Review

Progress in muscular dystrophy research with special emphasis on gene therapy

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Abstract: Duchenne muscular dystrophy (DMD) is an X-linked, progressive muscle-wasting disease caused by mutations in the *DMD* gene. Since the disease was described by physicians in the 19th century, information about the subject has been accumulated. One author (Sugita) was one of the coworkers who first reported that the serum creatine kinase (CK) level is elevated in progressive muscular dystrophy patients. Even 50 years after that first report, an elevated serum CK level is still the most useful marker in the diagnosis of DMD, a sensitive index of the state of skeletal muscle, and useful to evaluate therapeutic effects. In the latter half of this article, we describe recent progress in the therapy of DMD, with an emphasis on gene therapies, particularly exon skipping.

Keywords: Duchenne muscular dystrophy, dystrophin, exon skipping, out-of-frame mutation, clinical trial, antisense oligonucleotides

Introduction

Muscular dystrophy is not a single disease but a group of genetically heterogeneous muscle diseases marked by progressive wasting and weakness of the skeletal muscles, and sometimes involvement of cardiac and smooth muscle or other tissues. In 1851, Meryon reported boys with symptoms consistent with the diagnosis of muscular dystrophy,[1] and in 1868 in France, Duchenne published a detailed and systematic clinical, muscle pathological, and electrophysiological study of an "atrophie musculaire progressive", which is now generally recognized as Duchenne muscular dystrophy (DMD).[2] Its preva-

lence in the population is estimated to be 1.8–4.8 per 100,000. Although the gene responsible was identified in 1986[3),4)] and the underlying pathogenesis is understood to some extent,[5] there is no effective therapy at present other than corticosteroids. In this article, we review historical aspects of the research on DMD and discuss the therapies of the near future.

Biochemistry and diagnosis of DMD

Biochemical abnormalities in patients with muscular dystrophy were first reported by Sibley and Lehninger in 1949.[6] They determined serum aldolase activity in patients with muscular dystrophy, and reported increased serum aldolase activity in the patients. Sugita measured the serum aldolase levels of patients and confirmed that patients with muscular dystrophy had elevated serum aldolase activity; this finding was reported in a Japanese journal in 1958.[7] At the end of the same year, a middle-aged male was hospitalized at the University of Tokyo Hospital directed by Prof. Dr. Shigeo Okinaka. The patient had moderately atrophic extremities without positive tendon reflexes. Curiously, he did not show any sensory disturbance. Interestingly enough, he had a markedly elevated serum aldolase level. After much deliberate consideration, Prof. Okinaka diagnosed him with a motor neuronitis, however, at grand rounds, the attending

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Abbreviations: DMD: Duchenne muscular dystrophy; CK: creatine kinase; AO: antisense oligonucleotide; BMD: Becker muscular dystrophy; LNA: locked nucleic acid; PNA: peptide nucleic acid; ENA: ethylene-bridged nucleic acid; 2'-*O*-MePS AO: 2'-*O*-methyl phosphorothioate AO; PMO: phosphorodiamidate morpholino oligomer; PPMO: a cell-penetrating peptide-linked PMO; ESE: exonic splicing enhancer; nNOS: neuronal nitric oxide synthase; GRMD: golden retriever muscular dystrophy; CXMD_J: canine X-linked muscular dystrophy in Japan; AAV: adeno-associated virus.

physician told Prof. Okinaka that the patient was suffering from muscular dystrophy, based on the serum aldolase activities that Sugita measured. Prof. Okinaka became furious and told Sugita that it was not acceptable to make a diagnosis simply on the determination of something in the blood. In retrospect, Prof. Okinaka was correct; the patient was probably suffering from polymyositis rather than muscular dystrophy.

However, the incident led to one of the monumental achievements in the history of muscular dystrophy research due to the outstanding professional intuition of Dr. Setsuro Ebashi. When Dr. Momoi, a close friend of Dr. Ebashi since middle school, told him about the serum aldolase activity in muscular dystrophy, he pointedly asked Dr. Momoi, "Why do you determine a nonspecific enzyme such as aldolase in muscular diseases? You should look at the level creatine phosphokinase, which is more specific to skeletal muscle than aldolase." It was a stroke of genius! At his suggestion, we set up a team, headed by Dr. Ebashi and including Drs. Momoi, Toyokura, and Sugita, to study creatine phosphokinase (CPK; it was also known as creatine kinase or CK). Thus, first paper on serum CK activity in progressive muscular dystrophy was published by Ebashi and coworkers in 1959.[8] Among various neuromuscular disorders, they found increased serum CK levels in patients with muscular dystrophy, and it is now regarded as the most reliable laboratory test for muscular dystrophy. The discovery of the importance of serum CK opened the door for the recent myology research, in particular pathological studies including genetics and the exploration of treatments such as gene therapy.

Discovery of dystrophin and its localization at the muscle surface membrane

A pioneer application of positional cloning to human diseases appeared in 1986, when the gene responsible for DMD/Becker muscular dystrophy (BMD) was isolated by Dr. A.P. Monaco et al. of Dr. L. Kunkel's group.[3] The *DMD* gene is 2,500 kb long and consists of 79 exons covering 1% of the x-chromosome. It is transcribed to yield a 14 kb cDNA. In 1987, Dr. E.P. Hoffman et al. identified a 427 kD protein encoded by the *DMD* gene, and this protein was named "dystrophin",[4] which is absent from the skeletal muscle of most patients with DMD. Almost all cases of DMD showed an out-of-frame mutation. In contrast, most patients with BMD had an in-frame mutation. Using polyclonal antibodies against the

near N-terminal portion derived from the dystrophin cDNA, Dr. Kiichi Arahata et al. of the muscular dystrophy research group at National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan, identified a specific immunohistochemical reaction with peptides on the surface membrane of skeletal and cardiac muscle fibers that was absent in the muscles of DMD patients.[9] These results have been confirmed by other research groups. In symptomatic carriers of DMD, a distinct mosaic pattern of immunohistochemical staining of the surface membrane of the muscle fibers can be observed. BMD exhibits a positive but faint and patchy expression pattern of dystrophin with altered protein contents and molecular weights. Thus, it became clear that DMD and BMD are caused by fragility of the muscle surface membrane due to the lack of dystrophin.

Development of therapy for DMD

Drug treatment for DMD patients is currently restricted almost completely to corticosteroids (oxandrolone and prednisone), but a variety of therapeutic approaches to muscular dystrophies have been tested over the past few decades, and some of them show great promise (recently reviewed in Ref. 10, and current situation was summarized in Table 1). For successful application of viral vector-mediated gene therapy, there are still several hurdles to be overcome.[11] Pluri- or multipotent stem cell-based therapies are still in their immature stages, but currently some alternatives are progressing to clinical trials. Among several therapeutic approaches in preclinical or clinical stage, authors here focus on one of the most promising therapeutic approaches: exon skipping with antisense oligonucleotides (AOs).

Exon-skipping therapy using AOs

DMD is caused by the lack of dystrophin, most commonly as a result of frame-shift mutations. Deletions and duplications in the *DMD* gene result in out-of-frame mRNA, such as nonsense mutations in which a single base change alters a codon into a premature stop codon. Theoretically, in these cases, selective removal of the flanking exons can result in an in-frame mRNA transcript. Such an in-frame mRNA transcript can be translated into a quasi-dystrophin protein (reviewed in Ref. 12). AOs, which hybridize the sequences near the splice acceptor or donor sites as well as within exons, can alter gene expression *via* steric block interference with the splicing machinery, and thereby direct the exclusion

Table 1.   Clinical trials for DMD/BMD

Category	Interventions	Phase (ClinicalTrials.gov)
Drug	**Myostatin blocking**	
	MYO-029	Completed; not effective
	Read-through	
	PTC124	Completed; not effective
	Gentamicin	Completed; not effective
	Others	
	Pentoxifylline	Completed; not effective
	Idebenone	Phase III
	Ramipril vs. Carvedilol	Phase IV
	Coenzyme Q10 and prednisone	Phase III
	Coenzyme Q10 and lisinopril	Phase II/III
	Debio-025 (cyclosporine analogue)	Phase IIb
Gene therapy	**Exon skipping** (systemic delivery)	
	PRO051 (2′-O-MePS AO)(exon 51 skipping)	Phase III
	PRO044 (2′-O-MePS AO)(exon 44 skipping)	Phase I/II
	AVI-4658 (PMO)(exon 51 skipping)	Phase IIb
	AAV vector	
	rAAV2.5-CMV-Mini-Dystrophin	Phase I*
Cell therapy	**Satellite cells (myoblasts)**	Pending
	Mesoangioblasts	In preparation
	Induced pluripotent stem (iPS) cells	Experimental

Shown are representative ongoing or just finished clinical trials for DMD. Some have finished with disappointing results. For more information, please refer to the homepage presented by the U.S. National Institute of Health, 'ClinicalTrials.gov'.

*Unwanted immune responses to dystrophin have been reported at the 2010 meeting of the American Academy of Neurology (http://quest.mda.org/news/caution-immune-response-seen-dmd-gene-therapy).

of one or more exons in the final transcript, resulting in restoration of the reading frame of dystrophin mRNA and the expression of a shorter, truncated but functional dystrophin.

One of the pioneering researchers who tried to restore the reading frame of the mutated DMD transcripts was Dr. Masafumi Matsuo at Kobe University.[13] His group tried to skip exon 19 of the *DMD* gene in exon 20-deleted DMD patients, based on the idea of DMD Kobe, where exon 19 has been skipped due to a 52-bp deletion within the exon. Later, proof-of-concept studies by many groups followed *in vitro* and *in vivo* (reviewed in Ref. 14).

Chemistries of AOs

For maximal effects in exon-skipping therapy, the chemistry of AOs seems to be one of most critical factors. AOs used for exon skipping are usually 20–25 bases long and chemically synthesized. Various chemistries for AOs have been proposed to overcome the unstable nature of single-strand DNA or RNA molecules. Several modifications of AOs include bicyclic locked nucleic acid (LNA), peptide nucleic acid (PNA), ethylene-bridged nucleic acid (ENA), 2′-O-methyl phosphorothioate AO (2′-O-MePS AO), and phosphorodiamidate morpholino oligomer (PMO). Among them, 2′-O-MePS AO and PMO are the most frequently utilized because of their suitable properties (Fig. 1).

2′-O-MePS AO.   The structure of 2′-O-MePS AO is similar to that of RNA, but it is methylated at the 2′-OH position of the ribose ring. 2′-O-MePS AO is widely used because it is relatively easy to synthesize and cheap to produce. 2′-O-MePS AO is stable, has a high affinity to mRNA, and is also resistant to nucleases, however the low solubility of 2′-O-MePS AO in water prevents its use at higher dosages.

PMO.   PMO has a morpholine ring instead of a deoxyribose ring in DNA, and these artificial rings
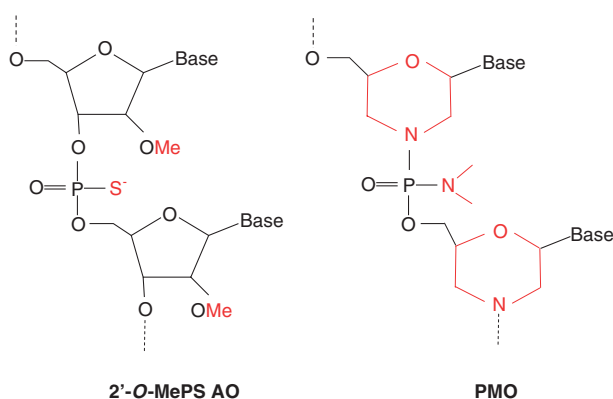
Fig. 1. Comparison of chemistry used in clinical trials. $2'$-O-MePS AO: $2'$-O-methyl phosphorothioate antisense oligonucleotides. PMO: phosphorodiamidate morpholino oligomers. PMO (AVI BioPharma, http://www.avibio.com/) has a different backbone (morpholino backbone) from RNA or DNA, enabling highly sequence-specific, stronger base pairing to the target RNA than RNA or DNA. Red highlights the differences in the chemistry from RNA or DNA.

are linked to each other through phosphorodiamidate, enabling highly sequence-specific, stronger base pairing to the target RNA than RNA or DNA. Importantly, PMO does not stimulate or activate Toll-like receptors and therefore does not activate innate immune responses. In addition, PMO is believed to be not recognized by cellular proteins and to be resistant to nuclease-mediated degradation. PMO has high solubility in water.

Other chemicals to improve efficiencies of delivery to whole-body musculature. Delivery of AOs to normal tissues *in vivo* is generally difficult because healthy tissues do not take up PMO or $2'$-O-MePS AO. Although the mechanisms are not fully understood, PMO can easily enter the nuclei of skeletal muscle of DMD patients. This might be because the dystrophin-deficient muscle membrane is inherently leaky due to absence of the dystrophin-glycoprotein complex.

However, systemic administration of $2'$-O-MePS AO or PMO failed to restore dystrophin expression in the heart, although it is again unclear why cardiac cells do not take up $2'$-O-MePS AO or PMO. To improve the efficacy of its introduction into cardiomyocytes, a PMO covalently conjugated with a designed cell-penetrating peptide (PPMO) was injected into dystrophin-deficient *mdx* mice. Systemic delivery of the novel PPMO restored dystrophin to almost normal levels in both cardiac and skeletal muscles in *mdx* mice.[15] Later, the same group reported that a PMO modified with an octaguanidi-

nium dendrimer, Vivo-Morpholino, also restored dystrophin expression in cardiac and skeletal muscles.[16] So far, no study has clearly demonstrated toxicity after systemic delivery or immune response to PPMO or Vivo-Morpholino.

AOs designs

To obtain efficient exon skipping while lowering the dose of AOs for clinical trials, the design of the AOs (base sequence) is important. In eukaryotic organisms, the gene is transcribed in the nucleus and introns are spliced out into mRNA, and then the mRNA is exported from the nucleus to the cytoplasm. Therefore, AOs must either enter the nucleus, where they bind to their target pre-mRNA sequences and get in the way of molecules that are otherwise involved in the splicing process, or they must alter the secondary structure folding of the pre-mRNA. AOs targeting exon-intron boundary sequences can often effectively induce exon skipping. On the other hand, when web-based software, such as ESEfinder (http://rulai.cshl.edu/tools/ESE), is used to design AO sequences to target an ESE, exon skipping is not always induced. Recently, Wee *et al.* have developed bioinformatic tools to optimize AOs sequences based on the pre-mRNA secondary structure.[17] Nevertheless, no single design tool is sufficient for designing the AOs, and often empirical analysis is required.

Proof of principle of exon skipping therapy in animal models

Cultured skeletal muscle cells from DMD patients are often used to evaluate the exon-skipping efficiency of newly-designed AOs.[18] However, to assess the therapeutic benefits, preclinical studies must be performed using animal models. In this section, studies using *mdx* mice and dystrophic dogs are described and discussed.

Exon skipping in *mdx* mice. The *mdx* mouse is a naturally occurring animal model that has a nonsense mutation in exon 23 of the *DMD* gene, resulting in a premature stop codon and complete absence of dystrophin.[19] *Mdx* mice show high levels of serum CK, active muscle degeneration/regeneration cycles, and loss of myofibers and fibrosis in the diaphragm, but still show mild, non-progressive muscle weakness of the limbs and only 20% reduction in life span. In this point, *mdx* might not be an ideal animal model for DMD, but due to the low cost of maintenance and short gestation times, many preclinical studies have been carried out using *mdx* mice.

Lu *et al.* reported the local administration of the AOs with the non-ionic polymer F127, which promotes intracellular uptake of 2′-*O*-MePS AO, to the skeletal muscles of 2-week-old *mdx* mice. The result showed that dystrophin together with β-dystroglycan, sarcoglycans, and nNOS was restored in 20% of the muscle fibers.[20] Furthermore, systemic administration of anti-sequences of the same 2′-*O*-MePS AO with F127 revealed that dystrophin was expressed in the skeletal muscle of the whole body. 2′-*O*-MePS AO produced no toxicity, but its expression did not reach a therapeutic level.[21] Likewise, Wells *et al.* reported that local administration of 2′-*O*-MeAO using electroporation restored dystrophin expression to up to 20% of the normal level.[22] Systemic induction of dystrophin expression by PMO administration reached up to 20% in whole body skeletal muscle.[23]

Dystrophic dogs as models for DMD. Muscular dystrophy in dogs was originally identified in golden retrievers and designated "golden retriever muscular dystrophy" (GRMD). GRMD shows progressive skeletal muscle weakness and atrophy as well as abnormal electrocardiographic findings and myocardial fibrosis like those seen in DMD. Because the phenotype and genetic background are closer to human DMD than those of the mouse model, the dystrophic dog is a useful model to examine pathogenesis and therapeutic strategies. However, the dogs are too large to be maintained conveniently; thus, we have established a colony of medium-sized beagle-based dystrophic dogs (canine X-linked muscular dystrophy in Japan: CXMD_J) at the National Center of Neurology and Psychiatry, Tokyo, by using artificial insemination of frozen GRMD semen.[24] Still, we have found that CXMD_J requires extra daily care, and therefore, is expensive.

Clinical features of CXMD_J. The level of serum CK in neonatal CXMD_J is very high, and 25–33% of the pups die of respiratory failure during the neonatal period, mainly due to severe degeneration and thus dysfunction of the diaphragm. Around the age of 2–3 months, atrophy and weakness of limb muscles appear; then, gait disturbance, joint contracture, macroglossia, and dysphasia appear in rapid progression until the dogs are 10 months of age; subsequently, the progression is retarded.[24]

Cardiac involvement of dystrophic dogs. Dystrophic dogs and DMD have similar cardiac involvement, including distinct deep Q-waves on the electrocardiogram and fibrosis of the left ventricular wall. The distinct deep Q-waves are generally ascribed to fibrosis in the posterobasal region of the left ventricular wall in DMD, but one report suggests that the deep Q-waves precede the development of fibrosis in CXMD_J.[25] Importantly, Purkinje fibers in dystrophic dogs showed remarkable vacuolar degeneration despite the absence of detectable fibrotic lesions in the ventricular myocardium. In the degenerated Purkinje fibers, Dp71, a C-terminal truncated isoform of dystrophin, was up-regulated at the sarcolemma. In addition, the calcium-dependent protease μ-calpain was found at the cell periphery near the sarcolemma or in the vacuoles. Utrophin, a homologue of dystrophin, was also highly up-regulated in the Purkinje fibers in the early stage. Together, the selective degeneration of Purkinje fibers may be associated with distinct deep Q-waves on electrocardiograms and the fatal arrhythmia seen in dystrophinopathy.[25]

Multi-exon skipping in dystrophic dogs. The dystrophic dogs have a point mutation at the intron 6 splice acceptor site in the canine *DMD* gene, resulting in skipping of exon 7 and a premature stop codon in exon 8. Thus, dystrophin is not produced in the affected dog. Recently, Yokota *et al.* reported systemic administration of three PMOs targeting exons 6 and 8 to convert an out-of-frame mutation to an in-frame mutation in CXMD_J.[26] The result showed that dystrophin was restored in the entire body skeletal muscle. The authors reported that the motor ability of treated dystrophic dogs was improved and that they showed no side effects. To the best of our knowledge, this is the first report that multi exon-skipping is feasible and effective in improving performance of dystrophic animals *in vivo*.

As reported in *mdx* mice, a combination of PMOs failed to restore the expression of dystrophin in cardiac muscle, even at a high dose in CXMD_J. To resolve this problem, modified PMOs (PPMO or Vivo-Morpholino) were tested (Yokota *et al.*, unpublished observation).

Viral vector-mediated exon skipping

The duration of the effects of AOs *in vivo* is limited; therefore, patients have to be injected with AOs weekly or monthly to maintain the therapeutic levels of dystrophin. An alternative strategy is to deliver AOs *via* adeno-associated virus (AAV) vector-mediated gene transfer. Based on an AAV vector-mediated approach, Goyenvalle *et al.* linked U7 small nuclear RNA to antisense sequences to achieve sustained dystrophin expression derived from skipped mRNA for more than 13 weeks in the limbs
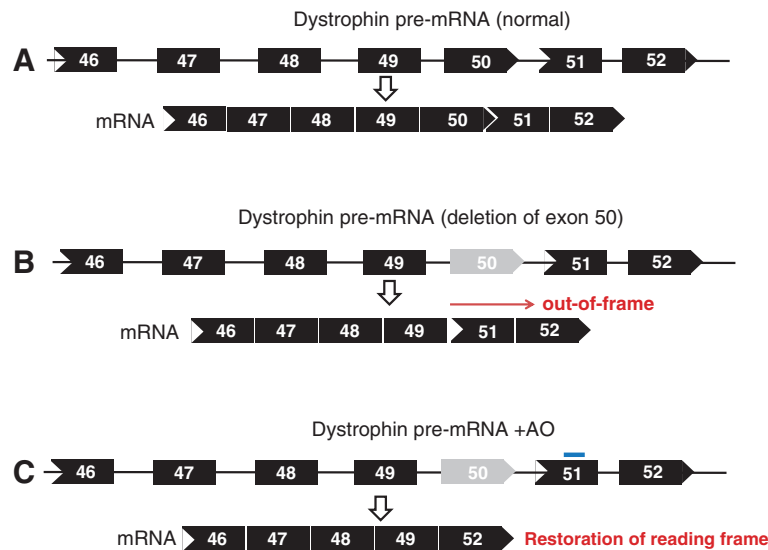
Fig. 2. Example of exon skipping therapy for DMD patients with deletion of exon 50. A. Normal dystrophin transcript and mRNA. B. Deletion of exon 50 disrupts the open reading frame, leading to a premature stop codon, unstable mRNA, and a truncated unstable protein. C. Targeted skipping of exon 51 using antisense oligonucleotides, such as AVI-4658 or PRO051 (blue line), restores the reading frame and produces a truncated but functional dystrophin that lacks exons 50 and 51.

of the *mdx* mouse.[27] Lentiviral vectors were also used to modify muscle stem cells expressing modified small nuclear RNAs that change splicing patterns of pre-mRNAs and correct the reading frame.[28],[29]

Test of AOs efficacy using patient cells

To apply exon skipping therapy to individuals, not only the deletion pattern in the genome but also the mRNA/cDNA level must be analyzed to determine the precise splicing pattern and to design therapeutic AOs. Subsequently, designed AOs must be tested *in vitro* on cells derived from the patient before clinical trials. Skin fibroblasts are easy to obtain, but the levels of DMD transcripts are low, and therefore the results tend to be variable. On the other hand, preparation of myoblasts through biopsy from dystrophic patients is invasive. Therefore, transformation of fibroblasts to myoblasts *in vitro* by forced MyoD expression is often used to test the efficacy of AOs.[30],[31]

Multiple exon skipping: theory and reality

We and other research groups tried to find the most effective target of exon skipping strategy among DMD patients. Theoretically, exon 51-skipping therapy is effective for up to 15% of DMD patients having a deletion, but large numbers of DMD patients will not benefit from it. We recently reported that three unrelated patients with a deletion of exons

45–55 showed very mild skeletal muscle involvement and were ambulant until his seventies in a patient.[32] Béroud *et al.* also described 15 asymptomatic or very mild patients with an exon 45–55 deletion.[33] In fact, the Leiden Muscular Dystrophy database indicates that the exon 45–55 deletion produced a BMD phenotype in more than 95% of the cases. Collectively, these observations indicate that approximately 60% of DMD patients having a deletion within the hot spot may be treatable with multi-exon skipping of exons 45–55. We recently tried exon 45–55 skipping by injection of a mixture of 10 PMOs in the anterior tibial muscle of *mdx*52 mice[34] and confirmed that multi-exon skipping is feasible, at least in mice (Aoki *et al.*, unpublished observation). However, the efficacy of multi-exon skipping was lower than that of single-exon skipping, possibly because many partially spliced products, many of which are again out-of-frame, are produced.

Phase I/II clinical trials of exon skipping for DMD

Based on the success of AOs-mediated exon skipping in animal models, clinical trials have been performed or are in progress for the skipping of exon 51 of the *DMD* gene (Fig. 2 and Table 1).

2′-*O*-MePS AO. Skipping of the exon 51 of the *DMD* gene with 2′-*O*-MePS AO, PRO051, has been already tested on DMD patients by a Dutch group.[35]

Four DMD patients received a dose of 0.8 mg of PRO051 injected into the tibialis anterior muscle. A biopsy performed 28 days later revealed restoration of sarcolemmal dystrophin in 64% to 97% of myofibers of each patient. Further, PRO051 did not evoke any clinically apparent adverse events.

PMO. Recently, a UK group reported that local injection of morpholino oligomer AVI-4658 successfully restored the expression of dystrophin in treated muscles of all seven DMD patients.[36] No adverse events related to AVI-4658 were observed. Based on these observations, systemic injection of AVI-4658 (Phase I and IIa) is now ongoing in the UK. At present, a Good Manufacturing Practice grade of PMO is produced exclusively by AVI Biopharma Inc. (http://www.avibio.com/).

Conclusions

Since the identification of dystrophin in 1987, various therapeutic approaches to DMD treatment have been evaluated, and now exon skipping, which is one of the most promising strategies, is in clinical trials. Because individual DMD patients have different mutations, exon skipping therapy requires a precise evaluation of mutations in the genome and the cDNA, and splicing patterns must be confirmed in each patient's muscle. In this point, exon skipping is a quite new, personalized therapeutic strategy. As clinicians and researchers involved in the study of muscular dystrophies, one of us for more than 50 years, we are pleased with recent progress in the field and hope that DMD patients benefit from this new therapy in the near future.

Acknowledgement

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Profile

Hideo Sugita was born in 1930. He was graduated from Faculty of Medicine, University of Tokyo in 1954. In 1959, Prof. S. Ebashi and coworkers, including Sugita discovered the increase in serum creatine kinase (CK) in muscular dystrophy. Even now, an elevated level of serum CK is the most useful marker in the diagnosis of muscular dystrophy. Since then, he had been concentrated his efforts to elucidate the pathogenesis of muscular dystrophy. In 1988, his research group at the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP) clarified that dystrophin, the gene product of Duchenne muscular dystrophy (DMD) was located along the surface membrane of the skeletal and cardiac muscles and absent in DMD muscles. He was promoted to Director General of the Institute in 1989 and devoted himself to the progress of neuroscience research, health and welfare of the patients suffering from mental, neurological and developmental disorders. He was awarded Uehara Prize in 1986 and The Takeda Prize for Medical Science in 1996. He was installed as the President of NCNP in 1994 and retired in 1998. Since 2009, he is the President of Japan Foundation for Neuroscience and Mental Health.