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## Gene therapy of amyotrophic lateral sclerosis\*\*

### Terapia genowa stwardnienia bocznego zanikowego

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#### Summary

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder affecting nearly half a million people worldwide. Approximately 35% of familial and 10% of sporadic cases are due to mutations in genes encoding for CuZn superoxide dismutase (*SOD1*), transactive response DNA-binding protein TDP-43 (*TARDP*) and fused in sarcoma/translocated in liposarcoma protein (*FUS/TLS*). These subpopulations of patients may be the first to profit from gene therapy. We here describe current directions of development of ALS treatment based on gene silencing.

Key words: amyotrophic lateral sclerosis (ALS), RNA interference, antisense oligonucleotides

#### Streszczenie

Stwardnienie boczne zanikowe (SLA) jest śmiertelną chorobą neurozwyrodnieniową dotyczącą niemal 500 000 osób na świecie. Około 35% przypadków rodzinnych i 10% sporadycznych jest spowodowanych mutacjami genu *SOD1* (kodującego miedziowo-cynkową dysmutazę ponadtlenkową), *FUS/TLS* (białko FUS/TLS) oraz *TARDP* (białko TDP-43). Te grupy chorych mają największą szansę skorzystania z terapii genowej. Artykuł opisuje główne kierunki rozwoju strategii leczniczych opartych na wyciszaniu genów w stwardnieniu bocznym zanikowym.

Słowa kluczowe: stwardnienie boczne zanikowe (SLA), interferencja RNA, digonukleotydy antysensowne

#### INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder affecting over 2500 people in Poland and nearly half a million people worldwide. It is sporadic in approximately 90% of cases (sporadic ALS, SALS). The remaining 10-15% of patients suffers from a familial condition mostly inherited as an autosomal dominant trait (familial ALS, FALS). From 12 to 23.5% of FALS and over 7% of SALS cases are due to mutations in a gene encoding for Cu, Zn-superoxide dismutase (*SOD1*), a free radical scavenging enzyme (1). Several new genes were found to be associated with classic ALS in recent years. They encode for transactive response (TAR) DNA-binding protein TDP-43 (*TARDP*), fused in sarcoma/translocated in liposarcoma protein (*FUS/TLS*), each responsible for 4% of FALS and 1% of SALS, angiogenin (*ANG*), optineurin (*OPTN*) and others (2, 3). The greatest number of mutations associated with ALS was found in the *SOD1* gene (for complete list please refer to <http://www.alsod.org>, <http://alsod1.iop.kcl.ac.uk/reports/mutations>). A vast major-

ity of over 155 causative *SOD1* mutations identified to date induce changes in stability of the enzyme. Some also influence its enzymatic activity. Experiments with the use of transgenic animals harboring human mutated *SOD1* have shown that mutations in the *SOD1* gene lead to a toxic gain of function of the *SOD1* protein. In transgenic mice, the disease phenotype directly correlates with the level of transgene expression (4). On the other hand, the animals deprived of wild type *SOD1* (wt *SOD1*) do not develop ALS-like phenotype. Overall, the experimental data leads to conclusion that genetic silencing of mutant *SOD1* may potentially decrease the mutation-induced toxicity and ameliorate the clinical phenotype (5).

The mutation in *TARDP*, *FUS/TLS*, *ANG* and *OPTN* associated with FALS, are also inherited in an autosomal dominant manner (with only single exceptions). Both mutated TDP-43 and FUS/TLS form intraneuronal and glial inclusions, what may suggest a toxic gain of protein function rather than its loss (6). In fact, increased expression of TDP-43 in the nuclei was proved toxic to

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neurons (7). On the other hand, a depletion of TDP-43 from mouse adult brain with antisense oligonucleotides resulted in numerous abnormal splicing events. It also lead to decrease of mRNA levels of several genes involved in synaptic activity, including *FUS/TLS* and progranulin (8). Silencing of either *TARDBP* or *FUS/TLS* by RNA interference was also shown to reduce the expression of histone deacetylase (HDAC) 6 mRNA in cell culture (9). The impact of such silencing on mammal physiology has not been evaluated. The lack of similar experiments designed for other genes, reduces their consideration as immediate candidates for gene therapy. More information on the physiological and pathological function of the newly discovered genes associated with ALS will arrive along with new *in vivo* studies (2).

For these reasons, at present, the gene therapy mostly orientates on patients who carry ALS-associated mutations in the *SOD1* gene. The autosomal dominant inheritance and toxic gain of protein function makes them prone to profit from therapy based on gene silencing. A specific reduction of expression of a target gene would assure the therapeutic effects. Two molecular mechanisms of potential use is gene silencing have been described to date. They are RNA interference and antisense oligonucleotides.

#### RNA INTERFERENCE (RNAi)

RNAi is a highly conserved cellular mechanism responsible for gene silencing. It is triggered by double stranded RNAs (dsRNAs) capable of degrading the complementary mRNA or blocking its translation. RNAi is a natural cell defense against viruses and excessive transposon mobilization. It also regulates gene expression during development (10, 11). DsRNAs may be synthesized within the nucleus as pre-microRNA (miRNA), or introduced to the cell by viral vectors or plasmids. In either case, dsRNAs are processed to small (21-23 nucleotide)-interfering RNAs (siRNAs) by RNase III-like enzyme, called Dicer (12). siRNAs are effector RNAs, which target cognate cellular mRNAs and recruit them to the cytosolic RNA-induced silencing complex (RISC), a multiprotein complex containing ribonuclease. Attachment to RISC leads to an ATP-dependent mRNA unwinding and binding a single stranded RNA to the target mRNA. The process ends by mRNA rapid degradation catalyzed by cellular exonucleases present in the cytoplasm or, dependent on the degree of complementarity, the inhibition of translation (12, 13). MiRNA are non coding RNAs responsible for gene regulation and not the protein synthesis. They are produced by Dicer from endogenous short hairpin RNAs (shRNA) precursors (12). After binding to protein complex they associate to ribosomes thus inhibiting translation of partially compatible mRNA. If miRNA is thoroughly complementary to the target RNA, it produces target degradation, just like in the case of exogenous siRNA. The possibility to use RNAi in gene therapy arouse from a study that permitted to trigger

gene silencing with the use of exogenous siRNAs (14). It's worth noticing that introducing ds RNAs longer than 23 nt provokes enhanced immune response mediated by interferons and inflammatory cytokines, which leads to global repression of translation and cell death (15). Since RNA interference is quite specific, it may be therapeutically used to repress expression of genes responsible for synthesis of mutated proteins with toxic properties (16). In light of our knowledge considering genetic background of ALS, and more particularly on ALSOD (ALS due to *SOD1* mutation), this disorder seems to be a perfect candidate for treatment using RNAi-based approach.

The biggest concern about the use of RNAi in therapy is to assure its efficient transport to target areas. siRNAs do not cross the blood-brain barrier and are highly unstable in biological fluids. They need to be introduced to the RISC in the cytoplasm by direct CSF/brain parenchyma infusion (anterograde transport). Since they are rapidly degraded after gene silencing (36-48 h), the potential effectiveness of this therapy implicates introduction of numerous siRNA copies. Unexpectedly, even very short siRNAs induce interferon response in a dose-dependent manner (17). The only solution of this issue seems to be a long-term repetitive siRNA administration, which may encounter important technical problems.

Micro and shRNAs can be transported to the nucleus by viral vectors or liposomal carriers following an intrathecal, intraparenchymal (anterograde) or intramuscular (retrograde) administration. It is not certain if their uptake by the target tissue is sufficient to assure clinical effectiveness. Attempts to transfer siRNA bound to liposomes injected intravenously into mice did not assure a sufficient uptake of RNA molecules into target tissues, but short peptides derived from a glycoprotein of rabies virus were able to deliver siRNA transvascularly into the brain (6, 18). Moreover, a recent study has shown that transgene expression mediated by adeno-associated virus (AAV) in the primate brain continues for at least 8 years without causing neuroinflammation or reactive gliosis (19). It still remains elusive if, in a long run, the viral vectors do not elicit neutralizing antibodies (6).

The lack of cell and tissue-specificity of vectors and liposomal carriers prevents their effective use to date. The uptake of effector RNAs into non-target tissues may impose important and long-lasting side-effects (12). Toxic liver injury and death was reported after intravenous injections of several adeno-associated virus-shRNAs vectors in animals. The effect was dose-dependent and probably resulted from the saturation of endogenous RNAi machinery (20). There was also a report of death of a cancer patient after adenovirus administration (21). New viral vectors ensuring higher specificity to CNS and not to peripheral organs (mostly liver) are being tested. There are also attempts to increase the tissue-specificity of other carriers. It can potentially be achieved by using recombinant molecules

combining liposomes, receptor-specific antibodies and/or ligands able to cross the blood-brain barrier or genetically modified stem cells capable of migrating within the brain or spinal cord (6). Even if these methods are found efficient, it is important to develop technical means to silence or remove the RNAi vector once the side effects have been encountered. One of the possibilities proposed recently is a genetic modification of polymerase III promoter, which is used by RNAi machinery, to be susceptible to repression by tetracyclin-repressor system (12).

As previously mentioned, effector RNAs can be delivered to the target gene by anterograde or retrograde transport. To date, the anterograde transport was used in three *in vivo* studies on transgenic model of ALS (22, 23, 24). When delivered intraparenchymally to the spinal cord of adult pre-symptomatic mice, siRNA-lentiviral vectors targeting *SOD1* gene were able to delay onset and progression of motor dysfunction (22). The construct reduced *SOD1* expression in both motor neurons and glial cells. As a result of constant intrathecal infusion of the chemically modified siRNA, the disease progression rate slowed down significantly (23). Chemically modified siRNA was proved to be stable for a prolonged period of time *in vivo*, diffuse to all CNS regions, cross the cell membrane and silence the expression of the mutant *SOD1* gene. Increase of the siRNAs concentration along with the reduction of the therapy period induced signs of toxicity, including decreased activity, poor grooming and weight loss of treated animals (23). Another genetic approach of a potential use not only in ALSOD, but also in ALS without *SOD1* mutation and in other neurodegenerative diseases was silencing of a proapoptotic *fas receptor* gene by RNAi. When introduced to transgenic animals with human *SOD1* mutation, it resulted in delaying the onset of motor deficits and increased survival (24).

The peripheral administration of viral RNAi vectors followed by their retrograde transport to the CNS is a challenging alternative. In an interesting study aiming to prevent ALS development in transgenic mice, a lentiviral vector encoding for RNAi targeting human *SOD1* was multi focally injected of into various muscle groups (25). The treatment of new born mice resulted in over 2-fold delay in the symptom onset and a significant increase of survival (by 80%). In another study, administration of adeno-associated virus 2 encoding siRNA against *SOD1* into one hindlimb of an adult presymptomatic mouse induced regional rescue of the motor neurons in the spinal cord with decreased *SOD1* expression (26). It resulted in preservation of limb strength for 50 days since administration (as compared to untreated muscles), which equals to approximately 90% of the transgenic animal lifespan. From the clinical point of view, it would be interesting to see if the efficacy of retrograde siRNA therapy is similar if started in symptomatic subjects. Establishment of injections protocol including choice of muscles, number of injection sites within a chosen muscle and efficient treatment frequency has not yet been established.

All these issues need to be resolved prior to attempt to introduce the therapy to humans.

#### ANTISENSE OLIGONUCLEOTIDES

Antisense oligonucleotides are synthetic single-stranded oligonucleotides able to bind target mRNA transcripts in a sequence-specific manner inhibiting their expression post-transcriptionally. In physiological conditions they are highly instable and rapidly degraded by exonucleases. Thus, their use in therapy implements primary chemical and tissue specific modifications. Once adjusted to a given tissue/cell type, antisense nucleotides can be administered to the CSF by osmotic pump at a given dose and infusion rate (27). Administration of oligonucleotides to the CSF of healthy animals allowed their widespread distribution and penetration to the brain parenchyma (13, 28). At the cellular level the 12-15 nucleotide-long synthetic sequences bind matching mRNA sequences of a mutated gene leading to its degradation by endogenous nucleic RNase H (29). The RNase H recognizes the DNA-mRNA heteroduplex and cleaves the mRNA molecule preventing its translation. Another mechanism involves steric blockade of ribosomes by the DNA-mRNA heteroduplex (30).

Beside the lack of stability, the biggest hurdles to overcome prior to introduce antisense oligonucleotides into clinics are their proinflammatory effect and the inability to pass the brain-blood barrier. The first can be overcome by dose reduction, while the latter requires invasive administration methods: either to the ventricles or directly to brain parenchyma. A number of advantages of this method assure its important place in gene therapy. First of all, the antisense oligonucleotides are relatively easy to manipulate. They do not activate the RNAi machinery, which prevents RISC saturation. Second, when chemically modified they are much more stable in biological fluids compared to siRNAs (half-life up to weeks). The administered dose (although higher compared to siRNA) may thus be reduced in order to prevent toxicity. Third, the CSF administration of antisense oligonucleotides assures widespread and efficient brain distribution (28). And last but not least, due to administration by routinely used neurosurgical procedure, their dose and infusion rate can be adjusted in case of side-effects (13).

Oligonucleotides targeting *SOD1* mRNA were administered to new born transgenic rats harboring human *SOD1* mutation (28). The therapy resulted in slowing the disease progression and extended the animals survival. Beside *SOD1*, there have been attempts to use antisense constructs targeting death-signaling p75 neurotrophin receptor. When administered intraperitoneally to adult *SOD1* mice, the oligonucleotides significantly delayed motor impairment and mortality of the animals (31). Repetitive intraperitoneal injections of an antisense sequence targeting the gene encoding for GluR3 subunit of the AMPA receptor delayed disease onset and extended survival of transgenic *SOD1* animals when

administered at pre-symptomatic stage (32). There is no data on potential efficacy of the studied constructs in ALS not associated with mutations in the *SOD1* gene.

The antisense technology has already been introduced into humans. It was proved to be safe in patients with cancer, autoimmune diseases and asthma (29). Due to the encouraging results from both human and transgenic studies, a group from Washington designed a clinical trial in patients with FALS due to *SOD1* mutations. The recently started phase I of a randomized, placebo-controlled study aimed to evaluate safety, tolerability, and pharmacokinetics of four dose levels of antisense oligonucleotide (ISIS333611) designed to inhibit *SOD1* mRNA. The antisense nucleotide was delivered to the CNS by intrathecal infusion of a 12 hour-duration. Treatment of the first 6 patients induced no serious adverse events. After the infusion, the drug was found in CSF and plasma consistently with levels predicted from non-human primate preclinical dosing studies (33). The European study is going to start enrolling patients in 2012.

## CONCLUSIONS

The genetic therapy made a great progress over the last two decades. For some incurable diseases the dream has nearly come true. Since rodents harboring mutant *SOD1* are the most common disease model for studying pathogenesis of ALS, it seemed natural that patients suffering from ALSOD would be the first to benefit from newly discovered therapies. The genetic therapy is an optimal solution for disorders with autosomal dominant mode of inheritance, in which the mutation is responsible for a toxic gain of function of encoded protein. Despite extensive research it still encounters several obstacles. Short interference RNAs are very effective in gene silencing, especially that they are able to react at lower concentrations compared to antisense oligonucleotides (12). The major challenge in using RNAi is assuring the efficient route of effector RNA administration to the nervous system and its

long term effect. The new approach should increase the siRNA stability without the risk of overburdening the RNA processing machinery. The viral vectors used to carry shRNA and microRNA should be equipped with elements permitting to halt their function in case of adverse effects. The tempting strategy of transporting effector RNA from muscles to the nervous system needs much more studies including determination of its efficiency in symptomatic individuals, a sufficient number of injected muscles and the administration frequency. At this point the antisense oligonucleotides seem to be an easier and safer treatment strategy. Their direct administration to the CSF or brain parenchyma can be assured by routinely used osmotic pumps. The administration to the CSF leads to widespread brain distribution (28). In ventral horns, the antisense oligonucleotides are taken up by both neuronal and non-neuronal cells. The biggest challenge is to increase the specificity of target cell delivery by modification of the carriers' properties. More efficient methods of increasing the particles stability are in progress (30).

Every month brings along more information on the function of TDP-43 and FUS/TLS, as well as the role of their genes mutations in pathogenesis of ALS. They may help increase the percentage of patients taking advantage of genetic therapy in near future. For all other patients with SALS and FALS without known genetic defects, the combination of either neurotrophic factors with viral vectors/non-viral carriers, or stem cells releasing neurotrophic factors might become an efficient solution (13).

*“Research into the cause of ALS and its treatment should be given a priority by major funding institutions, including drug industry (...). We desperately need new more effective drugs to treat ALS, but until the primary cause of sporadic ALS is unknown it is unlikely we will be able to cure this devastating disease.”*

*Prof. Hubert Kwieciński, MD PhD  
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