presence of a critical period when a sufficient amount of SMN protein is required during motor neuron development. In addition, the maturation of the blood brain barrier may hamper the transport of the SMN-expressing vectors to the target neurons, suggesting a finite period for efficient gene therapy.

In 2011, Dominguez and colleagues reported that they used postnatal day 1 systemic injection of self-complementary adeno-associated virus (scAAV9) vectors carrying a codon-optimized *SMN1* sequence and a chimeric intron placed downstream of the strong phosphoglycerate kinase (PGK) promoter (SMNopti) to overexpress the human SMN protein in a mouse model of severe SMA ($Smn^{-/-}SMN2^{+/+}SMN\Delta 7^{+/+}$) (Dominguez et al., 2011). Codon optimization is a gene optimization technology that can alter both naturally occurring and recombinant gene sequences to achieve the highest possible levels of productivity in any given expression system. This treatment increased life expectancy from 27 to over 340 days (median survival of 199 days) in mice that normally survive about 13 days. The systemic scAAV9 therapy mediated complete correction of motor function, prevented motor neuron death and rescued the weight loss. This study also showed sex differences in the responsiveness to the treatment. Male SMA mice displayed a lower body weight gain than age-matched control mice, whereas the body weight of females was not statistically different from the controls.

As for stem cell therapy, it has been shown that "spinal cord-derived stem cells" and "embryonic stem cell-derived neural stem cells" can differentiate into motor neurons in vivo (Corti et al., 2008; Corti et al., 2010). In addition, following intrathecal transplantation into SMA mice ($Smn^{-/-}SMN2^{+/+}SMN\Delta 7^{+/+}$), the administrated neural stem cells survived and migrated extensively to appropriate areas. Here, the transplanted embryonic stem cells were found to work by secreting soluble neuroprotection factors, such as glial-cell derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), and tumor growth factor (TGF)-α. These growth factors were confirmed to play a role in the improved functional recoveries of SMA mice following transplantation, showing an increase in myofiber number and size, axon length and body weight gain, suggesting that neural stem cell transplantation resulted in successful amelioration of behavioral end points and life span extension in SMA mice (Corti et al., 2010).

Gene and stem cell therapies may be very promising treatments for SMA patients, especially for pre-symptomatic patients. However, the efficacy of such therapies should further be tested in non-human primates before such approaches are applied to the patients, especially in considering if blood brain barrier function may hinder the delivery of therapeutic agents to the neurons (Tsai, 2012). Foust and colleagues investigated whether scAAV9 can traverse the blood–brain barrier in a nonhuman primate, *cynomolgus macaque* (Foust et al., 2010). They intravenously injected scAAV9 carrying the green fluorescent protein (GFP) gene on postnatal day 1, and demonstrated that scAAV9 crossed the blood brain barrier and reached motor neurons in the nonhuman primate model, suggesting that gene therapy targeting motor neurons can also be done in human.

Non-*SMN*-Targeting Strategies

Protection of motor neurons

Neuroprotection therapy with riluzole and gabapentin, which had originally been used for the patients with amyotrophic lateral sclerosis (ALS), was also applied to SMA patients (Russman et al., 2003; Merlini et al., 2003). Glutamate excitotoxicity may be an important factor in the pathogenesis of ALS since the cell bodies of motor neurons receive afferent innervation from glutamate neurons. Hence, pharmacologic agents that rescue glutamate excitotoxicity may be effective in slowing disease progression in ALS (van den Bosch, 2006). Similarly, glutamate excitotoxicity may also be an important factor in the pathogenesis of SMA. Riluzole inhibits the presynaptic release of glutamate, while gabapentin reduces the pool of releasable glutamate in the pre-synaptic neurons. Russman et al. (2003) reported the outcome of a clinical trial of riluzole in 10 patients with SMA type 1 (phase 1 trial with randomization of 2:1, i.e., 2 riluzole to 1 placebo). None of the subjects in this study experienced adverse effects. Even though the study sample size was small, some benefits of riluzole were suggested in the treated patients: three of the seven patients taking riluzole lived to more than 5, 4, and 2 years of age respectively with only BiPAP respiratory assistance at night. On the contrary, the placebo-controlled trials of gabapentin showed no significant benefit in motor function of the patients with SMA type 2/3 (Miller et al., 2001; Merlini et al., 2003).

Another group of compounds, β-lactam antibiotics, can also provide neuroprotection against glutamate-mediated excitotoxicty by increasing the expression level of the glutamate transporter EAAT2/GLT-1. However, to date these observations have only been demonstrated using model mice. A treated ALS mouse model showed a delay in loss of neurons and muscle strength, and increase in survival rate (Rothstein et al., 2005). Nizzardo and colleagues demonstrated that a β-lactam antibiotic, ceftriaxone, also ameliorated the neuromuscular phentotype in SMA mice ($Smn^{-/-}SMN2^{+/+}SMN\Delta 7^{+/+}$) (Nizzardo et al., 2011). Treatment with ceftriaxone increased general weight, muscle size, motor neuron numbers and NMJs, which are likely the reasons for the increased life span and muscle strength of the SMA mice. According to them, the neuroprotective effect of the β-lactam antibiotic in the SMA mice seems to be mediated not only through the process of increasing

EAAT2/GLT-1, but also by other mechanisms that increase transcription factor Nrf2 and SMN.

Thyrotropin-releasing hormone (TRH, L-pyroglutamyl-L-histidyl-L-prolinamide) has trophic effects on spinal motor neurons, and it has also been tried for ALS and SMA patients. TRH was administered intravenously (Takeuchi et al., 1994; Tzeng et al., 2000) or orally (Kato et al., 2009) to SMA patients in small clinical trials but only transient improvement was observed in some patients.

Olesoxime (TRO19622), a small molecule with a cholesterol-like structure, has protective properties for motor neurons. It targets proteins associated with the mitochondrial permeability pore (Bordet, 2007). Olesoxime has been granted orphan drug status for the treatment of ALS and SMA, and clinical trials for ALS and SMA have been started in the US and Europe (http://clinicaltrials.gov/ct2/show/NCT01285583; http://clinicaltrials.gov/ct2/show/NCT01302600). In December 2011, Trophos SA announced the results from the phase 3 study of olesoxime in 512 patients with ALS; olesoxime was well tolerated but did not demonstrate a significant increase in survival of patients receiving riluzole (Rilutek®). Olesoxime trials for SMA are still ongoing and the results are to be expected in 2013 (http://www.trophos.com/news/pr20111213.htm).

IGF-1 is reported to modulate multiple fundamental cellular processes, such as cellular growth, proliferation, and survival (Vardatsikos et al., 2009). Most recently, Tsai and colleagues reported that CNS-directed IGF-1 delivery could reduce motor neuron death in SMA mice ($Smn^{-/-}SMN2^{+/-}$) (Tsai et al., 2012). Murdocca and colleagues also reported the effects of IPLEX™ [recombinant human insulin-like growth factor 1 (rhIGF-1) combined with recombinant human IGF-1 binding protein 3 (rhIGFBP-3)] on a severe SMA mouse model ($Smn^{-/-}SMN2^{+/+}SMN\Delta 7^{+/+}$) (Murdocca et al., 2012). According to them, perinatal administration of IPLEX™ results in reduced degeneration of motor neurons, increased muscle fiber size and in amelioration of motor functions in SMA mice, suggesting this compound as a plausible therapeutic candidate to hinder the progression of the neurodegenerative process in SMA. However, it should be noted that CNS-directed IGF-1 delivery could not improve motor function in SMA mice (Tsai et al., 2012) and that IPLEX™ did not improve lifespan and body weight gain of the treated mice (Murdocca et al., 2012). IGF-1 may provide at least some beneficial effects on the survival of motor neurons. However, it is necessary to further study the systemic effect of IGF-1 administration before clinical application, because IGF-1 has multiple functions in various organs.

Protection of non-neuronal tissues

Nutrition may be critical for the care of SMA patients, especially SMA type 1 patients (Oskoui et al., 2007). The improved survival of SMA type 1 patients observed in recent years can be attributed to noninvasive pulmonary support and aggressive nutrition with gastrostomy feedings. Butchbach and colleagues observed that maternal diet can significantly modify survival and the motor neuron disease phenotype in SMA mice ($Smn^{-/-}SMN2^{+/+}SMN\Delta 7^{+/+}$). According to them, SMA mice from dams that were fed a higher fat diet survived longer than those from dams on a lower fat diet (Butchbach et al., 2010a). The effect of nutritional support on survival of SMA patients and model animals indicates that improvement of pathological conditions of non-neuronal tissues including muscles should be considered in SMA therapy.

Treatments that directly target muscles and improve muscle mass have been reported: inhibition of myostatin by overexpression of follistatin (Sumner et al., 2009) and expression of IGF-1 (Bosch-Marcé et al., 2011). Although inhibition of myostatin did not ameliorate motor function or survival of severe SMA mice ($Smn^{-/-}SMN2^{+/+}SMN\Delta 7^{+/+}$) (Sumner et al., 2009), overexpression of IGF-1 resulted in enlarged myofibers, but not in improvement of motor function (Bosch-Marcé et al., 2011). Murine IGF-1 administration had been proven to give different positive effects when it was expressed locally in muscle of SMA mice ($Smn^{-/-}SMN2^{+/+}SMN\Delta 7^{+/+}mIGF\text{-}1^{+/-}$) (Bosch-Marcé et al., 2011) or in motor neurons (Murdocca et al., 2012).

Recently, there has been emerging evidence that the RhoA/ROCK pathway may play an important role in the pathogenesis of SMA (Bowerman et al., 2009; Nölle et al., 2011) since SMN depletion leads to an increased activation of ROCK, a major regulator of actin dynamics. Bowerman et al. (2010) reported that ROCK inhibitors, Y-27632 and Fasudil, dramatically improved the survival of the $Smn^{2B/-}$ mice, an intermediate SMA mouse model. They emphasized that lifespan extension in SMA mice with ROCK inhibitors was accompanied by an improvement in the maturation of NMJs and an increase in muscle fiber size (Bowerman et al., 2010; Bowerman et al., 2012). However, Bowerman and colleagues showed that administration of Y-27632 had no beneficial effect on the $Smn^{-/-}$ mouse model with the most severe SMA phenotype. Here, they suggested that there may be a need for differential therapies for the different types of SMA severities and that the "one size fits all" approach may not be tenable (Bowerman et al., 2010).

Challenges in Clinical Trials

Despite the large number of candidate compounds evaluated, there has yet to be any effective drug treatment reported for all types of SMA (Wadman et al., 2012a; Wadman et al., 2012b). A number of clinical trials for SMA have already been conducted in the past decade, some of which are still ongoing (Miller et al., 2001; Mercuri et al., 2007; Pane et al., 2008;

Swoboda et al., 2009; Tiziano et al., 2010; Kissel et al., 2011). However, all clinical trials reported so far failed to show significant effectiveness of the therapeutic approaches, which may indicate the difficulties of designing clinical trials for this disorder. An adequate design should take into account the rarity of the patients, clinical disease heterogeneity (subtypes, onset age, sex, stage of disease progress, timing of enrollment, and intervention relative to disease progression), treatment plans (selection of the drug with possible ameliorating effects on the clinical symptoms, sufficient dose, and duration to see some measurable effects) and outcome measures [laboratory biomarkers including *SMN* transcript and SMN protein amounts, muscle mass and strength, motor function testing, respiratory function testing, MUNE, questionnaires for quality of life (QOL)] (Swoboda et al., 2007; Kissel et al., 2011). Clinical endpoints, i.e., the target outcome of the clinical trials: such as extension of the survival period in the patients with SMA type 1 (which will be discussed again below), improvement of motor function in the patients with SMA type 2, and extension of the walking period in the patients with SMA type 3, need to be specified. However, great subtlety may be required for the accurate evaluation of these outcomes. Even if a therapeutic approach could ameliorate the symptoms in some patients, these outcomes may not be detected if the trials are not adequately designed.

To address the challenges due to the rarity of SMA, Mercuri's group (2012) called for clinical trials to be carried out as large multicenter international trials. Such large-scale collaborations would increase the numbers of patients enrolled and would enable randomized placebo studies to be carried out. This approach could also overcome the problems due to clinical heterogeneity as a stratification method could be used to provide a fair evaluation of the treatments (Mercuri et al., 2012).

The selection of appropriate outcome measures to test the efficacy of a therapy remains one of the most difficult problems to be resolved. As for laboratory biomarkers, only *SMN* transcript or SMN protein levels have been established. However, determination of *SMN* transcript or SMN protein levels may not be enough, because these cannot be used to evaluate treatments targeting biochemical reactions downstream of SMN-related signaling (Crawford et al., 2012). Recently, metabolomics studies have suggested that some proteins and metabolites can be used as laboratory biomarkers to reflect responsiveness to treatment (Finkel et al., 2012). Further studies are still required for future clinical usage.

The Hammersmith Functional Motor Scale (HFMS) (Main et al., 2003), Modified HFMS (MHFMS) (Krosschell et al., 2006), and gross motor function measure (GMFM) (Nelson et al., 2006) have been established as standard measures of functional ability in children with SMA types 2 and 3 for use in longitudinal multicenter clinical trials. The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) may also be used for the evaluation of children with SMA type 1 (Glanzman et al., 2010). However, it is difficult to evaluate the actual change in motor scales in SMA patients with any motor function measurements. Thus, it is necessary for investigators in multicenter networks to share the test skills and scoring criteria in order to improve inter-rater reliability and objectivity. For that purpose, training of test skills and collaboration in the scoring criteria should be implemented across centers with different expertise (Mercuri et al., 2012).

In an SMA mouse model, extension of lifespan has been considered to reflect the effectiveness of therapeutic approaches. However, lifespan cannot be simply applied to evaluate the therapeutic approaches in human SMA patients because not only the administered therapy, but the type of supportive care including respiratory management can also change the lifespan of patients. In addition, the use of an artificial respirator in SMA type 1 management is still controversial. Such differences in clinical care may hamper simple comparison using lifespan outcomes in international clinical trials. The occurrence of death and the requirement for an artificial respirator may be considered as equivalent events when evaluating the efficacy of clinical trials in patients with SMA type 1 because improvement of motor scale cannot be expected from these patients (Oskoui et al., 2007; Mercuri et al., 2012). Currently, using lifespan as the only available outcome measure, is not ideal anymore. If it is possible to measure improvements in respiratory function or restoration of motor function, alternative outcome measures for SMA type 1 may become achievable. Highly effective therapies which will improve motor scale of patients with SMA type 1 can then be sought.

Conclusions

SMA is an incurable motor neuron disease with autosomal recessive inheritance. Molecular biology studies of SMA have been greatly advanced in two directions, namely diagnostic applications and pathophysiological studies, since the discovery of the SMN genes in 1995. Molecular diagnostics has enabled us not only to diagnose SMA in patients, but has also provided the ability to carry out carrier and newborn screening of SMA for populations. Pathophysiological studies have provided an improved understanding of the underlying pathogenesis of SMA, including alternative splicing of *SMN2*, aberrant splicing due to the defect of snRNPs, impairment of motor circuit formation and/or NMJ development, and dysregulation of cytoskeleton dynamics. To date, there has been no successful therapy for SMA, but an in-depth understanding of the pathophysiology underlying the disease

can offer useful insights for development of effective treatment approaches. Some therapeutic strategies have already been devised based on current pathophysiological knowledge of the disease, namely *SMN2*-targeting, *SMN1*-introduction and non-*SMN* targeting strategies. With multiple approaches in therapeutic strategies for SMA being pursued, some of which are already in clinical trials, it is expected that some candidate compounds may emerge as potential therapeutic agents in the near future. These exciting developments offer promising outcomes for SMA patients in overcoming this debilitating disease.

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References

Abbaszadegan, M. R., Keify, F., Ashrafzadeh, F., Farshchian, M., Khadivi-Zand, F., Teymoorzadeh, M. N., Mojahedi, F., Ebrahimzadeh, R. & Ahadian, M. (2011) Gene dosage analysis of proximal spinal muscular atrophy carriers using real-time PCR. *Arch Iranian Med* **14**, 188–1891.

ACOG (2009) ACOG committee opinion no. 432: spinal muscular atrophy. *Obstet Gynecol* **113**, 1194–1196.

Advis, J. P., Richard, J. S. & Ojeda, S. R. (1981) Hyperprolactinemia-induced precocious puberty: studies on the mechanism(s) by which prolactin enhances ovarian progesterone responsiveness to gonadothropins in prepubertal rats. *Endocrinology* **108**, 1333–1342.

Aguilar, R., Bellido, C., Sánchez-Criado, J. E. & Aguilar, E. (1988) Mechanisms of precocious puberty induced in male rats by pituitary grafts. *J Reprod Fertil* **83**, 879–883.

Akten, B., Kye, M. J., Hao, Le T., Wertz, M. H., Singh, S., Nie, D., Huang, J., Merianda, T. T., Twiss, J. L., Beattie, C. E., Steen, J. A. & Sahin, M. (2011) Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *Proc Natl Acad Sci USA* **108**, 10337–10342.

Andreassi, C., Angelozzi, C., Tiziano, F.D., Vitali, T., De Vincenzi, E., Boninsegna, A., Villanova, M., Bertini, E., Pini, A., Neri, G. & Brahe, C. (2004) Phenylbutyrate increases SMN expression in vitro: relevance for treatment of spinal muscular atrophy. *Eur J Hum Genet* **12**, 59–65.

Andreassi, C., Jarecki, J., Zhou, J., Coovert, D. D., Monani, U. R., Chen, X., Whitney, M., Pollok, B., Zhang, M., Androphy, E. & Burghes, A. H. (2001) Aclarubicin treatment restores SMN levels to cells derived from type I spinal muscular atrophy patients. *Hum Mol Genet* **10**, 2841–2849.

Angelozzi, C., Borgo, F., Tiziano, F. D., Martella, A., Neri, G. & Brahe, C. (2008) Salbutamol increases SMN mRNA and protein levels in spinal muscular atrophy cells. *J Med Genet* **45**, 29–31.

Anhuf, D., Eggermann, T., Rudnik-Schöneborn, S. & Zerres, K. (2003) Determination of *SMN1* and *SMN2* copy number using TaqMan technology. *Hum Mut* **22**, 74–78.

Arkblad, E. L., Darin, N., Berg, K., Kimber, E., Brandberg, G., Lindberg, C., Holmberg, E., Tulinius, M. & Nordling, M. (2006) Multiplex ligation-dependent probe amplification improves diagnostics in spinal muscular atrophy. *Neuromusc Disorder* **16**, 830–838.

Avila, A. M., Burnett, B. G., Taye, A. A., Gabanella, F., Knight, M. A., Hartenstein, P., Cizman, Z., Di Prospero, N. A., Pellizzoni, L., Fischbeck, K. H. & Sumner, C. J. (2007) Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy. *J Clin Invest* **117**, 659–671.

Azzouz, M., Le, T., Ralph, G. S., Walmsley, L., Monani, U. R., Lee, D. C., Wilkes, F., Mitrophanous, K. A., Kingsman, S. M., Burghes, A. H., & Mazarakis, N. D. (2004) Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. *J Clin Invest* **114**, 1726–1731.

Bäumer, D., Lee, S., Nicholson, G., Davies, J. L., Parkinson, N. J., Murray, L. M., Gillingwater, T. H., Ansorge, O., Davies, K. E., Talbot, K. & Bordet (2009) Alternative splicing events are a late feature of pathology in a mouse model of spinal muscular atrophy. *PloS Genet* **5**, e1000773.

Biondi, O., Branchu, J., Sanchez, G., Lancelin, C., Deforges, S., Lopes, P., Pariset, C., Lécolle, S., Côté, J., Chanoine, C. & Charbonnier, F. (2010) *In Vivo* NMDA ceceptor activation accelerates motor unit maturation, protects spinal motor neurons, and enhances SMN2 gene expression in severe spinal muscular atrophy mice. *Jour Neurosci* **30**, 11288–11299.

Bordet, T., Buisson, B., Michaud, M., Drouot, C., Galea, P., Delaage, P., Akentieva, N. P., Evers, A. S., Covey, D. F., Ostuni, M. A., Lacapere, J. J., Massaad, C., Schumacher, M., Steidl, E. M., Maux, D., Delaage, M., Henderson, C. E. & Pruss, R. M. (2007) Identification and characterization of cholest-4-en-3-one, oxime (TRO19622), a novel drug candidate for amyotrophic lateral sclerosis. *J Pharmacol Exp Ther* **322**, 709–720.

Bosch-Marcé, M., Wee, C. D., Martinez, T. L., Lipkes, C. E., Choe, D. W., Kong, L., Van Meerbeke, J. P., Musaro, A. & Sumner, C. J. (2011) Increased IGF-1 in muscle modulates the phenotype of severe SMA mice. *Hum Mol Genet* **20**, 1844–1853.

Bowerman, M., Anderson, C. L., Beauvais, A., Boyl, P. P., Witke, W. & Kothary, R. (2009) SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Mol Cell Neurosci* **42**, 66–74.

Bowerman, M., Beauvais, A., Anderson, C. L. & Kothary, R. (2010) Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Hum Mol Genet* **19**, 1468–78.

Bowerman, M., Murray, L. M., Boyer, J. G., Anderson, C. L. & Kothary, R. (2012) Fasudil improves survival and promotes skeletal muscle development in a mouse model of spinal muscular atrophy. *BMC medicine* **10**, 24.

Bowerman, M., Shafey, D. & Kothary, R. (2007) Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. *J Mol Neurosci* **32**, 120–131.

Brahe, C., Vitali, T., Tiziano, F. D., Angelozzi, C., Pinto, A. M., Borgo, F., Moscato, U., Bertini, E., Mercuri, E. & Neri, G. (2005) Phenylbutyrate increases SMN gene expression in spinal muscular atrophy patients. *Eur J Hum Genet* **13**, 256–259.

Branchu, J., Biondi, O., Chali, F., Collin, T., Leroy, F., Mamchaoui, K., Makoukji, J., Pariset, C., Lopes, P., Massaad, C., Chanoine, C., & Charbonnier, F. (2013) Shift from extracellular signal-regulated

kinase to AKT/cAMP responsive element-binding protein pathway increases survival-motor-neuron expression in Spinal Muscular Atrophy-like mice and patient cells. *J Neurosci* **33**, 4280–4294.

Brichta, L., Hofmann, Y., Hahnen, E., Siebzehnrubl, F. A., Raschke, H., Blümcke, I., Eyupoglu, I. Y. & Wirth, B. (2003) Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. *Hum Mol Genet* **12**, 2481–2489.

Brzustowicz, L. M., Lehner, T., Castilla, L. H., Penchaszadeh, G. K., Wilhelmsen, K. C., Daniels, R., Davies, K. E., Leppert, M., Ziter, F., Wood, D., Dubowitz, V., Zerres, K., Hausmanowa-Petrusewicz, I., Ott, J., Munsat, T. L. & Gilliam, T. C. (1990) Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2–13.3. *Nature* **344**, 540–541.

Burghes, A. H. & Beattie, C. E. (2009) Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? *Nat Rev Neurosci* **10**, 597–609.

Burnett, B. G., Muñoz, E., Tandon, A., Kwon, D. Y., Sumner, C. J., Fischbeck, K. H. (2009) Regulation of SMN protein stability. *Mol Cell Biol* **29**(5), 1107–1115.

Bussaglia, E., Clermont, O., Tizzano, E., Lefebvre, S., Burglen, L., Cruaud, C., Urtizberea, J. A., Colomer, J., Munnich, A., Baiget, M. & Melki, J. (1995) A frame-shift deletion in the survival motor neuron gene in Spanish spinal muscular atrophy patients. *Nat Genet* **11**, 335–337.

Butchbach, M. E., Rose, F. F., Jr., Rhoades, S., Marston, J., Mccrone, J. T., Sinnott, R. & Lorson, C. L. (2010a) Effect of diet on the survival and phenotype of a mouse model for spinal muscular atrophy. *Biochem Biophys Res Comm* **391**, 835–840.

Butchbach, M. E., Singh, J., Thorsteinsdottir, M., Saieva, L., Slominski, E., Thurmond, J., Andresson, T., Zhang, J., Edwards, J. D., Simard, L. R., Pellizzoni, L., Jarecki, J., Burghes, A. H. & Gurney, M. E. (2010b) Effects of 2,4-diaminoquinazoline derivatives on SMN expression and phenotype in a mouse model for spinal muscular atrophy. *Hum Mol Genet* **19**, 454–467.

Campbell, L., Potter, A., Ignatius, J., Dubowitz, V. & Davies, K. (1997) Genomic variation and gene conversion in spinal muscular atrophy: implications for disease process and clinical phenotype. *Am J Hum Genet* **61**, 40–50.

Carrel, T. L., McWhorter, M. L., Workman, E., Zhang, H., Wolstencroft, E.C., Lorson, C., Bassell, G. J., Burghes, A. H. & Beattie, C. E. (2006) Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis. *J Neurosci* **26**, 11014–11022.

Cartegni, L. & Krainer, A. R. (2002) Disruption of an SF2/ASF-dependent exonic splicing enhancer in *SMN2* causes spinal muscular atrophy in the absence of *SMN1*. *Nat Genet* **30**, 377–384.

Cartegni, L. & Krainer, A. R. (2003) Correction of disease-associated exon skipping by synthetic exon-specific activators. *Nat Struct Biol* **10**, 120–125.

Cauchi, R. J. (2010) SMN and Gemins: 'we are family' ... or are we?: insights into the partnership between Gemins and the spinal muscular atrophy disease protein SMN. *BioEssays* **32**, 1077–1089.

Chan, V., Yip, B., Yam, I., Au, P., Lin, C. K., Wong, V. & Chan, T. K. (2004) Carrier incidence for spinal muscular atrophy in southern Chinese. *J Neurol* **251**, 1089–1093.

Chang, J. G., Hsieh-Li, H. M., Jong, Y. J., Wang, N. M., Tsai, C. H. & Li, H. (2001) Treatment of spinal muscular atrophy by sodium butyrate. *Proc Natl Acad Sci USA* **98**, 9808–9813.

Chen, K. L., Wang, Y. L., Rennert, H., Joshi, I., Mills, J. K., Leonard, D. G. & Wilson, R. B. (1999) Duplications and de novo deletions of the SMNt gene demonstrated by fluorescence-based carrier testing for spinal muscular atrophy. *Am J Med Genet* **85**, 463–469.

Chen, T. H., Chang, J. G., Yang, Y. H., Mai, H. H., Liang, W. C., Wu, Y. C., Wang, H. Y., Huang, Y. B., Wu, S. M., Chen, Y. C., Yang, S. N. & Jong, Y. J. (2010) Randomized, double-blind, placebo-controlled trial of hydroxyurea in spinal muscular atrophy. *Neurology* **75**, 2190–2197.

Chen, T. H., Tzeng, C. C., Wang, C. C., Wu, S. M., Chang, J. G., Yang, S. N., Hung, C. H. & Jong, Y. J. (2011) Identification of bidirectional gene conversion between *SMN1* and *SMN2* by simultaneous analysis of SMN dosage and hybrid genes in a Chinese population. *J Neurol Sci* **308**, 83–87.

Chen, W. J., Dong, W. J., Lin, X. Z., Lin, M. T., Murong, S. X., Wu, Z. Y. & Wang, N. (2009) Rapid diagnosis of spinal muscular atrophy using high-resolution melting analysis. *BMC Med Genet* **10**, 45.

Chen, W. J., Wu, Z. Y., Lin, M. T., Su, J. F., Lin, Y., Murong, S. X. & Wang, N. (2007) Molecular analysis and prenatal prediction of spinal muscular atrophy in Chinese patients by the combination of restriction fragment length polymorphism analysis, denaturing high-performance liquid chromatography, and linkage analysis. *Arch Neurol* **64**, 225–231.

Cho, S., & Dreyfuss, G. (2010) A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity. *Gene Deves* **24**, 438–442.

Cifuentes-Diaz, C., Nicole, S., Velasco, M. E., Borra-Cebrian, C., Panozzo, C., Frugier, T., Millet, G., Roblot, N., Joshi, V. & Melki, J. (2002) Neurofilament accumulation at the motor endplate and lack of axonal sprouting in a spinal muscular atrophy mouse model. *Hum Mol Genet* **11**, 1439–1447.

Clermont, O., Burlet, P., Benit, P., Chanterau, D., Saugier-Veber, P., Munnich, A. & Cusin, V. (2004) Molecular analysis of SMA patients without homozygous *SMN1* deletions using a new strategy for identification of *SMN1* subtle mutations. *Hum Mut* **24**, 417–427.

Coady, T. H., Shababi, M., Tullis, G. E. & Lorson, C. L. (2007) Restoration of SMN function: delivery of a trans-splicing RNA re-directs *SMN2* pre-mRNA splicing. *Mol Ther* **15**, 1471–8.

Coady, T. H. & Lorson, C. L. (2010) Trans-splicing-mediated improvement in a severe mouse model of spinal muscular atrophy. *J Neurosci* **30**, 126–30.

Coovert, D. D., Le, T. T, McAndrew, P. E., Strasswimmer, J., Crawford, T.O., Mendell, J. R., Coulson, S. E., Androphy, E. J., Prior, T. W. & Burghes, A. H. (1997) The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet* **6**, 1205–1214.

Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., Saladino, F., Bordoni, A., Fortunato, F., Del Bo, R., Papadimitriou, D., Locatelli, F., Menozzi, G., Strazzer, S., Bresolin, N. & Comi, G. P. (2008) Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. *J Clin invest* **118**, 3316–3330.

Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., Simone, C., Falcone, M., Papadimitriou, D., Locatelli, F., Mezzina, N., Gianni, F., Bresolin, N. & Comi, G. P. (2010) Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice. *Brain* **133**, 465–481.

Crawford, T. O., Paushkin, S. V., Kobayashi, D. T., Forrest, S. J., Joyce, C. L., Finkel, R. S., Kaufmann, P., Swoboda, K. J., Tiziano, D., Lomastro, R., Li, R. H., Trachtenberg, F. L., Plasterer, T., Chen, K. S. & On Behalf of the Pilot Study of Biomarkers for Spinal Muscular Atrophy Trial, G. (2012) Evaluation of SMN Protein, Transcript, and Copy Number in the Biomarkers for

Spinal Muscular Atrophy (BforSMA) Clinical Study. *PloS one* **7**, e33572.

Cuscó, I., Barceló, M. J., Baiget, M. & Tizzano, E. F. (2002) Implementation of SMA carrier testing in genetic laboratories: comparison of two methods for quantifying the *SMN1* gene. *Hum Mut* **20**, 452–459.

Dobrowolski, S. F., Pham, H. T., Pouch Downes, F., Prior, T. W., Naylor, E. W. & Swoboda, K. J. (2012) Newborn screening for spinal muscular atrophy by calibrated short-amplicon melt profiling. *Clin Chem* **58**, 1033–1039.

Dominguez, E., Marais, T., Chatauret, N., Benkhelifa-Ziyyat, S., Duque, S., Ravassard, P., Carcenac, R., Astord, S., Pereira de Moura, A., Voit, T. & Barkats, M. (2011) Intravenous scAAV9 delivery of a codon-optimized SMN1 sequence rescues SMA mice. *Hum Mol Genet* **20**, 681–693.

Eggermann, T., Zerres, K., Anhuf, D., Kotzot, D., Fauth, C. & Rudnik-Schoneborn, S. (2005) Somatic mosaicism for a heterozygous deletion of the survival motor neuron (*SMN1*) gene. *Eur J Hum Genet* **13**, 309–313.

Fallini, C., Zhang H., Su Y., Silani V., Singer R.H., Rossol W. & Bassell, G.J. (2011) The survival motor neuron (SMN protein) interacts with the mRNA-binding protein HuD and regulates in primary motor neuron axons. *J Neurosci* **31**: 3914–3925.

Fallini, C., Bassell, G. J. & Rossoll, W. (2012) Spinal muscular atrophy: The role of SMN in axonal mRNA regulation. *Brain Res* **1462**, 81–92.

Farooq, F., Molina, F. A., Hadwen, J., Mackenzie, D., Witherspoon, L., Osmond, M., Holcik, M. & Mackenzie, A. (2011) Prolactin increases SMN expression and survival in a mouse model of severe spinal muscular atrophy via the STAT5 pathway. *J Clin Invest* **121**, 3042–3050.

Feldkötter, M., Schwarzer, V., Wirth, R., Wienker, T. F. & Wirth, B. (2002) Quantitative analyses of *SMN1* and *SMN2* based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet* **70**, 358–368.

Finkel, R. S., Crawford, T. O., Swoboda, K. J., Kaufmann, P., Juhasz, P., Li, X., Guo, Y., Li, R. H., Trachtenberg, F., Forrest, S. J., Kobayashi, D. T., Chen, K. S., Joyce, C. L., Plasterer, T. & On Behalf of the Pilot Study of Biomarkers for Spinal Muscular Atrophy Trial (2012) Candidate Proteins, Metabolites and Transcripts in the Biomarkers for Spinal Muscular Atrophy (BforSMA) Clinical Study. *PloS one* **7**, e35462.

Fischer, U., Liu, Q. & Dreyfuss, G. (1997) The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* **90**, 1023–1029.

Foust, K. D., Wang, X., Mcgovern, V. L., Braun, L., Bevan, A. K., Haidet, A. M., Le, T. T., Morales, P. R., Rich, M. M., Burghes, A. H. & Kaspar, B. K. (2010) Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nat Biotech* **28**, 271–274.

Garbes, L., Riessland, M., Hölker, I., Heller, R., Hauke, J., Tränkle, C., Coras, R., Blümcke, I., Hahnen, E. & Wirth, B. (2009) LBH589 induces up to 10-fold SMN protein levels by several independent mechanisms and is effective even in cells from SMA patients non-responsive to valproate. *Hum Mol Genet* **18**, 3645–3658.

Garbes, L., Heesen, L., Hölker, I., Bauer, T., Schreml, J., Zimmermann, K., Thoenes, M., Walter, M., Dimos, J., Peitz, M., Brüstle, O., Heller, R. & Wirth, B. (2013) VPA response in SMA is suppressed by the fatty acid translocase CD36. *Hum Mol Genet* **22**, 398–407.

Gennarelli, M., Lucarelli, M., Capon, F., Pizzuti, A., Merlini, L., Angelini, C., Novelli, G. & Dallapiccola, B. (1995) Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients. *Biochem Biophys Res Commun* **213**, 342–348.

Gilliam, T. C., Brzustowicz, L. M., Castilla, L. H., Lehner, T., Penchaszadeh, G. K., Daniels, R. J., Byth, B. C., Knowles, J., Hislop, J. E., Shapira, Y., Dubowitz V., Munsat, T. L., Ott, J. & Davies, K. E. (1990) Genetic homogeneity between acute and chronic forms of spinal muscular atrophy. *Nature* **345**, 823–825.

Gitlin, J. M., Fischbeck, K., Crawford, T. O., Cwik, V., Fleischman, A., Gonye, K., Heine, D., Hobby, K., Kaufmann, P., Keiles, S., Mackenzie, A., Musci, T., Prior, T., Lloyd-Puryear, M., Sugarman, E. A., Terry, S. F., Urv, T., Wang, C., Watson, M., Yaron, Y., Frosst, P. & Howell, R.R. (2010) Carrier testing for spinal muscular atrophy. *Genet Med* **12**, 621–622.

Glanzman, A. M., Mazzone, M., Main, M., Pelliccioni, M., Wood, J., Swoboda, K. J., Scott, C., Pane, M., Messina, S., Bertini, E., Mercuri, E. & Finkel, R. S. (2010) The Children's Hospital of Philadelphia infant test of neuromuscular disorders (CHOP INTEND): test development and reliability. *Neuromusc Disord* **20**, 155–161.

Gómez-Curet, I., Robinson, K. G., Funanage, V. L., Crawford, T. O., Scavina, M. & Wang, W. (2007) Robust quantification of the SMN gene copy number by real-time TaqMan PCR. *Neurogenet* **8**, 271–278.

Grzeschik, S. M., Ganta, M., Prior, T. W., Heavlin, W. D. & Wang, C. H. (2005) Hydroxyurea enhances *SMN2* gene expression in spinal muscular atrophy cells. *Ann Neurol* **58**, 194–202.

Gubitz, A. K., Feng, W., Dreyfuss, G. (2004) The SMN complex. *Exp Cell Res* **296**, 51–56.

Hahnen, E., Eyupoglu, I. Y., Brichta, L., Haastert, K., Tränkle, C., Siebzehnrubl, F. A., Riessland, M., Hölker, I., Claus, P., Romstock, J., Buslei, R., Wirth, B. & Blümcke, I. (2006) In vitro and ex vivo evaluation of second-generation histone deacetylase inhibitors for the treatment of spinal muscular atrophy. *J Neurochem* **98**, 193–202.

Hahnen, E., Forkert, R., Marke, C., Rudnik-Schoneborn, S., Schonling, J., Zerres, K. & Wirth, B. (1995) Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: evidence of homozygous deletions of the SMN gene in unaffected individuals. *Hum Mol Genet* **4**, 1927–1933.

Harada, Y., Sutomo, R., Sadewa, A. H., Akutsu, T., Takeshima, Y., Wada, H., Matsuo, M. & Nishio, H. (2002) Correlation between *SMN2* copy number and clinical phenotype of spinal muscular atrophy: three *SMN2* copies fail to rescue some patients from the disease severity. *J Neurol* **249**, 1211–1219.

Hastings, M. L., Berniac, J., Liu, Y. H., Abato, P., Jodelka, F. M., Barthel, L., Kumar, S., Dudley, C., Nelson, M., Larson, K., Edmonds, J., Bowser, T., Draper, M., Higgins, P. & Krainer, A. R. (2009) Tetracyclines that promote *SMN2* exon 7 splicing as therapeutics for spinal muscular atrophy. *Sci Transl Med* **1**, 5ra12.

Heier, C. R. & DiDonato, C. J. (2009) Translational readthrough by the aminoglycoside geneticin (G418) modulates SMN stability in vitro and improves motor function in SMA mice in vivo. *Hum Mol Genet* **18**, 1310–1322.

Hendrickson, B. C., Donohoe, C., Akmaev, V. R., Sugarman, E. A., Labrousse, P., Boguslavskiy, L., Flynn, K., Rohlfs, E. M., Walker, A., Allitto, B., Sears, C. & Scholl, T. (2009) Differences in *SMN1* allele frequencies among ethnic groups within North America. *J Med Genet* **46**, 641–644.

Hofmann, Y., Lorson, C. L., Stamm, S., Androphy, E. J. & Wirth, B. (2000) Htra2-beta 1 stimulates an exonic splicing enhancer and can restore full-length SMN expression to survival motor neuron 2 (*SMN2*). *Proc Natl Acad Sci USA* **97**, 9618–9623.

Hofmann, Y. & Wirth, B. (2002) hnRNP-G promotes exon 7 inclusion of survival motor neuron (SMN) via direct interaction with Htra2-beta1. *Hum Mol Genet* **11**, 2037–2049.

Hsieh-Li, H. M., Chang, J. G., Jong, Y. J., Wu, M. H., Wang, N. M., Tsai, C. H. & Li, H. (2000) A mouse model for spinal muscular atrophy. *Nat Genet* **24**, 66–70.

Hsu, Y. Y., Jong, Y. J., Tsai, H. H., Tseng, Y. T., An, L. M. & Lo, Y. C. (2012) Triptolide increases transcript and protein levels of survival motor neurons in human SMA fibroblasts and improves survival in SMA-like mice. *B J Pharmacol* **166**, 1114–1126.

Hua, Y., Sahashi, K., Hung, G., Rigo, F., Passini, M. A., Bennett, C. F. & Krainer, A. R. (2010) Antisense correction of *SMN2* splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev* **24**, 1634–1644.

Hua, Y., Sahashi, K., Rigo, F., Hung, G., Horev, G., Bennett, C. F. & Krainer, A. R. (2011) Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* **478**, 123–126.

Hua, Y., Vickers, T. A., Okunola, H. L., Bennett, C. F. & Krainer, A. R. (2008) Antisense masking of an hnRNP A1/A2 intronic splicing silencer corrects *SMN2* splicing in transgenic mice. *Am J Hum Genet* **82**, 834–848.

Hubers, L., Valderrama-Carvajal, H., Laframboise, J., Timbers, J., Sanchez, G. & Cote, J. (2010) HuD interacts with survival motor neuron protein and can rescue spinal muscular atrophy-like neuronal defects. *Hum Mol Genet* **20**, 553–579.

Iwahashi, H., Eguchi, Y., Yasuhara, N., Hanafusa, T., Matsuzawa, Y. & Tsujimoto, Y. (1997) Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. *Nature* **390**, 413–417.

Jablonka, S., Holtmann, B., Meister, G., Bandilla, M., Rossoll, W., Fischer, U. & Sendtner, M. (2002) Gene targeting of Gemin2 in mice reveals a correlation between defects in the biogenesis of U snRNPs and motoneuron cell death. *Proc Natl Acad Sci U S A* **99**, 10126–10131.

Jarecki, J., Chen, X., Bernardino, A., Coovert, D. D., Whitney, M., Burghes, A., Stack, J. & Pollok, B. A. (2005) Diverse small-molecule modulators of SMN expression found by high-throughput compound screening: early leads towards a therapeutic for spinal muscular atrophy. *Hum Mol Genet* **14**, 2003–2018.

Jodelka, F. M., Ebert, A. D., Duelli, D. M. & Hastings, M. L. (2010) A feedback loop regulates splicing of the spinal muscular atrophy-modifying gene, *SMN2*. *Hum Mol Genet* **19**, 4906–4917.

Kashima, T. & Manley, J. L. (2003) A negative element in *SMN2* exon 7 inhibits splicing in spinal muscular atrophy. *Nat Genet* **34**, 460–463.

Kashima, T., Rao, N. & Manley, J. L. (2007) An intronic element contributes to splicing repression in spinal muscular atrophy. *Proc Natl Acad Sci USA* **104**, 3426–3431.

Kato, Z., Okuda, M., Okumura, Y., Arai, T., Teramoto, T., Nishimura, M., Kaneko, H. & Kondo, N. (2009) Oral administration of the thyrotropin-releasing hormone (TRH) analogue, taltireline hydrate, in spinal muscular atrophy. *J Child Neurol* **24**, 1010–1012.

Kerruish, N. & Robertson, S. P. (2005) Newborn screening: new developments, new dilemmas. *J Med Ethics* **31**, 393–398.

Kinali, M., Mercuri, E., Main, M., De Biasia, F., Karatza, A., Higgins, R., Banks, L. M., Manzur, A. Y. & Muntoni, F. (2002) Pilot trial of albuterol in spinal muscular atrophy. *Neurology* **59**, 609–610.

Kissel, J. T., Scott, C. B., Reyna, S. P., Crawford, T. O., Simard, L. R., Krosschell, K. J., Acsadi, G., Elsheik, B., Schroth, M. K., D'anjou, G., Lasalle, B., Prior, T. W., Sorenson, S., Maczulski, J. A., Bromberg, M. B., Chan, G. M. & Swoboda, K. J. (2011) SMA CARNIVAL TRIAL PART II: a prospective, single-armed trial of L-carnitine and valproic acid in ambulatory children with spinal muscular atrophy. *PloS one* **6**, e21296.

Kolb, S. J. & Kissel, J. T. (2011) Spinal muscular atrophy: a timely review. *Arch Neurol* **68**, 979–984.

Kong, L., Wang, X., Choe, D. W., Polley, M., Burnett, B. G., Bosh-Marcé, M., Griffin, J. W., Rich, M. M. & Sumner, C. J. (2009). Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *J Neurosci* **29**, 842–851.

Kotani, T., Sutomo, R., Sasongko, T. H., Sadewa, A. H., Gunadi Minato, T., Fujii, E., Endo, S., Lee, M. J., Ayaki, H., Harada, Y., Matsuo, M. & Nishio, H. (2007) A novel mutation at the N-terminal of SMN Tudor domain inhibits its interaction with target proteins. *J Neurol* **254**, 624–630.

Krosschell, K. J., Maczulski, J. A., Crawford, T. O., Scott, C. & Swoboda, K. J. (2006) A modified Hammersmith functional motor scale for use in multi-center research on spinal muscular atrophy. *Neuromuscul Disord* **16**, 417–426.

Le, T. T., Pham, L. T., Butchbach, M. E., Zhang, H. L., Monani, U. R., Coovert, D. D., Gavrilina, T. O., Xing, L., Bassell, G. J. & Burghes, A. H. (2005) SMNDelta7, the major product of the centromeric survival motor neuron (*SMN2*) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum Mol Genet* **14**, 845–857.

Lee, T. M., Kim, S. W., Lee, K. S., Jin, H. S., Koo, S. K., Jo, I., Kang, S. & Jung, S. C. (2004) Quantitative analysis of *SMN1* gene and estimation of *SMN1* deletion carrier frequency in Korean population based on real-time PCR. *J Korean Med Sci* **19**, 870–873.

Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M., Paslier D. L., Frézal, J., Cohen, D., Weissenbach, J., Munnich, A., & Melki, J. (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **80**, 155–165.

Li, B. S., Wang, X. Y., Ma, F. L., Jiang B., Song, X. X. & Xu, A. G. (2011) Is high resolution melting analysis (HRMA) accurate for detection of human disease-associated mutations? A meta analysis. *PloS One* **6**, e28078.

Lim, S. R. & Hertel, K. J. (2001) Modulation of survival motor neuron pre-mRNA splicing by inhibition of alternative 3' splice site pairing. *J Biol Chem* **276**, 45476–45483.

Ling, K. K., Gibbs, R. M., Feng, Z. & Ko, C. P. (2012) Severe neuromuscular denervation of clinically relevant muscles in a mouse model of spinal muscular atrophy. *Hum Mol Genet* **21**, 185–195.

Little, S. E., Janakiraman, V., Kaimal, A., Musci, T., Ecker, J. & Caughey, A. B. (2010) The cost-effectiveness of prenatal screening for spinal muscular atrophy. *Am J Obs Gynecol* **202**, 253.e1-e7.

Liu, Q. & Dreyfuss, G. (1996) A novel nuclear structure containing the survival of motor neurons protein. *EMBO J* **15**, 3555–3565.

Lorson, C.L., Hahnen, E., Androphy, E. J. & Wirth, B. (1999) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc Natl Acad Sci USA* **96**, 6307–6311.

Lorson, C. L., Strasswimmer, J., Yao, J. M., Baleja, J. D., Hahnen, E., Wirth, B., Le, T., Burghes, A. H. & Androphy, E. J. (1998) SMN

oligomerization defect correlates with spinal muscular atrophy severity. *Nat Genet* **19**, 63–66.

Lotti, F., Imlach, W. L., Saieva, L., Beck, E. S., Haole, T., Li, D. K., Jiao, W., Mentis, G. Z., Beattie, C. E., McCabe, B. D. (2012) An SMN-dependent U12 splicing event essential for motor circuit function. *Cell* **151**, 440–454.

Lunn, M. R., Root, D. E., Martino, A. M., Flaherty, S. P., Kelley, B. P., Coovert, D. D., Burghes, A. H., Man, N. T., Morris, G. E., Zhou, J., Androphy, E. J., Sumner, C. J. & Stockwell, B. R. (2004) Indoprofen upregulates the survival motor neuron protein through a cyclooxygenase-independent mechanism. *Chem Biol* **11**, 1489–1493.

Main, M., Kairon, H., Mercuri, E. & Muntoni, F. (2003) The Hammersmith functional motor scale for children with spinal muscular atrophy: a scale to test ability and monitor progress in children with limited ambulation. *Eur J Paed Neurol* **7**, 155–159.

Makhortova, N. R., Hayhurst, M., Cerqueira, N., Sinor-Anderson, A. D., Zhao, W. N., Heiser, P. W., Arvanites, A. C., Davidow, L. C., Waldon, Z. O., Steen, J. A., Lam, K., Ngo, H. D. & Rubin, L. L. (2011) A screen for regulators of survival of motor neuron protein levels. *Nat Chem Biol* **7**, 544–552.

Matera, A. G., Terns, R. M. & Terns, M. P. (2007) Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat Rev. Mol Cell Biol* **8**, 209–220.

Matthijs, G., Devriendt, K. & Fryns, J. P. (1998) The prenatal diagnosis of spinal muscular atrophy. *Prenatal Diagnosis* **18**, 607–610.

Mattis, V. B., Ebert, A. D., Fosso, M. Y., Chang, C. W. & Lorson, C. L. (2009) Delivery of a read-through inducing compound, TC007, lessens the severity of a spinal muscular atrophy animal model. *Hum Mol Genet* **18**, 3906–3913.

Mattis, V. B., Rai, R., Wang, J., Chang, C. W., Coady, T. & Lorson, C. L. (2006) Novel aminoglycosides increase SMN levels in spinal muscular atrophy fibroblasts. *Hum Genet* **120**, 589–601.

McAndrew, P. E., Parsons, D. W., Simard, L. R., Rochette, C., Ray, P. N., Mendell, J. R., Prior, T. W. & Burghes, A. H. (1997) Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *Am J Hum Genet* **60**, 1411–1422.

Melki, J., Abdelhak, S., Sheth, P., Bachelot, M. F., Burlet, P., Marcadet, A., Aicardi, J., Barois, A., Carriere, J. P., Fardeau, M., Fontan, D., Ponsot, G., Billete, T., Angelini, C., Barbosa. C., Ferriere, G., Lanzi, G., Ottolini, A., Babron, M. C., Cohen, D., Hanauer, A., Cierget-Darpoux, F., Lathrop, M., Munnich, A. & Frezal, J. (1990a) Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. *Nature* **344**, 767–768.

Melki, J., Sheth, P., Abdelhak, S., Burlet, P., Bachelot, M. F., Lathrop, M. G., Frezal, J. & Munnich, A. (1990b) Mapping of acute (type I) spinal muscular atrophy to chromosome 5q12-q14. The French Spinal Muscular Atrophy Investigators. *Lancet* **336**, 271–273.

Mercuri, E., Bertini, E. & Iannaccone, S. T. (2012) Childhood spinal muscular atrophy: controversies and challenges. *Lancet Neurol* **11**, 443–452.

Mercuri, E., Bertini, E., Messina, S., Solari, A., D'amico, A., Angelozzi, C., Battini, R., Berardinelli, A., Boffi, P., Bruno, C., Cini, C., Colitto, F., Kinali, M., Minetti, C., Mongini, T., Morandi, L., Neri, G., Orcesi, S., Pane, M., Pelliccioni, M., Pini, A., Tiziano, F. D., Villanova, M., Vita, G. & Brahe, C. (2007) Randomized, double-blind, placebo-controlled trial of phenylbutyrate in spinal muscular atrophy. *Neurology* **68**, 51–55.

Merlini, L., Solari, A., Vita, G., Bertini, E., Minetti, C., Mongini, T., Mazzoni, E., Angelini, C. & Morandi, L. (2003) Role of gabapentin in spinal muscular atrophy: results of a multicenter, randomized Italian study. *J Child Neurol* **18**, 537–541.

Meyer, K., Marquis, J., Trub, J., Nlend Nlend, R., Verp, S., Ruepp, M. D., Imboden, H., Barde, I., Trono, D. & Schumperli, D. (2009) Rescue of a severe mouse model for spinal muscular atrophy by U7 snRNA-mediated splicing modulation. *Hum Mol Genet* **18**, 546–555.

Miller, R. G., Moore, D. H., Dronsky, V., Bradley, W., Barohn, R., Bryan, W., Prior, T. W., Gelinas, D. F., Iannaccone, S., Kissel, J., Leshner, R., Mendell, J., Mendoza, M., Russman, B., Samaha, F. & Smith, S. (2001) A placebo-controlled trial of gabapentin in spinal muscular atrophy. *J Neurol Sci* **191**, 127–131.

Miyajima, H., Miyaso, H., Okumura, M., Kurisu, J. & Imaizumi, K. (2002) Identification of a *cis*-acting element for the regulation of SMN exon 7 splicing. *J Biol Chem* **277**, 23271–23277.

Miyaso, H., Okumura, M., Kondo, S., Higashide, S., Miyajima, H. & Imaizumi, K. (2003) An intronic splicing enhancer element in survival motor neuron (SMN) pre-mRNA. *J Biol Chem* **278**, 15825–15831.

Morikawa, S., Harahap, I. S., Kaszynski, R. H., Yamamoto, T., Pramudya, D. K., Pham, H. T., Hartomo, T. B., Lee, M. J., Morioka, I., Nishimura, N., Yokoyama, N., Ueno, Y., Matsuo, M. & Nishio, H. (2011) Diagnosis of spinal muscular atrophy via high-resolution melting analysis symmetric polymerase chain reaction without probe: a screening evaluation for *SMN1* deletions and intragenic mutations. *Genet Test Mol Bio* **15**, 677–684.

Murdocca, M., Malgieri, A., Luchetti, A., Saieva, L., Dobrowolny, G., de Leonibus, E., Filareto, A., Quitadamo, M. C., Novelli, G. & Musaro, A. (2012) IPLEX administration improves motor neuron survival and ameliorates motor functions in a severe mouse model of spinal muscular atrophy. *Mol Med* **18**, 1076–1085.

Nelson, L., Owens, H., Hynan, L. S., Iannaccone, S. T., AmSMART group (2006) The gross motor function measure™ is a valid and sensitive outcome measure for spinal muscular atrophy. *Neuromuscul Disord* **16**, 374–380.

Nizzardo, M., Nardini, M, Ronchi, D., Salani, S., Donadoni, C., Fortunato, F, Colciago, G., Falcone, M., Simone, C., Riboldi, G., Govoni, A., Bresolin, N., Comi, G. C., Corti, S. (2011) Beta-lactam antibiotic offers neuroprotection in a spinal muscular atrophy model by multiple mechanisms. *ExpNeurol* **229**, 214–225.

Nollë, A., Zeug A., Van Bergeijk, J., Tönges, L., Gerhard, R., Brinkmann, H., Al Rayes, S., Hensel, N., Schill, Y., Apkhazava, D., Jablonka, S., O'mer, J., Srivastav, R. K., Baasner A., Lingor, P., Wirth, B., Ponimaskin, E., Niendenthal, R., Grothe, C. & Claus, P. (2011). The spinal muscular atrophy disease protein SMN is linked to the rho-kinase pathway via profilin. *Hum Mol Genet* **20**, 4865–4878.

Ogino, S., Leonard, D. G., Rennert, H., Ewens, W. J. & Wilson, R. B. (2002) Genetic risk assessment in carrier testing for spinal muscular atrophy. *Am J Med Genet* **110**, 301–307.

Ogino, S. & Wilson, R. B. (2002) Genetic testing and risk assessment for spinal muscular atrophy (SMA). *Hum Genet* **111**, 477–500.

Oprea, G. E., Kröber, S., Mcwhorter, M. L., Rossoll, W., Müller, S., Krawczak, M., Bassell, G. J., Beattie, C. E. & Wirth, B. (2008) Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* **320**, 524–527.

Orzalesi, M. & Danhaive, O. (2009) Ethical problems with neonatal screening. *Annali dell'Istituto superiore di sanita* **45**, 325–330.

Oskoui, M., Levy, G., Garland, C. J., Gray, J. M., O'Hagen, J., De Vivo, D. C. & Kaufmann, P. (2007) The changing natural history of spinal muscular atrophy type 1. *Neurology* **69**, 1931–1936.

Pagliardini, S., Giavazzi, A., Setola, V., Lizier, C., Di Luca, M., Debiasi, S. & Battaglia, G. (2000) Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet* **9**, 47–56.

Pane, M., Staccioli, S., Messina, S., D'amico, A., Pelliccioni, M., Mazzone, E.S., Cuttini, M., Alfieri, P., Battini, R., Main, M., Muntoni, F., Bertini, E., Villanova, M. & Mercuri, E. (2008) Daily salbutamol in young patients with SMA type II. *Neuromuscul Disord* **18**, 536–540.

Passini, M. A., Bu, J., Roskelley, E. M., Richards, A. M., Sardi, S. P., O'Riordan, C. R., Klinger, K. W., Shihabuddin, L. S., Cheng, S. H. (2010) **CNS**-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy. *J Clin Invest* **120**, 1253–1264.

Passini, M.A., Bu, J., Richards, A. M., Kinnecom, C., Sardi, S. P., Stanek, L. M., Hua, Y., Rigo, F., Matson, J., Hung, G., Kaye, E. M., Shihabuddin, L. S., Krainer, A. R., Bennett, C. F. & Cheng, S. H. (2011) Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci Transl Med* **3**, 72ra18.

Passon, N., Dubsky De Wittenau, G., Jurman, I., Radovic, S., Bregant, E., Molinis, C., Damante, G. & Lonigro, I.R. (2010) Quick MLPA test for quantification of *SMN1* and *SMN2* copy numbers. *Mol Cell Probes* **24**, 310–314.

Pedrotti, S., Bielli, P., Paronetto, M. P., Ciccosanti, F., Fimia, G. M., Stamm, S., Manley, J. L. & Sette, C. (2010) The splicing regulator Sam68 binds to a novel exonic splicing silencer and functions in SMN2 alternative splicing in spinal muscular atrophy. *EMBO J* **29**, 1235–1247.

Pellizzoni, L., Kataoka, N., Charroux, B. & Dreyfuss, G. (1998) A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell* **95**, 615–624.

Pellizoni, L. (2007) Chaperoning ribonucleoprotein biogenesis in health and disease. *EMBO Rep* **8**, 340–345.

Porensky, P. N., Mitrpant, C., Mcgovern, V. L., Bevan, A. K., Foust, K. D., Kaspar, B. K., Wilton, S. D. & Burghes, A. H. (2012) A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse. *Hum Mol Genet* **21**, 1625–1638.

Prior, T. W. (2007) Spinal muscular atrophy diagnostics. *J Child Neurol* **22**, 952–956.

Prior, T. W., Professional, Practice, Guidelines, Committee (2008) Carrier screening for spinal muscular atrophy. *Genet Med* **10**, 840–842.

Prior, T. W., Krainer, A. R., Hua, Y., Swoboda, K. J., Snyder, P. C., Bridgeman, S. J., Burghes, A. H. & Kissel, J. T. (2009) A positive modifier of spinal muscular atrophy in the *SMN2* gene. *Am J Hum Genet* **85**, 408–413.

Prior, T. W. (2010) Spinal muscular atrophy: newborn and carrier screening. *Obst and Gynecol Clin North Am* **37**, 23–36,

Prior, T. W., Snyder, P. J., Rink, B. D., Pearl, D. K., Pyatt, R. E., Mihal, D. C., Conlan, T., Schmalz, B., Montgomery, L., Ziegler, K., Noonan, C., Hashimoto, S. & Garner, S. (2010) Newborn and carrier screening for spinal muscular atrophy. *Am J Med Genet. Part A* **152A**, 1608–1616.

Pruss, R. M., Giraudon-Paoli, M., Morozova, S., Berna, P., Abitbol, J. L. & Bordet, T. (2010) Drug discovery and development for spinal muscular atrophy: lessons from screening approaches and future challenges for clinical development. *Future Med Chem* **2**, 1429–1440.

Pyatt, R. E., Mihal, D. C. & Prior, T. W. (2007) Assessment of liquid microbead arrays for the screening of newborns for spinal muscular atrophy. *Clin Chem* **53**, 1879–1885.

Riessland, M., Ackermann, B., Forster, A., Jakubik, M., Hauke, J., Garbes, L., Fritzsche, I., Mende, Y., Blümcke, I., Hahnen, E. & Wirth, B. (2010) SAHA ameliorates the SMA phenotype in two mouse models for spinal muscular atrophy. *Hum Mol Genet* **19**, 1492–1506.

Riessland, M., Brichta, L., Hahnen, E. & Wirth, B. (2006) The benzamide M344, a novel histone deacetylase inhibitor, significantly increases *SMN2* RNA/protein levels in spinal muscular atrophy cells. *Hum Genet* **120**, 101–110.

Rochette, C. F., Surh, L. C., Ray, P. N., McAndrew, P. E., Prior, T. W., Burghes, A. H., Vanasse, M. & Simard, L. R. (1997) Molecular diagnosis of non-deletion SMA patients using quantitative PCR of SMN exon 7. *Neurogenetics* **1**, 141–147.

Rossoll, W., Jablonka, S., Andreassi, C., Kroning, A. K., Karle, K., Monani, U. R. & Sendtner, M. (2003) Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *J Cell Biol* **163**, 801–812.

Rossoll, W., Kroning, A. K., Ohndorf, U. M., Steegborn, C., Jablonka, S. & Sendtner, M. (2002) Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Hum Mol Genet* **11**, 93–105.

Rothstein, J. D., Patel, S., Regan, M. R., Haenggeli, C., Huang, Y. H., Bergles, D. E., Jin, L., Dykes Hoberg, M., Vidensky, S., Chung, D. S., Toan, S.V., Bruijn, L. I., Su, Z. Z., Gupta, P. & Fisher, P. B. (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature* **433**, 73–77.

Russman, B. S., Iannaccone, S. T. & Samaha, F. J. (2003) A phase 1 trial of riluzole in spinal muscular atrophy. *Arch Neurol* **60**, 1601–1603.

Scarciolla, O., Stuppia, L., De Angelis, M. V., Murru, S., Palka, C., Giuliani, R., Pace, M., Di Muzio, A., Torrente, I., Morella, A., Grammatico, P., Giacanelli, M., Rosatelli, M. C., Uncini, A. & Dallapiccola, B. (2006) Spinal muscular atrophy genotyping by gene dosage using multiple ligation-dependent probe amplification. *Neurogenetics* **7**, 269–276.

Scheffer, H., Cobben, J. M., Mensink, R. G., Stulp, R. P., Van Der Steege, G. & Buys, C. H. (2000) SMA carrier testing–validation of hemizygous SMN exon 7 deletion test for the identification of proximal spinal muscular atrophy carriers and patients with a single allele deletion. *Eur J Hum Genet* **8**, 79–86.

Schrank, B., Gotz, R., Gunnersen, J. M., Ure, J. M., Toyka, K. V., Smith, A. G. & Sendtner, M. (1997) Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc Natl Acad Sci USA* **94**, 9920–9925.

Shababi, M., Glascock, J. & Lorson, C. L. (2011) Combination of SMN trans-splicing and a neurotrophic factor increases the life span and body mass in a severe model of spinal muscular atrophy. *Hum Gene Ther* **22**, 135–144.

Sheng-Yuan, Z., Xiong, F., Chen, Y. J., Yan, T. Z., Zeng, J., Li, L., Zhang, Y. N., Chen, W. Q., Bao, X. H., Zhang, C. & Xu, X. M. (2010) Molecular characterization of SMN copy number derived from carrier screening and from core families with SMA in a Chinese population. *Eur J Hum Genet* **18**, 978–984.

Singh, J., Salcius, M., Liu, S. W., Staker, B. L., Mishra, R., Thurmond, J., Michaud, G., Mattoon, D. R., Printen, J., Christensen,

J., Bjornsson, J. M., Pollok, B. A., Kiledjian, M., Stewart, L., Jarecki, J. & Gurney, M. E. (2008) DcpS as a therapeutic target for spinal muscular atrophy. *ACS Chem Biol* **3**, 711–722.

Singh, N. K., Singh, N. N., Androphy, E. J. & Singh, R. N. (2006) Splicing of a critical exon of human survival motor neuron is regulated by a unique silencer element located in the last intron. *Mol Cell Biol* **26**, 1333–1346.

Singh, N. N., Androphy, E. J. & Singh, R. N. (2004a) An extended inhibitory context causes skipping of exon 7 of *SMN2* in spinal muscular atrophy. *Biochem Biophys Res Commun* **315**, 381–388.

Singh, N. N., Androphy, E. J. & Singh, R. N. (2004b) In vivo selection reveals combinatorial controls that define a critical exon in the spinal muscular atrophy genes. *RNA* **10**, 1291–1305.

Singh, N. N., Singh, R. N. & Androphy, E. J. (2007) Modulating role of RNA structure in alternative splicing of a critical exon in the spinal muscular atrophy genes. *Nuc. Acids Res* **35**, 371–389.

Singh, N. N., Seo, J., Rahn, S. J. & Singh, R. N. (2012) A multi-exon-skipping detection assay reveals surprising diversity of splice isoforms of spinal muscular atrophy genes. *PloS One* **7**, e49595.

Skordis, L. A., Dunckley, M. G., Yue, B., Eperon, I. C. & Muntoni, F. (2003) Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates *SMN2* gene expression in patient fibroblasts. *Proc Natl Acad Sci USA* **100**, 4114–4119.

Smith, M., Calabro, V., Chong, B., Gardiner, N., Cowie, S. & Du Sart, D. (2007) Population screening and cascade testing for carriers of SMA. *Eur J Hum Genet* **15**, 759–766.

Strasswimmer, J., Lorson, C. L., Breiding, D. E., Chen, J. J., Le, T., Burghes, A. H. & Androphy, E. J. (1999) Identification of survival motor neuron as a transcriptional activator-binding protein. *Hum Mol Genet* **8**, 1219–1226.

Su, Y. N., Hung, C. C., Li, H., Lee, C. N., Cheng, W. F., Tsao, P. N., Chang, M. C., Yu, C. L., Hsieh, W. S., Lin, W. L. & Hsu, S. M. (2005) Quantitative analysis of *SMN1* and *SMN2* genes based on DHPLC: a highly efficient and reliable carrier-screening test. *Hum Mut* **25**, 460–467.

Su, Y. N., Hung, C. C., Lin, S. Y., Chen, F. Y., Chern, J. P., Tsai, C., Chang, T. S., Yang, C. C., Li, H., Ho, H. N. & Lee, C. N. (2011) Carrier screening for spinal muscular atrophy (SMA) in 107,611 pregnant women during the period 2005–2009: a prospective population-based cohort study. *PloS one* **6**, e17067.

Sugarman, E. A., Nagan, N., Zhu, H., Akmaev, V. R., Zhou, Z., Rohlfs, E. M., Flynn, K., Hendrickson, B. C., Scholl, T., Sirko-Osadsa, D. A. & Allitto, B. A. (2012) Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. *Eur J Hum Genet* **20**, 27–32.

Sukenik-Halevy, R., Pesso, R., Garbian, N., Magal, N. & Shohat, M. (2010) Large-scale population carrier screening for spinal muscular atrophy in Israel–effect of ethnicity on the false-negative rate. *Genet Testing Mol Biomarkers* **14**, 319–324.

Sumner, C. J., Huynh, T. N., Markowitz, J. A., Perhac, J. S., Hill, B., Coovert, D. D., Schussler, K., Chen, X., Jarecki, J., Burghes, A. H., Taylor, J. P. & Fischbeck, K. H. (2003) Valproic acid increases SMN levels in spinal muscular atrophy patient cells. *Ann Neurol* **54**, 647–654.

Sumner, C. J., Wee, C. D., Warsing, L. C., Choe, D. W., Ng, A. S., Lutz, C. & Wagner, K. R. (2009) Inhibition of myostatin does not ameliorate disease features of severe spinal muscular atrophy mice. *Hum Mol Genet* **18**, 3145–3152.

Sutomo, R., Akutsu, T., Takeshima, Y., Nishio, H., Sadewa, A. H., Harada, Y. & Matsuo, M. (2002) Rapid *SMN1* deletion test using DHPLC to screen patients with spinal muscular atrophy. *Am J Med Genet* **113**, 225–256.

Swoboda, K. J., Kissel, J. T., Crawford, T. O., Bromberg, M. B., Acsadi, G., D'anjou, G., Krosschell, K. J., Reyna, S. P., Schroth, M. K., Scott, C. B. & Simard, L. R. (2007) Perspectives on clinical trials in spinal muscular atrophy. *J Child Neurol* **22**, 957–966.

Swoboda, K. J., Prior, T. W., Scott, C. B., Mcnaught, T. P., Wride, M. C., Reyna, S. P. & Bromberg, M. B. (2005) Natural history of denervation in SMA: relation to age, *SMN2* copy number, and function. *Ann Neurol* **57**, 704–712.

Swoboda, K. J., Scott, C. B., Crawford, T. O., Simard, L. R., Reyna, S. P., Krosschell, K. J., Acsadi, G., Elsheik, B., Schroth, M. K., D'anjou, G., Lasalle, B., Prior, T. W., Sorenson, S. L., Maczulski, J. A., Bromberg, M. B., Chan, G. M., Kissel, J. T. & Project Cure Spinal Muscular Atrophy Investigators, N. (2010) SMA CARNIVAL trial part I: double-blind, randomized, placebo-controlled trial of L-carnitine and valproic acid in spinal muscular atrophy. *PloS one* **5**, e12140.

Swoboda, K. J., Scott, C. B., Reyna, S. P., Prior, T. W., Lasalle, B., Sorenson, S. L., Wood, J., Acsadi, G., Crawford, T. O., Kissel, J. T., Krosschell, K. J., D'anjou, G., Bromberg, M. B., Schroth, M. K., Chan, G. M., Elsheikh, B. & Simard, L. R. (2009) Phase II open label study of valproic acid in spinal muscular atrophy. *PloS one* **4**, e5268.

Takeuchi, Y., Miyanomae, Y., Komatsu, H., Oomizono, Y., Nishimura, A., Okano, S., Nishiki, T. & Sawada, T. (1994) Efficacy of thyrotropin-releasing hormone in the treatment of spinal muscular atrophy. *J Child Neurol* **9**, 287–289.

Taylor, J. E., Thomas, N. H., Lewis, C. M., Abbs, S. J., Rodrigues, N. R., Davies, K. E. & Mathew, C. G. (1998) Correlation of SMNt and SMNc gene copy number with age of onset and survival in spinal muscular atrophy. *Eur J Hum Genet* **6**, 467–474.

Thurmond, J., Butchbach, M.E., Palomo, M., Pease, B., Rao, M., Bedell, L., Keyvan, M., Pai, G., Mishra, R., Haraldsson, M., Andresson, T., Bragason, G., Thosteinsdottir, M., Bjornsson, J. M., Coovert, D. D., Burghes, A. H., Gurney, M. E. & Singh, J. (2008) Synthesis and biological evaluation of novel 2,4-diaminoquinazoline derivatives as *SMN2* promoter activators for the potential treatment of spinal muscular atrophy. *J Med Chem* **51**, 449–469.

Tiziano, F. D., Lomastro, R., Pinto, A. M., Messina, S., D'amico, A., Fiori, S., Angelozzi, C., Pane, M., Mercuri, E., Bertini, E., Neri, G. & Brahe, C. (2010) Salbutamol increases survival motor neuron (SMN) transcript levels in leucocytes of spinal muscular atrophy (SMA) patients: relevance for clinical trial design. *J Med Genet* **47**, 856–858.

Torres-Benito, L., Neher, M. F., Cano, R., Ruiz, R. & Tabares, L. (2011) SMN requirement for synaptic vesicle, active zone and microtubule postnatal organization in motor nerve terminals. *PloS one* **6**, e26164.

Tran, V. K., Sasongko, T. H., Hong, D. D., Hoan, N. T., Dung, V. C., Lee, M. J., Gunadi Takeshima, Y., Matsuo, M. & Nishio, H. (2008) *SMN2* and NAIP gene dosages in Vietnamese patients with spinal muscular atrophy. *Ped Int* **50**, 346–3451.

Tsai, L. K. (2012) Therapy Development for Spinal Muscular Atrophy in SMN Independent Targets. *Neural Plasticity* 2012, ID 456478, 1–13.

Tsai, L. K., Chen, Y. C., Cheng, W. C., Ting, C. H., Dodge, J. C., Hwu, W. L., Cheng, S. H., Passini, M. A. (2012) IGF-1 delivery to CNS attenuates motor neuron cell death but does not improve motor function in type III SMA mice. *Neurobiol Dis* **45**, 272–279.

Tzeng, A. C., Cheng, J., Fryczynski, H., Niranjan, V., Stitik, T., Sial, A., Takeuchi, Y., Foye, P., Deprince, M. & Bach, J. R. (2000) A

study of thyrotropin-releasing hormone for the treatment of spinal muscular atrophy: a preliminary report. *Am J Phy Med Rehab/Ass Acad Physiatrists* **79**, 435-440.

Van Den Bosch, L. (2006) The causes and mechanism of selective motor neuron death in amyotrophic lateral sclerosis. *Verh K Acad Geneeskd Belg* **68**, 249–269.

Van Der Steege, G., Grootscholten, P. M., Van Der Vlies, P., Draaijers, T. G., Osinga, J., Cobben, J. M., Scheffer, H. & Buys, C. H. (1995) PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. *Lancet* **345**, 985–986.

Van Meerbeke, J. P. & Sumner, C. J. (2011) Progress and promise: the current status of spinal muscular atrophy therapeutics. *Discovery Med* **12**, 291–305.

Van Meerbeke, J. P., Gibbs, R., Plasterer, H., Feng, Z., Lin, M.-Y., Wee, C., Xia, B., Jacques, V., Rusche, J. & Sumner, C. (2011) The therapeutic effects of RG3039 in severe spinal muscular atrophy mice and normal human volunteers. In: *41st Annual Society of Neuroscience Meeting) Program 558.04/Poster H9, Annual Society of Neuroscience Meeting,* November 15, 2011 ed. Washington, DC: Society for Neuroscience.

Vardatsikos, G., Sahu, A. & Srivastava, A. K. (2009) The insulin-like growth factor family: molecular mechanisms, redox regulation, and clinical implications. *Antioxid Redox Signal* **11**, 1165–1190.

Velasco, E., Valero, C., Valero, A., Moreno, F. & Hernandez-Chico, C. (1996) Molecular analysis of the SMN and NAIP genes in Spanish spinal muscular atrophy (SMA) families and correlation between number of copies of cBCD541 and SMA phenotype. *Hum Mol Genet* **5**, 257–263.

Vezain, M., Saugier-Veber, P., Goina, E., Touraine, R., Manel, V., Toutain, A., Fehrenbach, S., Frebourg, T., Pagani, F., Tosi, M. & Martins, A. (2010) A rare *SMN2* variant in a previously unrecognized composite splicing regulatory element induces exon 7 inclusion and reduces the clinical severity of spinal muscular atrophy. *Hum Mut* **31**, E1110–E1125.

Wadman, R. I., Bosboom, W. M., Van Der Pol, W. L., Van Den Berg, L. H., Wokke, J. H., Iannaccone, S. T. & Vrancken, A. F. (2012a) Drug treatment for spinal muscular atrophy type I. *Cochrane Database Syst Rev* **4**, CD006281.

Wadman, R. I., Bosboom, W. M., Van Der Pol, W. L., Van Den Berg, L. H., Wokke, J. H., Iannaccone, S. T. & Vrancken, A. F. (2012b) Drug treatment for spinal muscular atrophy types II and III. *Cochrane Database Syst Rev* **4**, CD006282.

Weihl, C. C., Connolly, A. M. & Pestronk, A. (2006) Valproate may improve strength and function in patients with type III/IV spinal muscle atrophy. *Neurology* **67**, 500–501.

Wen, H. L., Lin, Y. T., Ting, C. H., Lin-Chao, S., Li, H. & Hsieh-Li, H. M. (2010) Stathmin, a microtubule-destabilizing protein, is dysregulated in spinal muscular atrophy. *Hum Mol Genet* **19**, 1766–1778.

Wirth, B., Brichta, L., Schrank, B., Lochmuller, H., Blick, S., Baasner, A. & Heller, R. (2006a) Mildly affected patients with spinal muscular atrophy are partially protected by an increased *SMN2* copy number. *Hum Genet* **119**, 422–428.

Wirth, B., Brichta, L., Hahnen, E. (2006b) Spinal muscular atrophy: from gene to therapy. *Semin Pediatr Neurol* **13**, 121–131.

Wirth, B., Herz, M., Wetter, A., Moskau, S., Hahnen, E., Rudnik-Schoneborn, S., Wienker, T. & Zerres, K. (1999) Quantitative analysis of survival motor neuron copies: identification of subtle *SMN1* mutations in patients with spinal muscular atrophy, genotype-phenotype correlation, and implications for genetic counseling. *Am J Hum Genet* **64**, 1340–1356.

Wolstencroft, E. C., Mattis, V., Bajer, A. A., Young, P. J. & Lorson, C. L. (2005) A non-sequence-specific requirement for SMN protein activity: the role of aminoglycosides in inducing elevated SMN protein levels. *Hum Mol Genet* **14**, 1199–1210.

Yoon, S., Lee, C. H. & Lee, K. A. (2010) Determination of *SMN1* and *SMN2* copy numbers in a Korean population using multiplex ligation-dependent probe amplification. *Korean J Lab Med* **30**, 93–96.

Zerres, K. & Davies, K. E. (1999) 59th ENMC International Workshop: Spinal Muscular Atrophies: recent progress and revised diagnostic criteria 17–19 April 1998, Soestduinen, The Netherlands. *Neuromuscul Disord* **9**, 272–278.

Zhang, H. L., Pan, F., Hong, D., Shenoy, S. M., Singer, R. H. & Bassell, G. J. (2003) Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *J Neurosci* **23**, 6627–6637.

Zhang, H., Xing, L., Rossol, W., Wichterle, H., Singer, R. H, Bassel, G. J. (2006) Multiprotein complexes of the survival motor neuron protein SMN with gemins traffic to neuronal processes and growth cones of motor neurons. *Neurobiol Dis* **26**, 8622–8632.

Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M. & Dreyfuss, G. (2008) SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell* **133**, 585–600.

Zou, T., Yang, X., Pan, D., Huang, J., Sahin, M. & Zhou, J. (2011) SMN deficiency reduces cellular ability to form stress granules, sensitizing cells to stress. *Cell Mol Neurobiol* **31**, 541–550.

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COMMENTARY

PLS3 expression and SMA phenotype: a commentary on correlation of PLS3 expression with disease severity in children with spinal muscular atrophy

Hisahide Nishio

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MODIFIERS OF SMA PHENOTYPE

Spinal muscular atrophy (SMA; OMIM 253300) is an autosomal recessive neuromuscular disorder characterized by the loss of motor neurons.[1] It is clinically heterogeneous and can be classified into three subtypes depending on the age of onset and achievement of motor milestones: SMA type 1 (a severe type with onset before the age of 6 months, patients are unable to sit without support), SMA type 2 (an intermediate type with onset before the age of 18 months, patients are unable to stand or walk without support) and SMA type 3 (a mild type with onset after the age of 18 months, patients are able to stand and walk independently until the disease progresses).[1]

Causative mutations of SMA are mainly homozygous deletions of the *SMN1* gene located on chromosome 5q13. The gene product, the SMN protein, has critical roles in a variety of cellular activities.[1] The *SMN2* gene, an almost identical homolog of *SMN1*, is retained in all SMA patients and produces low levels of SMN protein, but does not fully compensate for mutated *SMN1*. *SMN2* is now considered to be a modifier of the SMA phenotype, as high copy numbers of *SMN2* ameliorate the clinical severity in SMA patients.[1] However, some asymptomatic individuals inherit the same *SMN* genotype (homozygous *SMN1* deletion and identical *SMN2* copy numbers) as their affected siblings. The presence of such SMA-discordant families suggests the influence of modifier genes other than *SMN2*.[2]

H Nishio is at Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, Kobe, Japan
E-mail: nishio@med.kobe-u.ac.jp

PLS3 EXPRESSION IN FEMALE SMA PATIENTS

Oprea *et al.*[3] identified six SMA-discordant families with eight fully asymptomatic females who shared the same *SMN* genotype as their affected siblings. The authors found that *PLS3*, a gene encoding the actin-bundling protein plastin 3 (PLS3, T-plastin or T-fimbrin; MIM300131, Xq23), was highly expressed in lymphoblastoid cell lines from unaffected female siblings. They also obtained experimental evidence that the overexpression of *PLS3* rescues the axonal growth defect associated with low-SMN levels in motor neurons of SMA-mouse embryos and zebrafish. They concluded that PLS3 (or *PLS3*) may be a gender-specific SMA modifier.

To examine whether *PLS3* is a gender-specific modifier, Stratigopoulos *et al.*[4] analyzed 88 SMA patients (41 males and 47 females), and found that in postpubertal female patients, the amount of the *PLS3* transcript was highest in type 3 patients, followed by type 2 patients and lowest in type 1 patients. In these SMA patients, *PLS3* expression was related to *SMN2* copy number, gross motor function measure and clinical subtype. Interestingly, *PLS3* expression in either pre- or postpubertal male patients or prepubertal female patients did not correlate with clinical subtype or *SMN2* copy number. The authors concluded that *PLS3* is an age- and/or puberty-specific and sex-specific modifier of the SMA phenotype.

In this issue of the journal, Cao *et al.*[5] also suggested that *PLS3* is a gender-specific modifier for SMA phenotype, based on their analysis of 65 SMA patients (36 males and 29 females) and 59 healthy controls (31 males and 28 females). According to this study, among the older female patients (>3 years of age), *PLS3* expression was significantly higher in type 3 than type 2, which is consistent with the results of Stratigopoulos *et al.*, although the age cut-off levels differed between the two studies. Cao *et al.* demonstrated two important findings in this article. The first was that *PLS3* expression is age-dependent and decreases in type 1–2 SMA patients and healthy controls above 3 years of age, suggesting that the patient's age should always be considered when evaluating *PLS3* expression. The second was that *PLS3* expression of type 3 patients who can walk is higher than in patients unable to do so, suggesting that it could be used as a biomarker of disease progression. However, as stated in the report, this will be necessary to verify in a larger sample.

PERSPECTIVE ON SMA TREATMENT

The relationships between Smn protein levels (or *SMN2* copy number) and Pls3 protein levels (or *PLS3* expression) in motor neurons are still poorly understood and studies have shown conflicting results. For example, PLS3 was suggested to be expressed independently of SMN (or *SMN2* copy number) in the report by Oprea *et al.*,[3] which described unaffected siblings of the patients, and that by Ackermann *et al.*[6] using SMA mice. By contrast, the study of Hao *et al.*[7] on zebrafish showed that Pls3 levels were dependent on Smn levels.

Hao *et al.* demonstrated that human *PLS3* overexpression was able to rescue neuromuscular junction defects in SMA mutants

of transgenic zebrafish, suggesting that decreased PLS3 contributes to SMA motor phenotypes. Indeed, when Smn protein levels were severely decreased in SMA mutants, they found that PLS3 translation was compromised, leading to SV2 presynaptic defects at the neuromuscular junction. In addition, as human SMN expression increased the Pls3 level in SMA mutants, this led them to conclude that Pls3 levels are dependent on Smn levels.

Ackermann *et al.* generated a conditional PLS3-overexpressing mouse bred into an SMA background to show that PLS3 overexpression restored defects in motor neurons and neuromuscular junctions. In this study, they also observed that Pls3 was not decreased in the brain and spinal cord of SMA mice compared with controls. This finding suggests that Pls3 levels are not regulated by Smn levels. Thus, it was postulated that PLS3 has an Smn-independent ameliorative action on the phenotype of SMA mice.

As PLS3 expression restores the motor neuron and rescues neuromuscular junction defects, it could be a potential therapeutic target for SMA treatment.[3,6] This has already been hypothesized by Bowerman *et al.*[8] who have suggested possible treatment strategies including the upregulation of PLS3 to target actin cytoskeletal dynamics.

CONCLUSIONS

Cao *et al.*[5] confirmed that *PLS3* expression modifies the phenotype of female SMA patients and suggested that its expression in peripheral blood cells will be a useful biomarker of disease progression in female patients with SMA. Investigation of the modifiers including PLS3 expression, *SMN2* copy numbers and additional unknown factors will help our understanding of the mechanism underlying SMA pathology (Figure 1), and lead to the establishment of new treatments for SMA.

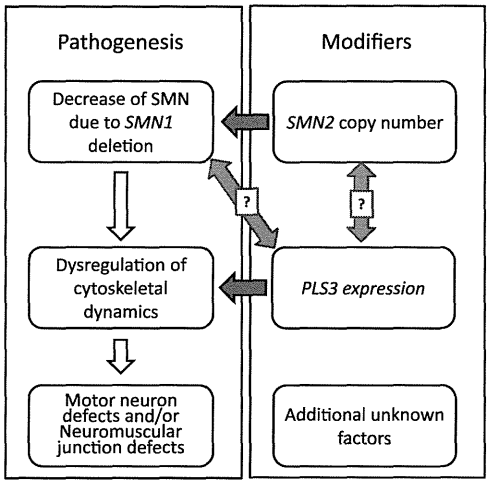

Figure 1 Modifiers of phenotype in female SMA patients. *SMN2* copy number and *PLS3* expression are recognized as phenotype modifiers in female SMA patients. Both the SMN and PLS3 proteins are involved in the regulation of cytoskeleton dynamics. However, the relationship between SMN protein levels (or *SMN2* copy number) and PLS3 protein levels (or *PLS3* expression) remain to be elucidated. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

1 Nurputra, D. K., Lai, P. S., Harahap, N. I. F., Morikawa, S., Yamamoto, T. & Nishimura, N. Spinal Muscular Atrophy: From Gene Discovery to Clinical Trials. *Ann. Hum. Genet.* **77,** 435–463 (2013).

2 Prior, T. W., Swoboda, K. J., Scott, H. D. & Hejmanowski, A. Q. Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. *Am. J. Med. Genet.* **130 A,** 307–310 (2004).

3 Oprea, G. E., Kröber, S., McWhorter, M. L., Rossoll, W., Müller, S., Krawczak, M. *et al.* Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* **320,** 524–527 (2008).

4 Stratigopoulos, G., Lanzano, P., Deng, L., Guo, J., Kaufmann, P., Darras, B. *et al.* Association of plastin 3 expression with disease severity in spinal muscular atrophy only in postpubertal females. *Arch. Neurol.* **67,** 1252–1256 (2010).

5 Cao, Y. Y., Qu, Y. J., Bai, J. L., Jin, Y. W., Wang, H. & Song, F. Correlation of PLS3 expression with disease severity in children with spinal muscular atrophy. *J. Hum. Genet.* **58** (2013). Q2

6 Ackermann, B., Kröber, S., Torres-Benito, L., Borgmann, A., Peters, M., Hosseini Barkooie, S. M. *et al.* Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. *Hum. Mol. Genet.* **22,** 1328–1347 (2013).

7 Hao, L. T., Wolman, M., Granato, M. & Beattie, C. E. Survival motor neuron affects plastin 3 protein levels leading to motor defects. *J. Neurosci.* **32,** 5074–5084 (2012).

8 Bowerman, M., Anderson, C. L., Beauvais, A., Boyl, P. P., Witke, W. & Kothary, R. SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Mol. Cell Neurosci.* **42,** 66–74 (2009).

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Original article

Intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some spinal muscular atrophy (SMA) patients

Tomoto Yamamoto [a,b], Hideyuki Sato [a], Poh San Lai [c], Dian Kesumapramudya Nurputra [a], Nur Imma Fatimah Harahap [a], Satoru Morikawa [a,b], Noriyuki Nishimura [a,b], Takashi Kurashige [d], Tomohiko Ohshita [d], Hideki Nakajima [e], Hiroyuki Yamada [f], Yoshinobu Nishida [f], Soichiro Toda [g], Jun-ichi Takanashi [g], Atsuko Takeuchi [h], Yumi Tohyama [i], Yuji Kubo [j], Kayoko Saito [j], Yasuhiro Takeshima [b], Masafumi Matsuo [k], Hisahide Nishio [a,b,*]

[a] *Department of Community Medicine and Social Health Care, Kobe University Graduate School of Medicine, Kobe, Japan*
[b] *Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan*
[c] *Department of Paediatrics, Yong Loo Lin School of Medicine, NUHS, National University of Singapore, Singapore*
[d] *Department of Clinical Neuroscience and Therapeutics, Hiroshima University, Graduate School of Biomedical and Health Sciences, Hiroshima, Japan*
[e] *Department of Clinical Neuroscience and Neurology, Nagasaki University Graduate School of Biomedical Science, Nagasaki, Japan*
[f] *Department of Pediatrics, Hyogo Prefectural Tsukaguchi Hospital, Amagasaki, Hyogo, Japan*
[g] *Department of Pediatrics, Kameda Medical Center, Kamogawa, Chiba, Japan*
[h] *Kobe Pharmaceutical University, Kobe, Japan*
[i] *Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, Himeji, Japan*
[j] *Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan*
[k] *Department of Medical Rehabilitation, Kobe Gakuin University, Kobe, Japan*

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Abstract

Background: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by deletion or intragenic mutation of *SMN1*. SMA is classified into several subtypes based on clinical severity. It has been reported that the copy number of *SMN2*, a highly homologous gene to *SMN1*, is associated with clinical severity among SMA patients with homozygous deletion of *SMN1*. The purpose of this study was to clarify the genotype-phenotype relationship among the patients without homozygous deletion of *SMN1*. *Methods:* We performed molecular genetic analyses of *SMN1* and *SMN2* in 112 Japanese patients diagnosed as having SMA based on the clinical findings. For the patients retaining *SMN1*, the PCR or RT-PCR products of *SMN1* were sequenced to identify the mutation. *Results:* Out of the 112 patients, 106 patients were homozygous for deletion of *SMN1*, and six patients were compound heterozygous for deletion of one *SMN1* allele and intragenic mutation in the retained *SMN1* allele. Four intragenic mutations were identified in the six patients: p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32 and p.Tyr277Cys. To the best of our knowledge, all mutations except p.Trp92Ser were novel mutations which had never been previously reported. According to our observation, clinical severity of the six patients was determined by the type and location of the mutation rather than *SMN2* copy number. *Conclusion: SMN2* copy number is not always associated with clinical severity of SMA patients, especially SMA patients retaining one *SMN1* allele.

* Corresponding author. Address: Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-Cho, Chuo-Ku, Kobe 650-0017, Japan. Tel.: +81 78 382 5540; fax: +81 78 382 5559.
E-mail address: nishio@med.kobe-u.ac.jp (H. Nishio).

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Keywords: Spinal muscular atrophy; *SMN1*; *SMN2*; Copy number; Intragenic mutation

1. Introduction

Spinal muscular atrophy (SMA) is a common neuromuscular disease characterized by degeneration of lower motor neurons, leading to the axial and limb weakness associated with muscle atrophy. The incidence of the disease has been estimated at 1 in 10,000 newborns, with an expected carrier frequency of 1 in 50 [1]. Based on molecular epidemiological analysis using *SMN1* copy number, the worldwide carrier frequency of SMA is 1 in 40–70, suggesting a disease incidence of 1 in 6000–20,000 [2].

SMA is classified into four subtypes depending on the age of disease onset and the achievement of motor milestones [3]: namely, type 1 (severe form; onset age of 0–6 months old, unable to sit unaided), type 2 (intermediate form; onset age of <18 months old, unable to stand or walk unaided), type 3 (mild form; onset age of >18 months old, able to stand or walk unaided), and type 4 (milder form; onset age of >21 years old, able to stand or walk unaided).

All SMA subtypes have been mapped to chromosomal region 5q11.2–13.3 [4–7] and the survival motor neuron gene (*SMN*) and neuronal apoptosis-inhibitory protein gene (*NAIP*) were cloned as SMA-causing gene candidates [8,9]. The *SMN* gene exists as two highly homologous copies, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [8]. It is now established that SMA is caused by deletions or intragenic mutations of *SMN1*. *SMN1* is homozygously deleted in more than 90% of SMA patients [8,10], and deleteriously mutated in the remaining patients [8,11]. On the other hand, *NAIP*-deletion has been found only in 50% of type 1 patients, and much less frequently in type 2 and 3 patients. The presence or absence of *NAIP* may be associated with the clinical severity of SMA [9,10].

Increased *SMN2* copy number is related to improved survival outcomes and maintenance of motor function [12–16]. Both *SMN* genes, *SMN1* and *SMN2*, differ by only five nucleotides [8]. Of the five nucleotide differences between the two *SMN* genes, only one is present in the coding region at position +6 of exon 7 in *SMN1* (c.840C) and *SMN2* (c.840T). Although this mutation is translationally silent, the C-to-T transition alters the splicing pattern in *SMN2* exon 7 [17]. *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces ~90% of exon7-lacking (△7) *SMN2* transcripts and ~10% of FL-*SMN2* transcripts [18]. It is expected that high *SMN2* copy number may produce a large amount of FL-*SMN2* to compensate for the loss of *SMN1* to some degree.

However, most phenotype-genotype correlation studies have been conducted only in SMA patients with a complete loss of *SMN1*. The relationship between *SMN2* copy number and clinical severity are yet to be clarified in SMA patients retaining one *SMN1* allele. In this study, to understand the modifying factors in determining the clinical phenotype of SMA patients retaining one *SMN1* allele, we conducted a mutation analysis and investigated the contribution of *SMN2* copy number to the clinical severity in such patients.

2. Patients and methods

2.1. Patients

All 112 Japanese patients (51 males and 61 females) fulfilled the diagnostic criteria defined by the International SMA Consortium [19]. Here, patients with onset before 20 years old was classified into type 3, and those with onset after 21 years old was classified into type 4 [3]. Informed consent was obtained from these patients and/or their parents. This study project including genetic analysis was approved by the Ethical Committee of the Kobe University Graduate School of Medicine, Japan.

In this study, six patients (Patients 1–6) retaining one allele of *SMN1* exon 7, were found to carry intragenic mutations in *SMN1*. Patients 1 (female) and 2 (male) were type 1 patients reported previously to have one *SMN1* allele [20]. Patient 3 was a 19-day-old male with SMA type 1, referred to us because of respiratory insufficiency and swallowing difficulties. Patient 4 was a 7-year-old female with type 2 SMA. She was first diagnosed as having SMA type 2 close to type 3 because she could sit unaided and stand while holding onto something (such as a wall or table) for support. However, she rapidly lost such abilities at 2 years old. Finally, she was bound to artificial ventilator because of respiratory insufficiency at 3 years old. Patient 5 was a 13-year-old male with type 3 SMA, who had pain and heaviness in legs during exercise since the age of 11 years. He later developed symptoms including waddling gait, muscle weakness and atrophy in quadriceps, and attenuated patellar tendon reflex. Patient 6 was a 19-year-old female with type 3 SMA, who had noticed muscle weakness during swimming exercise at the age of 13 years. She gradually lost her running ability and could no

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longer run as fast as the other classmates in her high school days.

2.2. SMN and NAIP deletion test

Genomic DNA was extracted from 3 ml of whole blood using a DNA extraction kit, SepaGene (Sanko Junyaku, Tokyo, Japan). For the *SMN* and *NAIP* deletion test, PCR and enzyme digestion reactions were performed according to the method of van der Steege et al. [21]. Exon 5 of the *NAIP* gene was detected using the PCR method of Roy et al. [9]. Here we adopted "exon 5" as a widely accepted exon number, although this exon has been denoted as "exon 4" by Chen et al. [22].

2.3. Copy number analysis of the SMN genes using real time PCR method

We determined the copy numbers of the *SMN* genes based on the real-time PCR method of Tran et al. [23]. Cystic fibrosis trans-membrane regulator gene (*CFTR* gene) was used as a reference gene for the relative quantification of copy numbers.

2.4. Messenger RNA analysis

For the assignment of the mutation to *SMN1* or *SMN2*, mRNA analysis was performed. Total RNA was extracted from leukocytes using the acid guanidiumthiocyanate-phenol–chloroform method. *SMN1* and *SMN2* mRNA species were amplified by reverse transcriptase (RT)-PCR method [16,24]. A new primer, ex1-F (5′- GCT ATG GCG ATG AGC A GC GGC –3′), was designed for this study. The mRNA species encompassing exons 1–8 were amplified using primers ex1-F and 541C1120 [8], and the mRNA species encompassing exons 1–7 were amplified using primers ex1-F and 541C770 [8].

2.5. Nucleotide sequencing

The amplified PCR or RT-PCR products of *SMN* exons were purified and sequenced directly or after subcloning. The sequencing reaction was performed using a dye terminator cycle-sequencing kit (Life Technologies Corporation, Carlsbad, CA). The reaction product was electrophoresed on an ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA).

2.6. Computational algorithms

We predicted the mutation effects on the protein function using three computational algorithms: Sorting Intolerant from Tolerant amino acid substitutions (SIFT) [25], Polymorphism Phenotyping-2 (PolyPhen-2) [26], and Grantham score difference (Align-GVGD) [27].

2.7. Statistics

The correlation of copy number of *SMN2* with the clinical subtypes was compared by chi-square test and *t*-test. *P*-value of less than 0.05 was considered to indicate a significant difference. The software used for statistical analysis was Statistical Program for Social Science (SPSS) Version 16 (IBM Corporation, Paulo Alto, US).

3. Results

3.1. SMN1 and NAIP deletion test

SMN1 exon 7-deletion (herein after referred to as *SMN1*-deletion) was found in almost all SMA patients, regardless of clinical subtypes: 106 out of 112 (95%) patients with SMA in this study had *SMN1*-deletion and 6 patients (5%) had subtle mutations in *SMN1*. Out of 106 *SMN1*-deleted patients, 48 (45%) were type 1, 35 (33%) were type 2, 19 (18%) were type 3, and 4 (4%) were type 4 (Table 1).

In our study, 96 of 106 (91%) *SMN1*-deleted patients had deletion of *SMN1* exon 8. However, the other 10 patients (9.0%) retained *SMN1* exon 8. We confirmed that these patients had at least one copy of the hybrid gene with *SMN2* exon 7 and *SMN1* exon 8 using direct sequencing analysis of the PCR fragment amplified with the common primers for *SMN1* and *SMN2*.

NAIP exon 5-deletion (herein after referred to as *NAIP*-deletion) was always accompanied by *SMN1*-deletion (Table 1). In addition, *NAIP*-deletion was much more frequent in SMA type 1 than SMA non-type 1. *NAIP*-deletion was found in 29 out of 48 (60%) patients with *SMN1*-deleted SMA type 1, while it was found in only 8 out of 58 (14%) patients with *SMN1*-deleted SMA types 2, 3 and 4.

3.2. SMN2 copy number and clinical severity in patients with SMN1-deletion

We determined the *SMN2* copy numbers of all the patients enrolled in this study using the real-time PCR method. For the analysis of *SMN2* copy number and clinical severity, the"*SMN2* exon 7-*SMN1* exon 8 hybrid" gene is regarded as *SMN2*.

A significant relationship between *SMN2* copy number and clinical severity was observed in this study (Table 2). 38 out of 48 (79%) patients with *SMN1*-deleted SMA type 1 showed one copy or two copies of *SMN2*, 34 out of 35 (97%) patients with *SMN1*-deleted SMA type 2 showed three copies of *SMN2*, 18 out of 19 (95%) patients with *SMN1*-deleted SMA type 3 showed three or four copies of *SMN2*, and 3 out of 4 (75%) patients with *SMN1*-deleted SMA type 4 showed four copies of *SMN2*.

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Table 1
SMN1 and *NAIP* deletion test ($n = 112$).

SMN1		*NAIP*	Type 1	Type 2	Type 3	Type 4	Total
Exon 7	Exon 8	Exon 5					
Del	Del	Del	29	6	1	1	37
Del	Del	Non-del	17	24	15	3	59
Del	Non-del	Non-del	2	5	3	0	10
Non-del	Non-del	Non-del	3	1	2	0	6
Total			51	36	21	4	112

Table 2
Clinical severity and *SMN2* copy number in patients with homozygous *SMN1*-deletion ($n = 106$).

	1 copy	2 copies	3 copies	4 copies	Mean	(SD)
Type 1	1	37	10	0	2.18	(0.44)
Type 2	0	1	34	0	2.97	(0.17)
Type 3	0	1	13	5	3.18	(0.51)
Type 4	0	0	1	3	3.80	(0.40)
Total	1	39	58	8		

Table 3
Clinical severity and *SMN2* copy number in patients retaining one *SMN1* allele ($n = 6$).

	Sex	Onset	Type	*SMN2* copy number	Nucleotide change (exon)	Amino acid change	Domain	References
Patient 1	F	5m	1	3	c.275 G > C (exon 3)	p.Trp92Ser	Tudor	[20]
Patient 2	M	6m	1	3	c.275 G > C (exon 3)	p.Trp92Ser	Tudor	[20]
Patient 3	M	0m	1	2	c.819_820 insT (exon 6)	p.Thr274Tyr fsX32	C-terminal	This study
Patient 4	F	12m	2	1	c.830 A > G (exon 6)	p.Tyr277Cys	C-terminal	This study
Patient 5	M	11y	3	1	c.5 C > T (exon 6)	p.Ala2Val	N-terminal	This study
Patient 6	F	13y	3	1	c.5 C > T (exon 1)	p.Ala2Val	N-terminal	This study

3.3. *SMN2* copy number and clinical severity in patients retaining one *SMN1* allele

In this study, we identified four different intragenic mutations in *SMN1* of six patients without *SMN1*-deletion (Patients 1–6) (Table 3). All of them were compound heterozygous for deletion of one *SMN1* allele and an intragenic point mutation of the other *SMN1* allele. The intragenic mutations included three missense mutations and one frame-shift mutation: c. 5C > T (p.Ala2Val) in exon 1, c. 275G > C (p.Trp92Ser) in exon 3, c.819_820insT (p.Thr274TyrfsX32) in exon 6, and c.830 A > G (p.Tyr277Cys) in exon 6. Three of the mutations, p.Ala2Val, p.Thr274TyrfsX32 and p.Tyr277Cys, are novel ones which have never been previously reported.

We predicted the effect of the missense mutations on the protein function using three computational algorithms: SIFT [25], PolyPhen-2 [26], and Align-GVGD [27]. All three types of missense mutation were predicted to damage the protein function.

Interestingly, the observed phenotype of patients carrying an intragenic mutation deviated from the expected correlations with the *SMN2* copy number (Table 3 and Fig. 1): type 3 patients with p.Ala2Val (Patients 5 and 6) carried only a single copy of *SMN2*, while type 1 patients with p.Trp92Ser (Patients 1 and 2) carried as many as 3 copies of *SMN2*. These findings suggested that intragenic mutations in *SMN1* influence the clinical phenotype more significantly than *SMN2* copy numbers in some patients.

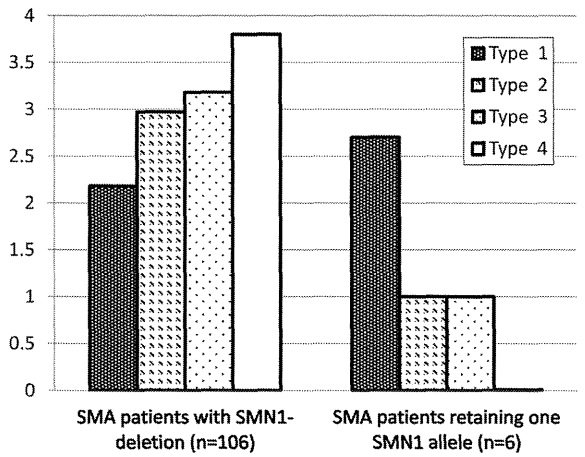

Fig. 1. Mean *SMN2* copy numbers in SMA patients. Patients with *SMN1*-deletion ($n = 106$) carried zero copies of *SMN1*. Patients retaining one *SMN1* allele ($n = 6$) which harbored intragenic mutations: p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32 and p.Tyr277Cys.

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4. Discussion

The identification of intragenic mutations, especially missense mutations, may help us to further elucidate the function of SMN and the pathogenic mechanism of SMA. In this study, we identified four different intragenic *SMN1* mutations in six SMA patients without *SMN1*-deletion. These intragenic mutations were p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32, and p.Tyr277Cys.

The p.Ala2Val mutation, which is located in the N-terminal domain, has never been reported until now. Our two patients with p.Ala2Val were unrelated. However, another mutation in the same location, p.Ala2Gly, has previously been reported in three SMA patients; these patients were also unrelated individuals, but had the possibility of sharing an ancestral origin [28]. All patients with p.Ala2Gly carried only one *SMN2* copy, and two of them showed mild phenotype (type 3). The mutation effect of p.Ala2Val, as well as p.Ala2Gly, may be much less deleterious than other missense mutations identified in this study. However, *SMN2* may not be dispensable in these patients. The mild SMA mutation, p.Ala2Gly, by itself cannot rescue $Smn^{-/-}$ mice, suggesting that homomer of the mutant SMN is not functional [29]. According to the Workman et al. [30], the heteromer of mutant SMN and FL-SMN from a single copy of *SMN2* must have some function.

We previously reported the p.Trp92Ser mutation in two unrelated patients [20]. This mutation is located in the Tudor domain to which other proteins bind. [31]. Many of them are involved in small nuclear ribonucleoprotein (snRNP) biogenesis. SMN Tudor domain preferentially binds symmetric dimethylated arginine (sDMA) of Sm proteins which constitute Sm core of snRNP [32]. We have already reported that the binding ability of the mutated SMN with p.Trp92Ser to SmB and fibrillarin was reduced to half of normal levels [20]. Most recently, Tripsianes et al. [33] examined the relationship between mutated Tudor domain and the binding capacity to sDMA *in vitro*. According to them, p.Trp92Ser mutant was unfolded, as judged by fingerprint NMR spectra analysis, and did not bind sDMA [33].

The p.Thr274TyrfsX32 mutation is a frameshift mutation arising from a single nucleotide insertion in exon 6 and results in a truncated SMN protein lacking the C-terminal domain of *SMN*. A new isoform of SMN, axonal SMN (a-SMN), is expected to be produced in the patient, because a-SMN is a truncated, alternatively spliced isoform of *SMN1*, originating from the retention of intron 3 [37,38]. Although the role of a-SMN in the pathogenesis of SMA has not been clarified yet, the disease severity of the patient with this mutation suggests that a-SMN functions were not enough to fully compensate for the deleterious mutation.

The p.Tyr277Cys mutation is located in the C-terminal domain of SMN known as the YG box, which is essential for oligomerization or self-association of SMN [31]. Oligomerization defect destroys the function of SMN and correlates with clinical severity of SMA [34]. Many other mutations in the same domain have been frequently reported [35,36], although the p.Tyr277Cys mutation has not been reported up to now.

An interesting question arises as to which factor contributes more significantly to clinical phenotype in SMA, *SMN1* intragenic mutation or *SMN2* copy number. According to our analysis of the patients with homozygous *SMN1*-deletion (Table 2 and Fig. 1), increased *SMN2* copy number was associated with milder phenotype, which was compatible with previous reports [12–16]. However, the phenotype of patients without *SMN1*-deletion was not related to their *SMN2* copy number (Table 3 and Fig. 1). In our study, p.Ala2Val was found in two type 3 patients with one *SMN2* copy, p.Trp92Ser in two type 1 patients with three *SMN2* copies, p.Thr274TyrfsX32 in one type 1 patient with two *SMN2* copies, and p.Tyr277Cys in one type 2 patients with one *SMN2* copy. According to our findings, *SMN1* intragenic mutations appear to contribute much more significantly to clinical severity than *SMN2* copy numbers in some patients.

Since our patients carry various intragenic *SMN1*mutations, the next question is whether *SMN2* copy number effect is present or absent among the patients with the same *SMN1* mutation. Using the data of the SMA patients with missense mutations described in a review paper of Sun et al. [36], we analyzed the relationship between *SMN2* copy number and clinical severity in eleven patients with p.Tyr272Cys in *SMN1*. We observed that higher *SMN2* copy number was correlated with reduced disease severity: patients with three *SMN2* copies showed milder phenotype than the patients with one *SMN2* copy number. Thus, we speculate that *SMN2* copy number effect is present when the *SMN1* mutation is the same in the patients.

In conclusion, *SMN2* copy number is not always associated with clinical severity of SMA patients, especially SMA patients without *SMN1*-deletion. In these patients, clinical severity in SMA caused by *SMN1* mutations may be determined by the type and location of the intragenic mutation. Intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some spinal muscular atrophy patients.

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References

[1] Prior TW, Snyder PJ, Rink BD, Pearl DK, Pyatt RE, Mihal DC, et al. Newborn and carrier screening for spinal muscular atrophy. Am J Med Genet A 2010;152A:1608–16.
[2] Nurputra DK, Lai PS, Harahap NI, Morikawa S, Yamamoto T, Nishimura N, et al. Spinal muscular atrophy: from gene discovery to clinical trials. Ann Hum Genet 2013;77:435–63.
[3] Kolb SJ, Kissel JT. Spinal muscular atrophy: a timely review. Arch Neurol 2011;68:979–84.
[4] Brzustowicz LM, Lehner T, Castilla LH, Penchaszadeh GK, Wilhelmsen KC, Daniels R, et al. Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. Nature 1990;344:540–1.
[5] Gilliam TC, Brzustowicz LM, Castilla LH, Lehner T, Penchaszadeh GK, Daniels RJ, et al. Genetic homogeneity between acute and chronic forms of spinal muscular atrophy. Nature 1990;345:823–5.
[6] Melki J, Abdelhak S, Sheth P, Bachelot MF, Burlet P, Marcadet A, et al. Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. Nature 1990;344:767–8.
[7] Melki J, Sheth P, Abdelhak S, Burlet P, Bachelot MF, Lathrop MG, et al. Mapping of acute (type I) spinal muscular atrophy to chromosome 5q12–q14. The French spinal muscular atrophy investigators. Lancet 1990;336:271–3.
[8] Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell 1995;80:155–65.
[9] Roy N, Mahadevan MS, McLean M, Shutler G, Yaraghi Z, Farahani R, et al. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell 1995;80:167–78.
[10] Hahnen E, Forkert R, Marke C, Rudnik-Schöneborn S, Schönling J, Zerres K, et al. Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: evidence of homozygous deletions of the *SMN* gene in unaffected individuals. Hum Mol Genet 1995;4:1927–33.
[11] Wirth B. An update of the mutation spectrum of the survival motor neuron gene (*SMN1*) in autosomal recessive spinal muscular atrophy (SMA). Hum Mutat 2000;15:228–37.
[12] Velasco E, Valero C, Valero A, Moreno F, Hernández-Chico C. Molecular analysis of the SMN and NAIP genes in Spanish spinal muscular atrophy (SMA) families and correlation between number of copies of *cBCD541* and SMA phenotype. Hum Mol Genet 1996;5:257–63.
[13] Coovert DD, Le TT, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, et al. The survival motor neuron protein in spinal muscular atrophy. Hum Mol Genet 1997;6:1205–14.
[14] McAndrew PE, Parsons DW, Simard LR, Rochette C, Ray PN, Mendell JR, et al. Identification of proximal spinal muscular atrophy carriers and patients by analysis of *SMNT* and *SMNC* gene copy number. Am J Hum Genet 1997;60:1411–22.
[15] Taylor JE, Thomas NH, Lewis CM, Abbs SJ, Rodrigues NR, Davies KE, et al. Correlation of *SMNt* and *SMNc* gene copy number with age of onset and survival in spinal muscular atrophy. Eur J Hum Genet 1998;6:467–74.
[16] Harada Y, Sutomo R, Sadewa AH, Akutsu T, Takeshima Y, Wada H, et al. Correlation between *SMN2* copy number and clinical phenotype of spinal muscular atrophy: three *SMN2* copies fail to rescue some patients from the disease severity. J Neurol 2002;249:1211–9.
[17] Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the *SMN* gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci USA 1999;96:6307–11.
[18] Jodelka FM, Ebert AD, Duelli DM, Hastings ML. A feedback loop regulates splicing of the spinal muscular atrophy-modifying gene, *SMN2*. Hum Mol Genet 2010;19(4):906–17.
[19] Zerres K, Davies KE. 59th ENMC International workshop: spinal muscular atrophies: recent progress and revised diagnostic criteria 17–19 April 1998, Soestduinen, The Netherlands. Neuromuscul Disord 1999;9:272–8.
[20] Kotani T, Sutomo R, Sasongko TH, Sadewa AH, Gunadi, Minato T, et al. A novel mutation at the N-terminal of SMN Tudor domain inhibits its interaction with target proteins. J Neurol 2007;254:624–30.
[21] van der Steege G, Grootscholten PM, van der Vlies P, Draaijers TG, Osinga J, Cobben JM, et al. PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. Lancet 1995;345:985–6.
[22] Chen Q, Baird SD, Mahadevan M, Besner-Johnston A, Farahani R, Xuan J, et al. Sequence of a 131-kb region of 5q13.1 containing the spinal muscular atrophy candidate genes *SMN* and *NAIP*. Genomics 1998;48:121–7.
[23] Tran VK, Sasongko TH, Hong DD, Hoan NT, Dung VC, Lee MJ, et al. *SMN2* and *NAIP* gene dosages in Vietnamese patients with spinal muscular atrophy. Pediatr Int 2008;50:346–51.
[24] Nishio H, Ishikawa Y, Lee MJ, Fujii M, Kanda F, Jinnai K, et al. Decreased expression of full-length mRNA for *cBCD541* does not correlate with spinal muscular atrophy phenotype severity. Neurology 1997;48:1266–70.
[25] Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4:1073–81.
[26] Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248–9.
[27] Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, et al. Comprehensive statistical study of 452 *BRCA1* missense substitutions with classification of eight recurrent substitutions as neutral. J Med Genet 2006;43:295–305.
[28] Parsons DW, McAndrew PE, Iannaccone ST, Mendell JR, Burghes AH, Prior TW. Intragenic *telSMN* mutations: frequency, distribution, evidence of a founder effect, and modification of the spinal muscular atrophy phenotype by *cenSMN* copy number. Am J Hum Genet 1998;63:1712–23.
[29] Monani UR, Pastore MT, Gavrilina TO, Jablonka S, Le TT, Andreassi C, et al. A transgene carrying an A2G missense mutation in the *SMN* gene modulates phenotypic severity in mice with severe (type I) spinal muscular atrophy. J Cell Biol 2003;160:41–52.
[30] Workman E, Saieva L, Carrel TL, Crawford TO, Liu D, Lutz C, et al. A *SMN* missense mutation complements *SMN2* restoring snRNPs and rescuing SMA mice. Hum Mol Genet 2009;18:2215–29.
[31] Coady TH, Lorson CL. SMN in spinal muscular atrophy and snRNP biogenesis. Wiley Interdiscip Rev RNA 2011;2:546–64.
[32] Liu K, Guo Y, Liu H, Bian C, Lam R, Liu Y, et al. Crystal structure of TDRD3 and methyl-arginine binding characterization of TDRD3, SMN and SPF30. PLoS One 2012;7:e30375.
[33] Tripsianes K, Madl T, Machyna M, Fessas D, Englbrecht C, Fischer U, et al. Structural basis for dimethylarginine recognition by the Tudor domains of human SMN and SPF30 proteins. Nat Struct Mol Biol 2011;18:1414–20.
[34] Lorson CL, Strasswimmer J, Yao JM, Baleja JD, Hahnen E, Wirth B, et al. SMN oligomerization defect correlates with spinal muscular atrophy severity. Nat Genet 1998;19:63–6.

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[35] Talbot K, Ponting CP, Theodosiou AM, Rodrigues NR, Surtees R, Mountford R, et al. Missense mutation clustering in the survival motor neuron gene: a role for a conserved tyrosine and glycine rich region of the protein in RNA metabolism? Hum Mol Genet 1997;6:497–500.

[36] Sun Y, Grimmler M, Schwarzer V, Schoenen F, Fischer U, Wirth B. Molecular and functional analysis of intragenic *SMN1* mutations in patients with spinal muscular atrophy. Hum Mutat 2005;25:64–71.

[37] Setola V, Terao M, Locatelli D, Bassanini S, Garattini E, Battaglia G. Axonal-SMN (a-SMN), a protein isoform of the survival motor neuron gene, is specifically involved in axonogenesis. Proc Natl Acad Sci USA 2007;104:1959–64.

[38] Locatelli D, d'Errico P, Capra S, Finardi A, Colciaghi F, Setola V, et al. Spinal muscular atrophy pathogenic mutations impair the axonogenic properties of axonal-survival of motor neuron. J Neurochem 2012;121:465–74.

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