

SHORT REPORT

Abolishing Trp53-dependent apoptosis does not benefit spinal muscular atrophy model mice

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Spinal muscular atrophy (SMA) is the most common genetic motoneuron degenerative disorder, but the mechanism(s) of motoneuron death is unclear. Previously, a direct interaction between tumor-suppressive TP53 protein and the SMA determinant gene product, survival motor neuron protein, was identified and therefore it has been suggested that a mechanism of TP53-dependent apoptosis plays an important role in motoneuron degeneration in SMA. We used our SMA model mice, generated by a combination of knockout and transgenic techniques, to decipher the role of TP53 protein in the motoneuron degeneration in SMA. We detected a significant increase of Trp53 expression in the spinal cord of SMA-like mice compared to their normal littermates. After crossing SMA-like mice with *Trp53* knockout mice, the progeny Trp53-deficient SMA-like mice did not show milder disease severity or longer lifespan compared to SMA littermates with wild-type *Trp53* genes. Our studies provide *in vivo* evidence indicating that Trp53-dependent apoptosis does not play a crucial role in motoneuron degeneration in SMA-like mice.

European Journal of Human Genetics (2006) 14, 372–375. doi:10.1038/sj.ejhg.5201556; published online 4 January 2006

Keywords: Trp53-dependent apoptosis; spinal muscular atrophy; motoneuron degeneration; SMA model mice

Introduction

Spinal muscular atrophy (SMA), an autosomal recessive motoneuron degenerative disorder, is caused by degeneration of the spinal motoneurons leading to muscular paralysis and atrophy.¹ SMA patients are clinically categorized, on the basis of age of disease onset and clinical course, into three types: SMA 1 (the most severe, Werdnig–Hoffmann disease, MIM253300), SMA 2 (the intermediate, MIM253550), and SMA 3 (the mildest, Kugelberg–Welander disease, MIM253400).² Mutations of the SMA determinant gene, *survival motor neuron (SMN1)* gene, are found in about 96% of human SMA patients.³ Although the relationship between SMA and the *SMN1* gene product has been known

for years, the neurodegeneration pathways responsible for SMA motoneuron death remain unclear.¹ Previously, we created a SMA mouse model by a combination of knockout and transgenic techniques.⁴ The offspring SMA-like mice with homologous mouse *Smn* gene knockouts and hemizygous human *SMN2* transgenes (genotype: *Smn*^{-/-} *SMN2*^{+/-}), genotypically and phenotypically mimic SMA patients, and they can be categorized into three clinical types in the same way as human patients according to the phenotypes and survival times.⁴ Therefore, these SMA-like mice are suitable for use in studying the mechanism of SMA motoneuron degeneration.

Tumor-suppressive TP53 protein, which can induce cell cycle arrest and apoptosis, performs a critical role in the regulation of neuronal differentiation and apoptosis.^{5,6} TP53 can induce neuronal death through a Bax-dependent pathway by directly transactivating proapoptotic Bax expression.^{7,8} Recently, TP53 was reported to directly interact with SMN protein,⁹ and therefore TP53-dependent

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Received 8 June 2005; revised 11 November 2005; accepted 15 November 2005; published online 4 January 2006

apoptotic pathways were suggested to play a crucial role in the motoneuron loss in SMA disease.^{9,10} To test this hypothesis, in the present study we used our SMA model mice to cross the *Trp53* knockout mice.¹¹ The offspring *Trp53*-deficient SMA mice did not show milder disease severity or longer lifespan than their SMA littermates with wild-type *Trp53* genes, but some of them showed different phenotypes and neurodegeneration in the brain stems. This constitutes *in vivo* evidence against the hypothesis that TP53-dependent apoptotic pathways play a crucial role in motoneuron loss in SMA disease.

Materials and methods

Mice

Transgenic mice containing two copies of human *SMN2* genes⁴ were crossed with heterozygous mouse *Smn* knockout mice with or without *SMN2* transgenes were intercrossed to generate SMA-like mice (genotypes: *Smn*^{-/-}*SMN2*^{+/-}). We generated the double heterozygous knockout mice (*Smn* and *Trp53*) with or without *SMN2* transgenes by crossing homozygous *Trp53* knockout mice (P53N4-M, Taconic Farm) with the *Smn*^{+/-}*SMN2*^{+/-} mice. We obtained SMA-like mice with different *Trp53* genotypes by intercrossing double heterozygous mice with and without *SMN2* transgenes. Mice were supplied with sterile water ad libitum and rodent pellets, and were under the care of the animal facility of the Institute of Molecular Biology, Academia Sinica. All procedures were approved by the Academia Sinica Animal Care and Use Committee, Master Protocol # RMIrBIMBLH2001132.

We analyzed the genotypes of *Smn* knockout, *SMN2* transgenic, and *Trp53* knockout mice by polymerase chain reaction (PCR) analysis. Mouse tail genomic DNAs were prepared as previously described.⁴ The three specific PCR primers (S1, S2, and H1) were used for detection of the wild-type and knockout *Smn* alleles, and the primer pair (3F and 3B) were used to identify the human *SMN2* transgenes.⁴ The three specific primers (primer 4b, primer 6, and primer in the *polIII* promoter region) were used to identify the wild-type and knockout *Trp53* alleles.¹¹ For statistical analyses of life spans of mice, differences were compared between two groups by the Student's *t*-test. The results were considered to be significant when $P < 0.05$.

Western blotting, histopathological and immunostaining analyses

We isolated spinal cords from three different clinical types of SMA-like mice and their normal littermates that had been freshly killed. Total proteins were extracted from the spinal cords using extraction buffer containing 0.4% Nonidet P-40, 0.1% sodium dodecylsulfate (SDS), and proteinase inhibitors (Complete™ proteinase inhibitor Cocktail, Roche Biomedical). Equal amounts of extracted proteins were loaded and proteins were separated by 12%

SDS–polyacrylamide gel electrophoresis.¹² After transferring to polyvinylidene difluoride membranes (Millipore), we blocked the membranes in TBST buffer containing non-skimmed milk. All antibodies used were purchased from Santa Cruz Biotechnology. Adequate dilutions as per the manufacturer's suggestion for anti- β -actin (I-19), anti-p53 (FL-393), and anti-SMN (N-19) antibodies were reacted. Secondary antibodies conjugated with horseradish peroxidase were then used. We detected the reactions by chemiluminescence (Chemiluminescence Reagent Plus, PerkinElmer Life Sciences) and signals were quantified by computer densitometer (Amersham Pharmacia Biotech). The expression levels of *Trp53* were compared with β -actin. Increases of *Trp53* expression in different clinical types of SMA-like mice were obtained and compared with values for their normal littermates. These increases were indicated as mean values \pm SD from three independent experiments.

After killing mice, brains and spinal cords were removed and fixed in 10% neutral-buffered formalin (Fisher Scientific) and then embedded in paraffin. Thick transverse sections, 6 μ m, were stained with hematoxylin and eosin⁴ and observed under light microscope (ZEISS, Axioskop2). The images were captured electronically by CoolSNAP-pro, Media Cybernetic, and were analyzed using Image Pro® Plus version 4.1.

Results and discussion

In order to investigate the relationship between *Trp53* protein and SMA motoneuron death, we analyzed the expression levels of *Trp53* protein within spinal cords of type 1 SMA-like mice and their normal littermates by Western blotting. Dramatically lower SMN levels (about one-tenth) were detected in the spinal cord extracts from type 1 SMA-like mice in comparison with the spinal cord extracts of their normal age-matched littermates, and levels of β -actin were detected as an internal control (Figure 1a). We found a significant increase in *Trp53* expression (2.78 ± 0.43 fold) in type 1 SMA-like mice in comparison with their normal littermates (Figure 1a). Furthermore, a significant increase in *Trp53* expression (1.96 ± 0.36 fold) was also found in type 2 SMA-like mice but not in type 3 SMA-like mice (1.11 ± 0.41 fold) in comparison with their normal littermates (Figure 1b). Since the more severe SMA-like mice showed the more significant increase in *Trp53* expression in their spinal cords, the increase in *Trp53* expressions correlated with the disease severities of SMA in SMA-like mice.

To determine the *in vivo* role of *Trp53* protein in SMA motoneuron degeneration, we crossed knockout-transgenic mice (genotype: *Smn*^{+/-}*SMN2*^{+/-}) with *Trp53* knockout mice (genotype: *Trp53*^{-/-})¹¹ to generate double heterozygous knockout mice (genotype: *Smn*^{+/-}*Trp53*^{+/-}) with or without *SMN2* transgenes. The *Trp53*^{-/-} mice were deficient in *Trp53* protein expression, but SMN protein expression was not affected (Figure 1c). This result indicated

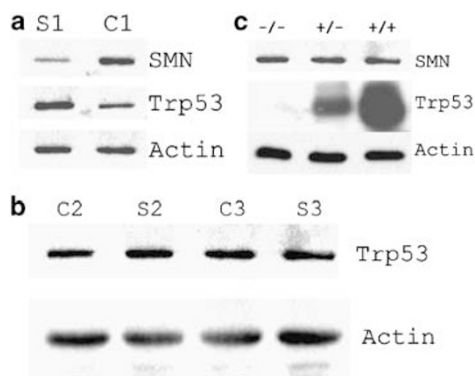


Figure 1 Analysis of protein expression levels in the mouse spinal cords. (a) Expression levels of Trp53, SMN, and β -actin were detected in the spinal cords of type 1 SMA-like mice (S1) and age-matched normal littermates (C1). (b) Expression levels of Trp53 and β -actin were detected in the spinal cords of type 2 (S2) and type 3 (S3) SMA-like mice and their age-matched normal littermates (C2 and C3). (c) Expression levels of Trp53, SMN, and β -actin were detected in the spinal cords of homozygous ($-/-$) and heterozygous ($+/-$) *Trp53* knockout mice and their wild-type littermate ($+/+$).

that Trp53 did not regulate the expression of SMN protein. We then crossed *Smn*^{+/-}*Trp53*^{+/-}*SMN2*^{-/-} mice with *Smn*^{+/-}*Trp53*^{+/-}*SMN2*^{+/-} mice to generate SMA-like mice (genotype: *Smn*^{-/-}*SMN2*^{+/-}) with different *Trp53* genotypes. After breeding and genotyping more than a thousand offspring mice, we obtained the survival data of SMA-like mice with *Trp53*^{-/-} and *Trp53*^{+/-} genotypes bred by the same parents. Totals of 11 pups with type 1, six pups with type 2, and six pups with type 3 SMA were obtained with *Trp53*^{-/-} genotype; in addition, 14 pups with type 1, 16 pups with type 2, and four pups with type 3 SMA were obtained with *Trp53*^{+/-} genotype. Although the theoretical probabilities of breeding SMA-like mice with *Trp53*^{-/-} or *Trp53*^{+/-} genotypes are the same, the total number of *Trp53*^{-/-}SMA mice ($n=23$) obtained was much lower than *Trp53*^{+/-}SMA mice ($n=34$). This might be because of embryonic or neonatal lethality caused by Trp53 deficiency. Furthermore, we obtained over 20 dead neonatal mice containing *Trp53* knockout but not *Smn* knockout genotypes (data not shown); we can conclude these mice died because of Trp53 deficiency but not SMA. The average lifespan of type 1 *Trp53*^{-/-}SMA mice was 6.73 ± 1.45 days, which was significantly shorter than type 1 *Trp53*^{+/-}SMA mice with 8.57 ± 1.03 days ($P < 0.03$). The decrease in average lifespan of type 1 *Trp53*^{-/-}SMA mice might be the result of the neonatal lethality caused by Trp53 deficiency. Furthermore, the lifespans of type 3 *Trp53*^{-/-}SMA mice (from 2 to 5 months) were also shorter than lifespans of type 3 *Trp53*^{+/-}SMA mice (from 6 to 15 months). Type 3 *Trp53*^{-/-}SMA mice showed various tumor formations, as previously presented.¹³ Since Trp53 deficiency may result in spontaneous tumor formation,^{11,13} the shorter lifespan of type 3 *Trp53*^{-/-}SMA mice can probably be attributed to tumor formation. However, the average lifespan of type 2

Trp53^{-/-}SMA mice (17.33 ± 3.63 days) was significantly longer than lifespan of type 2 *Trp53*^{+/-}SMA mice (13.38 ± 2.21 days) ($P < 0.04$). Therefore, the survival curves of *Trp53*^{-/-}SMA and *Trp53*^{+/-}SMA mice were inter-crossed (Figure 2). The probabilities of survival for mice were higher before postnatal day 10 and lower after postnatal day 11 in the *Trp53*^{+/-}SMA than in the *Trp53*^{-/-}SMA mice groups. From these results, we can not conclude that Trp53 deficiency prolongs the lifespan of SMA-like mice.

Type 2 *Trp53*^{-/-}SMA mice that showed severe atrophy of the tail and lower limbs, as well as bleeding from the lower abdomen were obtained (Figure 3a). However, these phenotypes were not found in type 2 *Trp53*^{+/-}SMA mice. Furthermore, type 3 *Trp53*^{-/-}SMA mice showed neuronal degeneration in their brain stems that were not detected in their type 3 *Trp53*^{+/-}SMA littermates (Figures 3b–e). These results suggest the possibility that Trp53 deficiency in SMA-like mice changed the pathophysiology and symptoms of SMA disease. The mechanism of this change needs to be further analyzed.

We previously found *in vivo* evidence that showed that Bax-dependent apoptosis played a crucial role in motoneuron degeneration in SMA (Tsai *et al*, unpublished data). Here, we detected an increase in Trp53 expression in spinal cords of type 1 and type 2 SMA-like mice compared to normal littermates (Figure 1a and b). An increase in Trp53 expression was also detected in the spinal motoneurons of amyotrophic lateral sclerosis 1 (ALS1, MIM105400) model mice.¹⁴ Furthermore, TP53 can directly interact with SMN protein; a decrease in SMN protein expression in SMA patients inhibited TP53-SMN complex formation, thus promoting the translocation of TP53 into nucleolus.⁹ Therefore, a possible mechanism for motoneuron loss by TP53-dependent apoptosis has been suggested for both ALS and SMA

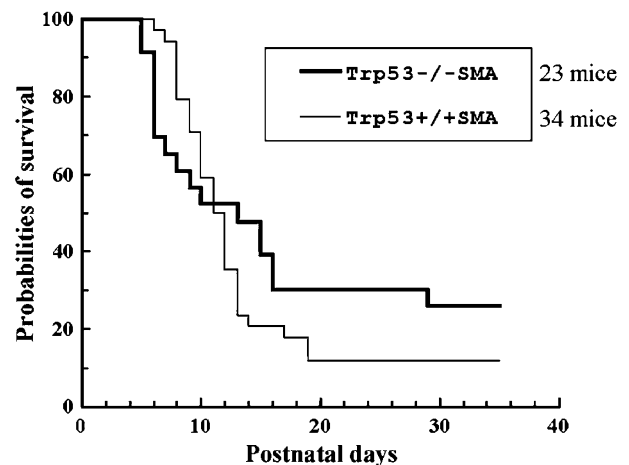


Figure 2 Survival curves of offspring SMA-like mice with different *Trp53* genotypes. Survival curves of SMA littermates with *Trp53*^{+/-} and *Trp53*^{-/-} genotypes were presented as Kaplan–Meier plots. Numbers of mice obtained were as indicated.

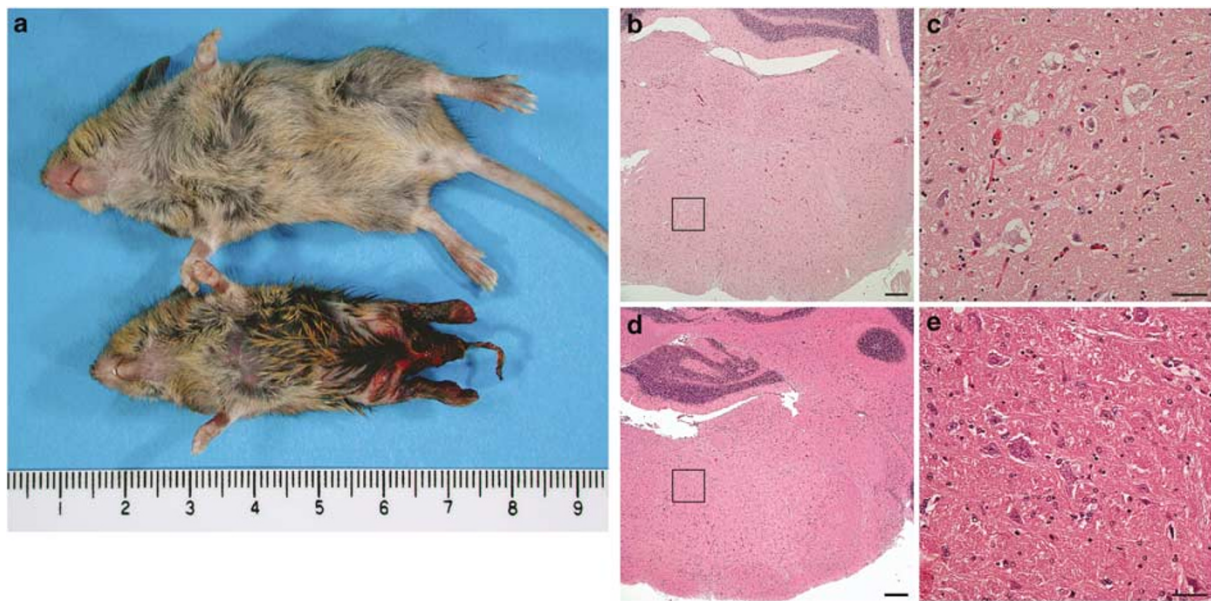


Figure 3 Phenotypes and histopathological analysis of Trp53-deficient SMA-like mice and their wild-type or SMA littermates. (a) Images of a type 2 Trp53-deficient SMA-like mouse and its normal littermate at 22 postnatal days. Histopathological findings of cross-sections from the brain stems of a 3-month-old type 3 *Trp53*^{-/-} SMA mouse (b and c) and its type 3 *Trp53*^{+/+} SMA littermate (d and e). Panel c and panel e are enlarged figures of the boxed regions from panel b and panel d, respectively. Vesicles to vacuolar degenerations of motoneurons in the brain stem can be seen (c). Scale bars: 250 μm (b and d); 40 μm (c and e).

diseases.^{9,14} However, our results, obtained by crossing SMA-like mice with *Trp53* knockout mice, do not support this hypothesis. Although type 2 *Trp53*^{-/-} SMA mice showed a longer lifespan than their type 2 SMA littermates with wild-type *Trp53* genes, Trp53 deficiency was detrimental to the survival of both type 1 and type 3 SMA-like mice. In addition, Trp53 deficiency might cause alteration of the phenotypes and pathophysiology of type 2 and type 3 SMA-like mice (Figure 3). If Trp53-dependent apoptosis played a crucial role in motoneuron degeneration of SMA-like mice, Trp53 deficiency would protect the spinal motoneurons from death and result in Trp53-deficient SMA-like mice living longer. However, we can not obtain this conclusion from this study. Our results do not support the hypothesis that inhibition of Trp53-dependent apoptosis shows beneficial effect on a SMA model mice, although this does not rule out a different role for Trp53 in SMA patients.

Acknowledgements

We thank Dr K Deen for his critical reading of manuscript and Ms. Ping-Jui Chen for her great help in the preparation of mouse tail DNAs. This work was supported in part by the National Health Research Institute (Grant NHRI-EX92-9029SP).

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