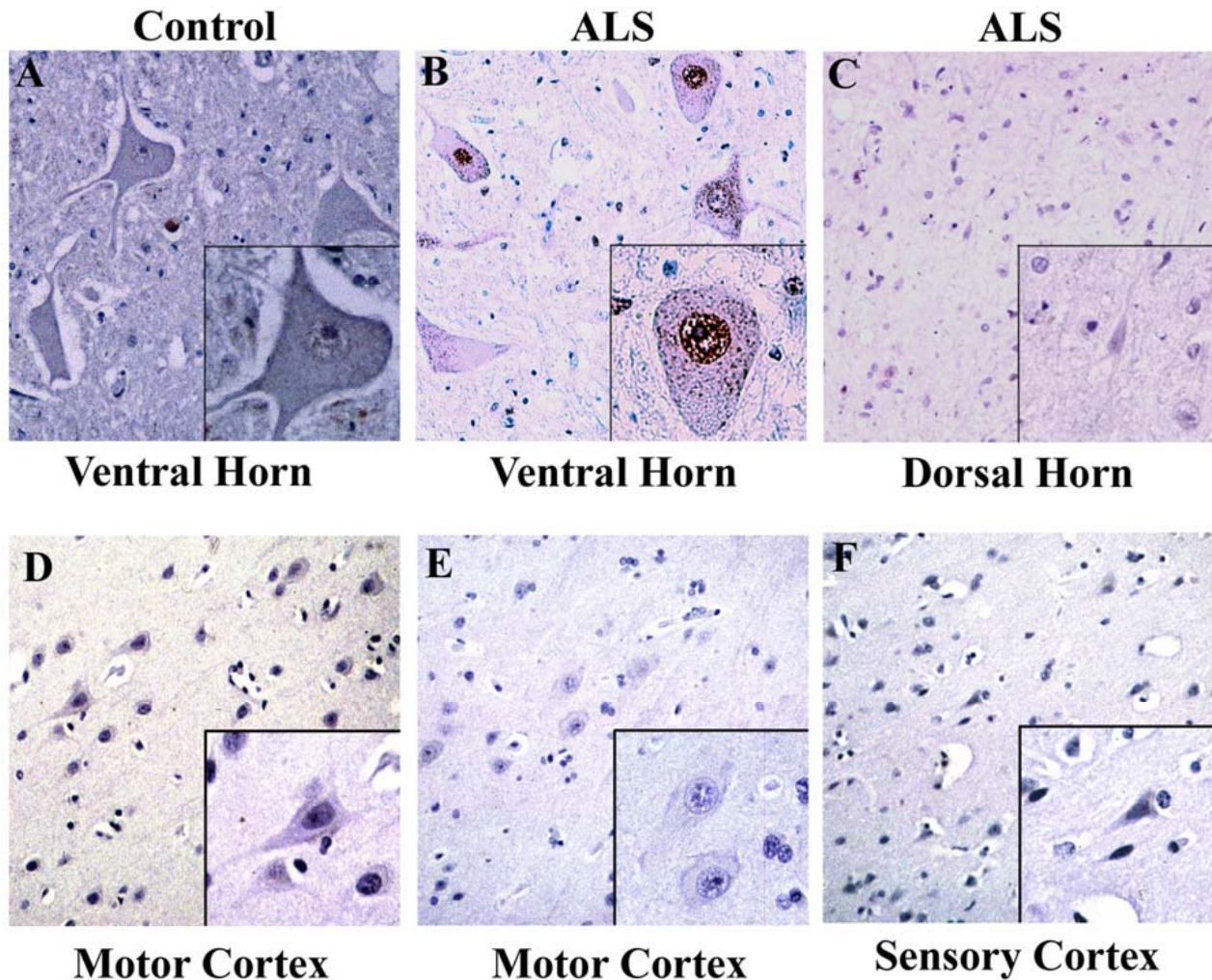


**Fig. (1).** Increased p53 and cell death markers in ALS spinal cord. Immunoblot analysis of lumbar spinal cord tissue extracts. **A)** Top panel: Lane assignments for p53, p73 and Bax are as follows: “+” indicates positive control, lanes 1 to 5 are control cases and lanes 6 to 12 are ALS cases. The arrows on the BAX blot indicate immunoreactive bands of 19- and 21-kDa. Bottom panel: Lanes for Fas and Caspase immunoblots are as follows: “+” indicates positive control, lanes 1 to 3 are control cases and lanes 4 to 9 are ALS cases. **B)** Quantification (mean  $\pm$  SEM) of the immunoblots. Black bars represent control cases (n=10) and the gray bars are ALS cases (n=18). Statistical analysis was performed using single-factor ANOVA and asterisks (\*\*) indicate  $p \leq 0.05$ .

### Increased p53 in ALS Spinal Cord But Not Motor Cortex

Immunoblotting of tissue extracts presents the caveat of having a mixed population of cells. Therefore we utilized immunohistochemistry to analyze cell specific and sub-cellular localization of p53 in ALS tissues. Paraffin-embedded sections from lumbar spinal cord and motor cortex from the same cases were analyzed by light microscopy.

In control subjects, 38% of lumbar spinal cord motor neurons were p53 immunoreactive, and the protein was predominately located in the cytoplasm (Fig. 2A). Approximately 84% (from a total of 200) of the lumbar spinal cord motor neurons in ALS cases exhibited abundant nuclear accumulation of p53 (Fig. 2B). There was negligible or absent p53 immunostaining in the dorsal horn sensory neurons (Fig. 2C). In addition, p53 immunoreactivity was



**Fig. (2).** Increased p53 immunoreactivity in ALS spinal cord motor neurons. Lumbar spinal cord (A-C) and motor cortex (D-F) tissues from 18 ALS and 9 non-neurological disease controls were immunostained for p53 using a phospho-specific anti-p53 antibody. Each panel is at X200 magnification with the insets at X400. Relative to controls (panels A and D), there is abundant nuclear p53 in ALS spinal cord (B) but not motor cortex (E). Panels C and F are the internal controls for the respective regions.

absent in both the cortical motor and sensory neurons of ALS and the age-matched controls (Figs. 2D-F). We failed to detect altered p73 levels in ALS spinal cord or motor cortex (data not shown).

#### Sub-Cellular Distribution of Apoptotic Death Markers in ALS Spinal Cord and Motor Cortex

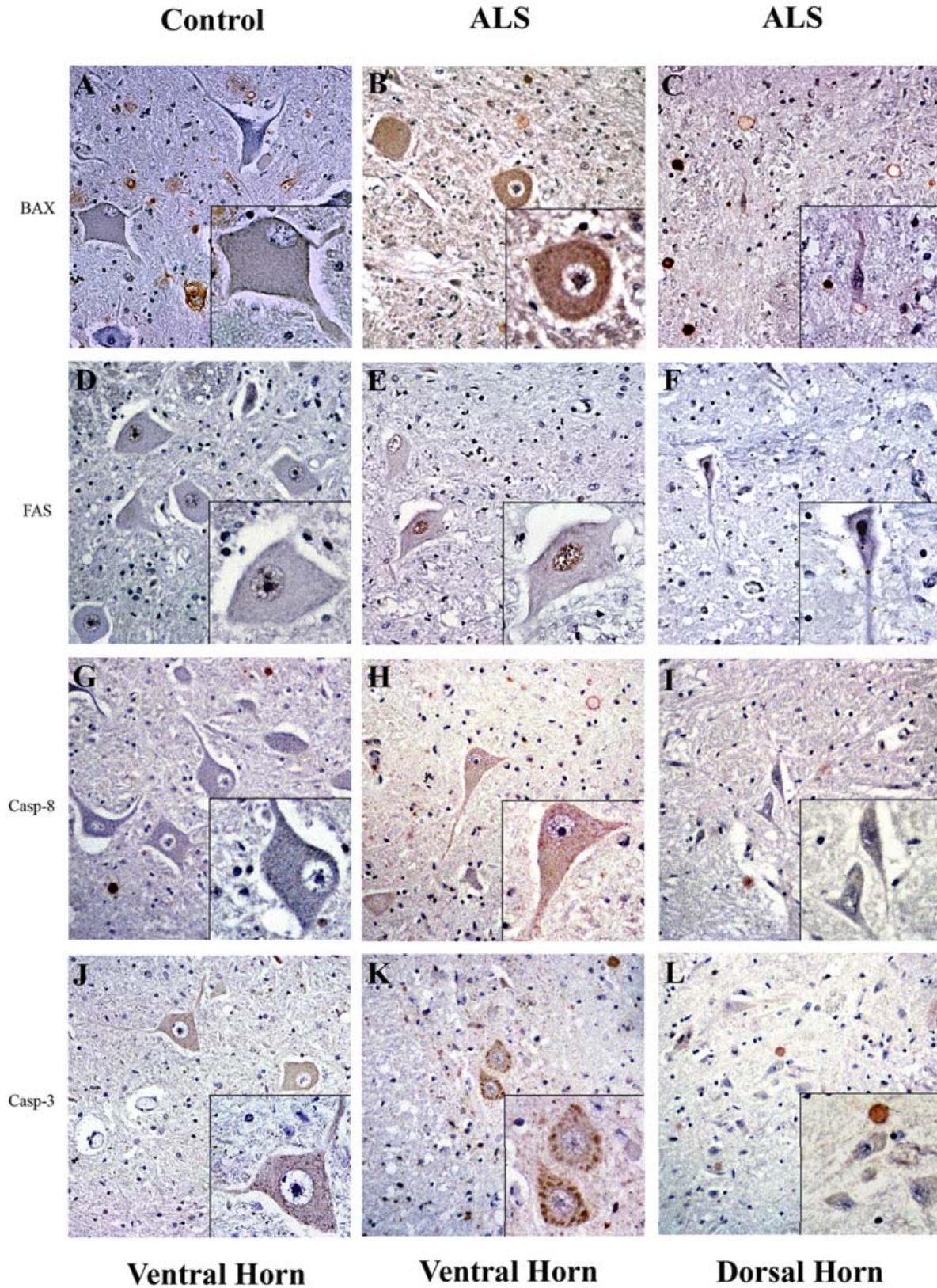
We next analyzed the distribution of other pro-apoptotic proteins by immunohistochemistry. Bax was elevated in ALS lumbar ventral motor neurons (cytoplasm and nucleus) but not in the dorsal sensory neurons (Figs. 3A-C). We also observed increased levels of Fas in the nucleus of ALS spinal motor neurons (Figs. 3D-F). Caspase-8, a downstream effector of Fas, and caspase-3 were both increased in ALS spinal motor neurons (Figs. 3G-L).

In the motor cortex, we noted Bax immunoreactive cells but not pyramidal neurons (data not shown). Fas immunoreactivity was negligible throughout the motor cortex (Fig. 4A). Weak caspase-8 immunostaining was

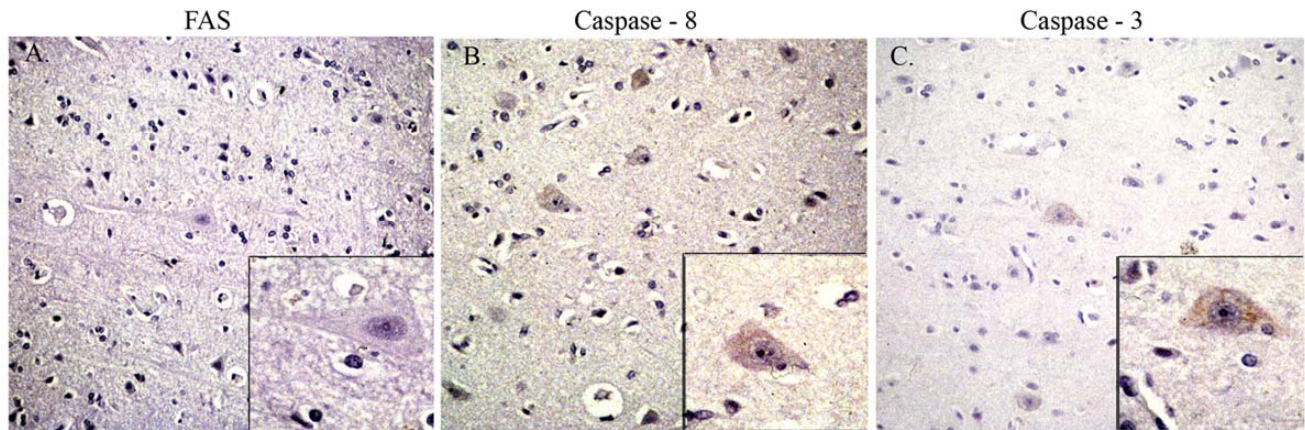
observed in neurons that did not differ between control and ALS (Fig. 4B). In ALS subjects, caspase-3 immunoreactivity was evident in a few neurons of the motor cortex (Fig. 4C), but it was not different from that of control subjects (data not shown).

#### Co-Localization of G1 to S Phase Regulators and Apoptotic Death Markers in ALS Spinal Cord and Motor Cortex

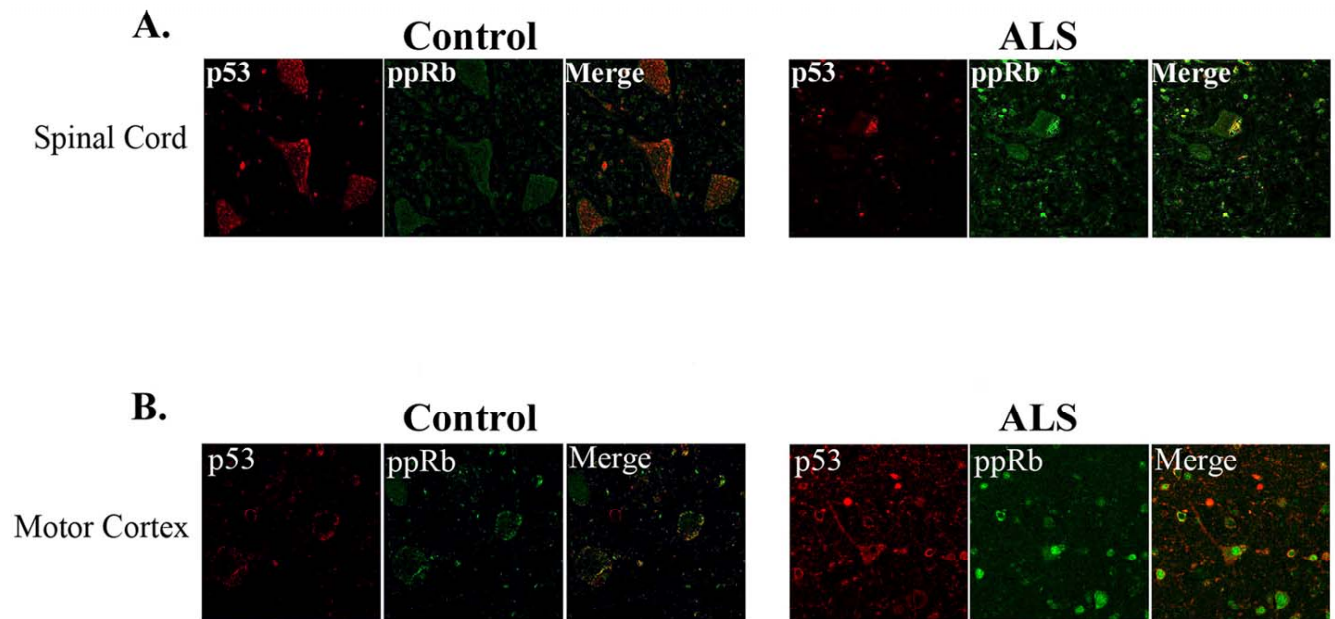
We next determined if G1 to S phase cell cycle regulators co-localize with p53 in ALS spinal and cortical motor neurons. In ALS, 68% of spinal cord motor neurons that displayed increased ppRb also had increased levels of E2F-1 (data not shown) and 61% of ppRb positive spinal motor neurons were also p53 immunopositive (Fig. 5A). Nuclear co-localization was specific to ALS and not present in control cases, though cytoplasmic p53 was evident in the control lumbar spinal cord. Within the motor cortex, p53 did not co-localize with ppRb (Fig. 5B).



**Fig. (3).** Increased immunoreactivity of cell death markers in spinal cord motor neurons. Lumbar spinal cord tissues from 18 ALS and 9 non-neurological disease controls were immunostained for BAX (A-C), Fas (D-F), caspase-8 (G-I) and caspase-3 (J-L). Each panel is at X200 magnification with the insets at X400. Relative to controls, there is increased BAX (B), nuclear Fas (E), caspase-8 (H) and caspase-3 (K) in ALS spinal cord motor neurons. Panels C, F, I and L represent immunostaining of sensory neurons of the ALS spinal cords.



**Fig. (4).** Cell death markers in cortical motor neurons. Motor cortex tissues from 18 ALS and 10 non-neurological disease controls were immunostained for Fas (A), caspase-8 (B) and caspase-3 (C). There was negligible staining for each protein in motor neurons of the cortex. Each panel is at X200 magnification with the insets from the same cases at X400.



**Fig. (5).** Co-localization of ppRb and p53 in motor neurons of ALS spinal cord. Tissue sections were immunostained for p53 and ppRb by double-label confocal microscopy. The secondary antibodies were either conjugated to a red dye (Cy5) or a green dye (Alexa green). While p53 co-localized with ppRb in the nucleus of spinal motor neurons (A), nuclei of cortical motor neurons lacked p53 but contain abundant ppRb (B). All panels are at X400 magnification.

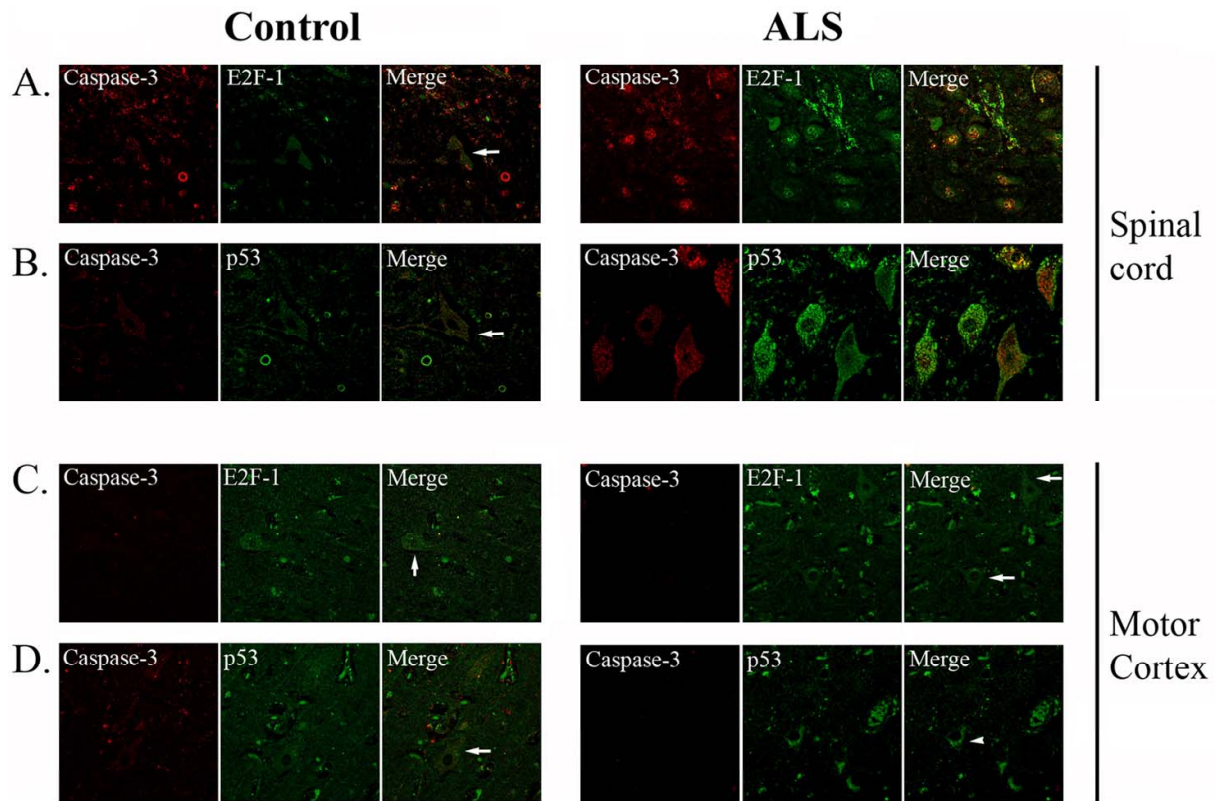
Caspase-3 immunoreactivity was present in 22% of E2F1 positive spinal motor neurons and 32% of p53 positive motor neurons across 12 ALS cases (Figs. 6A, B). Motor neurons positive for activated caspase-3 in ALS subjects were not widespread, i.e., not all motor neurons were positive for caspase-3. Spinal motor neurons in control cases lack E2F-1 immunoreactivity and exhibited low levels of caspase-3 (Fig. 6A). E2F1 positive neurons in the motor cortex of ALS patients lacked caspase 3 immunoreactivity (arrows in Fig. 6C). The p53 and caspase-3 levels were negligible or absent in cortical motor neurons (Fig. 6D).

We next examined Fas and caspase-8 in the spinal cord of control and ALS subjects (Fig. 7). While caspase-8 immunoreactivity was observed in small cells in control subjects, we noted strong Fas immunoreactivity

predominantly in the nucleus of motor neurons in ALS and co-localization with caspase-8 (Fig. 7). In cortical motor neurons, there was negligible staining for either protein in all cases (data not shown).

#### DNA Fragmentation in ALS Spinal Motor Neurons

In addition to activated caspase-3, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end-labeling (TUNEL) staining was used as a measure of nicked or fragmented DNA, a characteristic feature of apoptosis. There were increased numbers of TUNEL positive motor neurons in ALS spinal cord (Figs. 8A, B) but not the motor cortex (Figs. 8C, D). Sections were analyzed to determine if ALS spinal motor neurons that exhibited increased ppRb or p53 were positive for TUNEL staining. We determined that



**Fig. (6).** E2F-1 and p53 co-localize with caspase-3 in ALS spinal motor neurons. Tissue sections of lumbar spinal cord (A and B) and motor cortex (C and D) from 11 ALS and 6 age-matched controls were labeled with the combinations of p53 or E2F-1 (Alexa green conjugated secondary antibody) and active caspase-3 (Cy5 conjugated secondary antibody). Arrows in the control panels indicate representative motor neurons. E2F-1 and p53 co-localize with caspase-3 in spinal motor neurons of ALS patients. All panels are at X400 magnification.

approximately 25% of the ppRb positive motor neurons and 30% of p53 positive motor neurons had fragmented DNA detected by TUNEL (arrowheads in Fig. 8A, B, respectively). We also identified ppRb or p53 positive but TUNEL negative ALS motor neurons (asterisk in Fig. 8A inset). Approximately 75% of the spinal motor neurons that exhibited TUNEL also contain nuclear p53. Motor neurons of control subjects lack TUNEL staining (Fig. 8). In the motor cortex, approximately 64% of the large pyramidal neurons were positive for ppRb (Fig. 8C, arrow) while only 9% were p53 positive (Fig. 8D). There were TUNEL positive small cells in the gray matter of the cortex but not in any large pyramidal neurons (arrows and arrowheads in Figs. 8C, D).

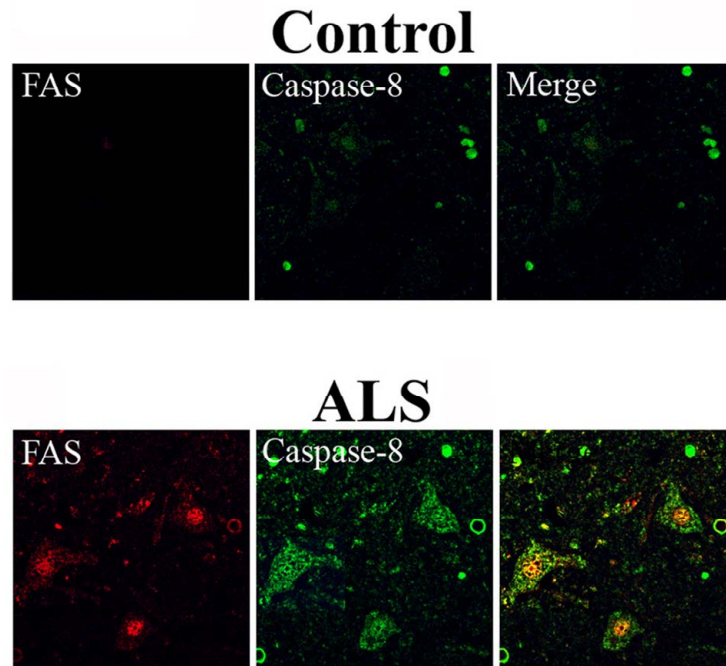
#### DNA-Binding Activity of p53

To determine the functional status of p53 in ALS, DNA binding activity was analyzed using nuclear extracts of spinal cord and motor cortex and radiolabeled oligonucleotide containing the consensus p53 binding site. DNA binding was monitored by gel mobility shift assays (EMSA) (Fig. 9). Competition experiments with excess unlabeled wild-type or mutant oligonucleotide demonstrated the specificity of the complex observed by EMSA (Fig. 9A). The p53 DNA binding activity in the spinal cord or motor cortex exhibited no significant difference between control and ALS (Figs. 9B-D).

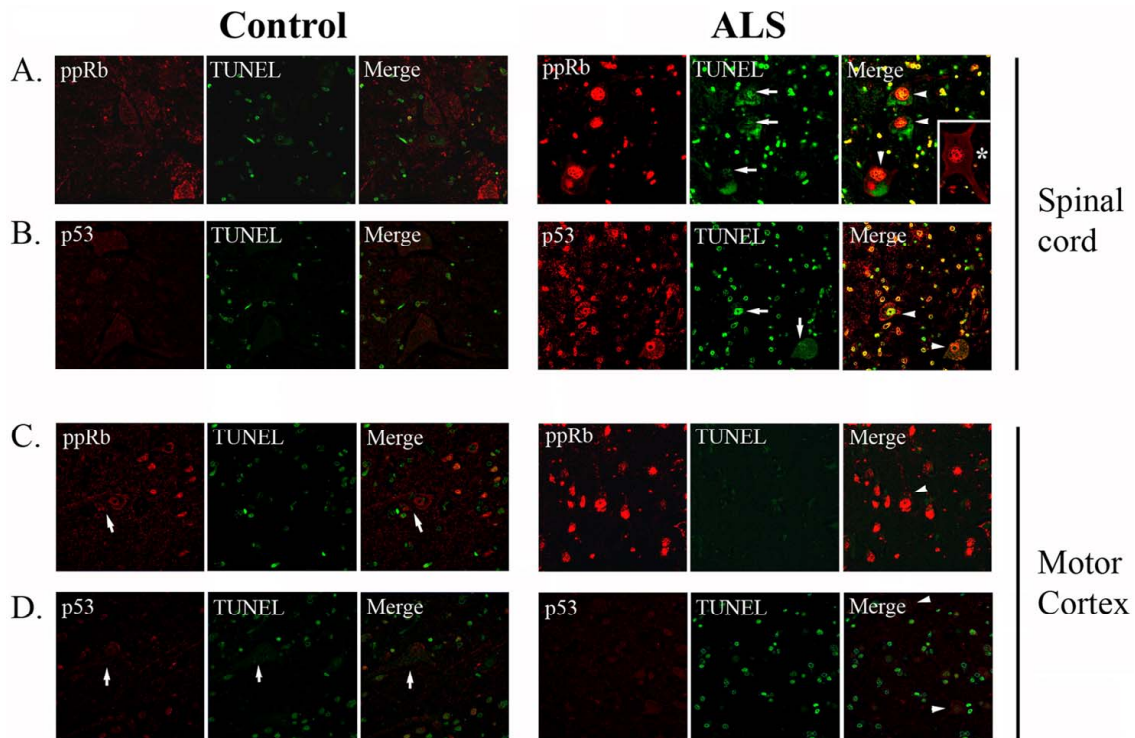
#### DISCUSSION

The balance between pro- and anti-apoptotic proteins modulates cell survival and influences the mode of cell death. Prior studies have suggested that motor neurons enter an apoptotic mode of cell death during ALS [17]. In this study we report altered levels and co-localization of p53 and pro-apoptotic proteins in ALS post-mortem tissues. Interestingly, p53 protein levels were increased in the ALS spinal motor neurons but not neurons of the motor cortex. Fas and caspase-8 immunoreactivity was also evident specifically in spinal motor neurons of ALS patients (Fig. 7).

The role of p53 in ALS has been questioned due to results obtained from transgenic SOD1 mice studies. Loss of p53 in p53<sup>-/-</sup>/G93A SOD1<sup>+</sup> double transgenic mice did not alter disease onset or progression [27]. A similar study using transgenic mice containing a low copy number of the G93A SOD1 mutant transgene reported identical results [28]. However SOD1 induced motor neuron cell death may occur *via* p53 independent mechanisms and not mimic conditions in sporadic ALS patients. Depending on the cell type, over-expression of p53 can result in either cell growth arrest or apoptosis [29-32]. Conversely, p53 knockout induces resistance to apoptosis [33, 34]. Thymocytes from these mice are resistant to DNA damaging agents such as ionizing radiation and topoisomerase II inhibitors (etoposide) but not

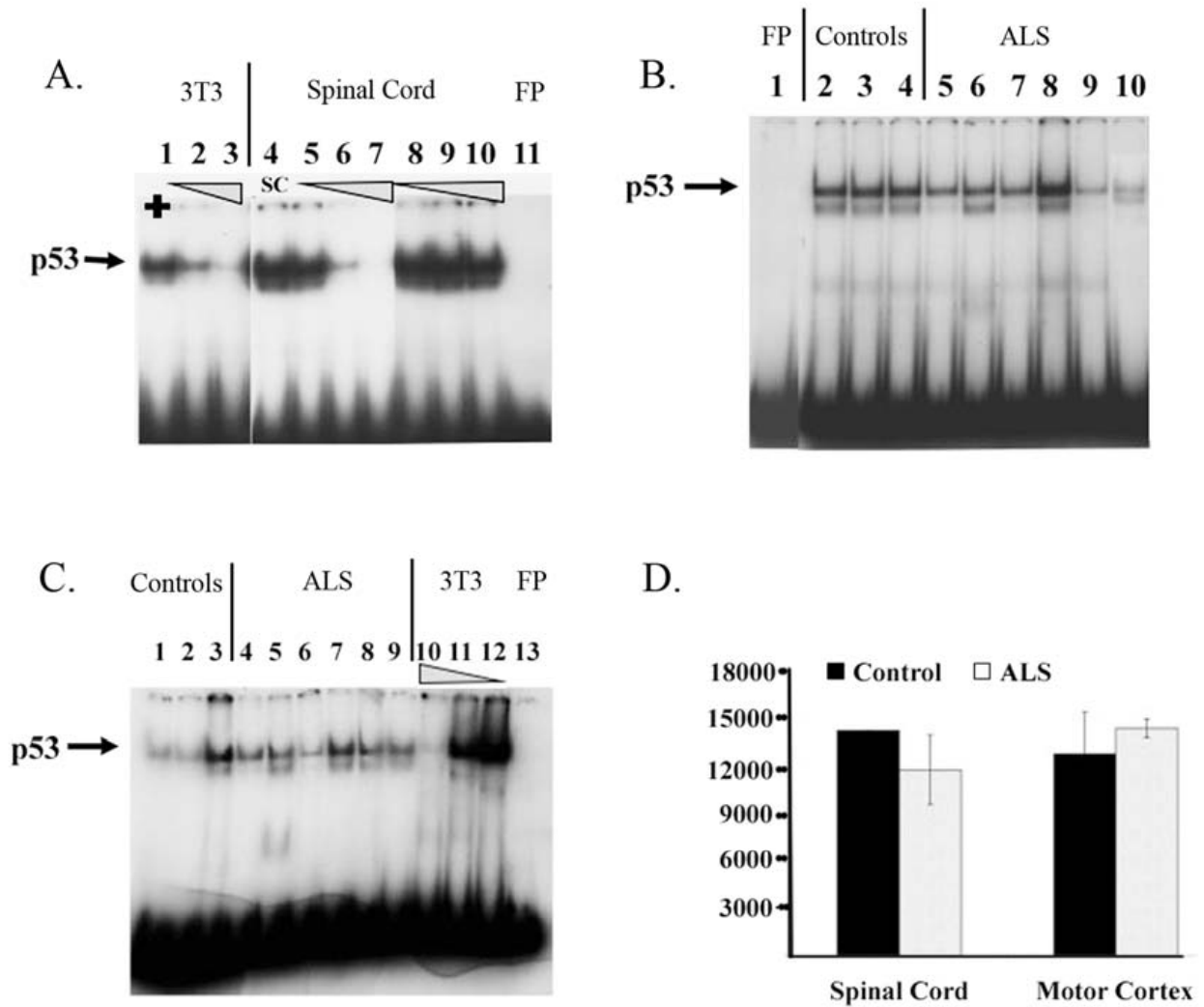


**Fig. (7).** Fas and Caspase-8 co-localize in spinal motor neurons. Lumbar spinal cord sections from 11 ALS and 6 age-matched controls were labeled with anti-caspase-8 (Alexa green conjugated secondary antibody) and anti-Fas (Cy5 conjugated secondary antibody) antibodies. In ALS motor neurons, Fas (red signal) is present in the nucleus and co-localized with caspase-8.



**Fig. (8).** Increased DNA fragmentation in spinal but not cortical motor neurons. Lumbar spinal cord (**A** and **B**) and motor cortex (**C** and **D**) sections from 11 ALS and 6 age-matched controls were double-labeled for TUNEL (FITC-conjugated) and p53 or ppRb (Cy-5 conjugated). Sections from all control subjects exhibited negligible co-localization of ppRb or p53 and TUNEL within neurons. Arrows in **A** and **B** indicate TUNEL positive spinal motor neurons. TUNEL positive motor neurons were also ppRb or p53 immunoreactive in the spinal cord (Merge panels; arrowheads). The asterisk in the inset depicts a ppRb(+) but TUNEL(-) ALS motor neuron. The cortical motor neurons in ALS cases were ppRb positive (**C**) and p53 negative (**D**). Motor neurons in panels **C** and **D** appear negative for TUNEL (arrows indicate control motor neurons and arrowheads indicate ALS motor neurons).





**Fig. (9).** No change in p53 DNA binding activity in ALS motor neurons. Nuclear extracts from lumbar spinal cord and motor cortex were used for electrophoretic mobility shift assays (EMSA). **(A):** Competition EMSA using radiolabeled p53 oligonucleotide and either extract from NIH3T3 cells as a positive control (lanes 1 to 3) or spinal cord nuclear extracts (25µg; lanes 4 to 7) indicating a concentration dependent competition (lanes 2 – 3 and 5 – 7) when excess cold oligonucleotides were used in the binding reaction. The complex remains unaltered when excess unrelated oligonucleotides (lanes 8 – 10) were utilized in the binding reaction. Lane assignments for competition assays were as follows: Lanes 2 and 3: 3T3 extract with excess unlabeled p53 oligonucleotide (3ng and 100ng, respectively); Lanes 5 to 7: spinal cord extract with excess unlabeled p53 oligonucleotide (3ng, 30ng and 100ng, respectively); Lanes 8 to 10: spinal cord extract with excess unlabeled unrelated oligonucleotide (30ng, 100ng and 300ng, respectively); Lane 11: Free radiolabeled probe alone (FP). **(B):** EMSA of control or ALS spinal cord nuclear extract and radiolabeled p53 oligonucleotide. Lane 1 is free probe alone; Lanes 2 to 4 are Control subjects; Lanes 5 to 10 are ALS cases. **(C):** EMSA of control or ALS motor cortex nuclear extract and radiolabeled p53 oligonucleotide. Lanes 1 to 3 are Control subjects; Lanes 4 to 9 are ALS subjects; Lanes 10 to 12 are 3T3 extracts with 100ng excess unlabeled p53 oligonucleotide (lane 10), 3ng excess unlabeled p53 oligonucleotide (lane 11), and radiolabeled p53 oligonucleotide without competitor (lane 12). Lane 13 is free probe alone. **(D):** Densitometry measurement of the p53:DNA complex in each lane using NIH 1.58 software indicates unaltered levels of DNA binding activity in either the spinal cord or motor cortex. Controls are denoted in black bars (n=3) and ALS cases in stippled bars (n=6). There is no statistically significant alteration in DNA binding activity.

to cell death induced by glucocorticoids or phorbol ester [35]. This suggests that both p53-dependent and independent cell death pathways exist.

We reported an aberrant activation of G1 to S phase regulators in ALS motor neurons marked by altered levels of ppRb and E2F-1 [26]. Our currently study indicates the co-localization of these cell cycle regulators with nuclear p53.

While p53 is negatively regulated by direct protein-protein interactions with modulators such as mdm-2, it is stabilized by the action of CDK inhibitors such as p14<sup>ARF</sup>. Furthermore, post-translational modifications such as phosphorylation can enhance the transcriptional activity of p53 [36]. Our immunoblot results indicate increased levels of phosphorylated p53 in the nuclear fractions of spinal cord

tissue extracts. In a prior study, protein levels of nuclear p53 and its DNA binding activity were increased in affected CNS regions of ALS patients [18]. However, we found no significant difference in the DNA binding activity of p53 in ALS spinal cord despite its enhanced nuclear localization. This result is likely due to the fact that the nuclear extracts used for EMSA are generated from a heterogeneous cell population within the tissue.

In normal physiologic conditions p53 has a very short half-life and is rapidly degraded. The presence of elevated levels of p53 in ALS patients suggests that the protein is aberrantly stabilized either by the down-regulation of proteins that negatively regulate p53 such as mdm2 or by reduced degradation of p53 *via* the proteasomal machinery. It is well documented that the proteasome machinery is compromised in neurodegenerative diseases such as ALS with concomitant increases in aggregate formations [37-39]. It is possible that p53 transcripts are stabilized in ALS through RNA binding proteins or that post-translational modification of p53 or p53 binding proteins can alter p53 stability and its consequent activity.

The TAR DNA binding protein (TDP-43) is a nuclear ribonucleoprotein (hnRNP) that functions in pre-mRNA splicing, transcription and stability of mRNA [40]. TDP-43 has been localized to cytoplasmic inclusions in ALS and frontotemporal lobar degeneration (FTLD) patients [41]. While transgenic mice expressing mutant human SOD1 fail to exhibit cytoplasmic TDP-43 inclusions (nor altered p53), the wobbler mouse exhibits TDP-43 accumulation and inclusions in the cytoplasm. Loss of TDP-43 increases cyclin-dependent kinase 6 (cdk6) levels, pRb hyperphosphorylation, and apoptosis. We observed increased nuclear ppRb in ALS motor neurons and co-localization of p53 and ppRb (Fig. 5). We propose that p53 participates in cell death induced by TDP-43 accumulation in cytoplasmic inclusions. Further studies are required to examine the link between TDP-43, p53 and motor neuron cell death.

We further investigated the correlation between p53 subcellular distribution and markers of motor neuron cell death. p53 co-localized with activated caspase-3 immunoreactivity and TUNEL staining (Figs. 6, 8). Altered subcellular distribution of p53 could be in response to DNA damage to induce re-entry into the cell cycle. DNA damage can activate proteins that induce cell cycle arrest and/or DNA repair. However, extensive DNA damage induces neuronal apoptosis. The presence of p53 in the nucleus of motor neurons that apparently lack dendritic processes with a rounded cell body suggest that nuclear accumulation of p53 may correspond to cell atrophy and death.

Our p53 data in the spinal cord of ALS patients contrasts that obtained in the motor cortex of these same subjects. Surprisingly, neurons of the motor cortex exhibited negligible p53 immunoreactivity. p53 was undetectable by immunoblot and its DNA binding activity unaffected by ALS. This suggests that motor neuron loss is p53-dependent in spinal cord motor neurons and p53-independent in upper motor neurons of the motor cortex. In addition, studies in adult mice and neuronal cell cultures show evidence for localized transcription-independent mechanism of p53 action in the synapses [42]. Oxidative and excitotoxic insults increased the levels of active (phosphorylated) p53 in

isolated cortical synaptosomes, which preceded the loss of synapsin I. This suggests that p53 can affect the dysfunction and degeneration of synapses independent of modulating gene expression.

Irrespective of the mechanism that induces cell death, apoptosis involves the induction of pro-apoptotic genes with downstream activation of caspases. Our data suggests the activation of apoptosis downstream of cell cycle activation. Both mitochondrial mediated (increased Bax and caspase-3) and death receptor-mediated pathways (increased Fas, caspase-8 and caspase-3) may be involved in lower motor neuron death in ALS. Active caspase-3 immunoreactivity was detected in punctate structures in the cytoplasm and the nucleus of spinal motor neurons. This data is consistent with prior studies in the mutant SOD1 transgenic mouse model of ALS that also implicates caspase induced apoptosis as a mechanism of motor neuron cell death [43, 44]. We also used DNA fragmentation by TUNEL staining as a measure of apoptosis in motor neurons. It is noteworthy to find that there was increased evidence for fragmented DNA in the spinal cord but not the motor cortex. In the spinal cord, the presence of p53 immunoreactivity in 75% of the TUNEL positive motor neurons suggests that DNA damage results in p53 accumulation. However only 30% of the p53 positive neurons were TUNEL positive, suggesting that p53 stabilization/activation may occur *via* other means unrelated to DNA damage. While TUNEL does not differentiate between single-strand (necrosis) and double-strand (apoptosis) DNA breaks, our results do indicate the presence of nicked DNA that co-localizes to the G1 to S phase regulators. These observations coupled with the presence of activated caspase-3 lend further support for an apoptotic mode of cell death in the spinal cord.

Fas-mediated cell death has been reported in cultured motor neurons from mutant SOD1 transgenic mice [45, 46]. Increased Fas ligand has been observed in the mutant SOD1 transgenic mice and spinal cord tissue of ALS patients [47]. In addition, there is evidence for p53 to mediate apoptosis through the Fas-mediated death receptor pathway [4]. Hence it is possible that p53 might act *via* both the caspase driven apoptotic pathway and Fas receptor activation. Fas expression has also been shown to occur in neurons entering the cell cycle to regulate neuronal death during the cell cycle and is tied to cell cycle checkpoints [48]. However, it was intriguing to find Fas receptor in the nucleus of spinal cord motor neurons and co-localized with caspase-8. We performed peptide blocking and other antibody control experiments to demonstrate the specificity of our results. The mode of Fas nuclear translocation or its nuclear function is unknown. However, Fas has been observed in the nucleus of motor neurons after spinal cord injury in an ischemia model system and suggested to participate in DNA damage and apoptosis of motor neurons [49]. Thus from this study we can conclude that a caspase-dependent apoptotic modes of cell death occur in the spinal cord motor neurons but not in neurons of the motor cortex. Although these findings contradict earlier reports wherein alterations of apoptotic proteins were evident both in the spinal cord and motor cortex, our data supports the hypothesis that multiple pathways are involved in motor neuron cell death during ALS.

In conclusion, we report alterations in the G1 to S phase transition of cell cycle with concomitant increases in p53 levels in the spinal cord of ALS patients that may induce an apoptotic mode of spinal motor neuron cell death involving both caspase-3 and Fas. We failed to observe similar alterations of this apoptotic mechanism in the motor cortex. Therefore, these findings suggest differential mechanisms of neuronal cell death in ALS spinal cord and motor cortex.

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#### ABBREVIATIONS

ALS	=	Amyotrophic lateral sclerosis
EMSA	=	Electrophoretic mobility shift assay
TUNEL	=	Terminal TdT transferase nick-end labeling

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