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REVIEW ARTICLE

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CHALLENGES TO GENE THERAPY FOR DUCHENNE MUSCLE DYSTROPHY

Key words: Duchenne Muscular Dystrophy, DMD gene, dystrophin complex, gene therapy.

ABSTRACT

Muscular myopathies are a heterogeneous group of disorders caused by a number of factors. Many muscular myopathies are of genetic origin, especially muscular dystrophies (MDs) caused by mutations in any of the large number of genes (Emery 2002). Duchenne and Becker muscular dystrophies are allelic forms with incidence of 1 in 3500 live male births. The disease is associated with a mutation in the Duchenne Muscular Dystrophy (DMD) gene, mapped to Xp21. The high rate of mutation involves deletions (60%) – comprising up to several exons, duplications (6%) and point mutations (34%). Molecular-genetic diagnostics includes analysis of deletions of promoter and 19 exons, located in two "hot-spot" regions, by multiplex PCR. Development in gene therapy has made it possible to administer treatment for diseases such as dystrophies, and now such therapies have entered the clinical phase of experiment. Reducing the size of enormous dystrophin gene from 2.4 Mb to 3.5 kb without any loss of functionality has also become possible. Such a shortened gene remains active and enables transduction of muscle tissue with minimal immunological response and toxic side-effects. There are a few main directions in which the DMD gene therapy is heading; however, the use of "naked" DNA seems particularly promising.

INTRODUCTION

There are nearly forty types of neurodegenerative diseases, classified into two categories (neuropathies and myopathies) depending on the affected tissue. Among myopathies the following can be distinguished: muscular dystrophy, inflammation-based myopathies, metabolic myopathies and myopathies caused by disorders of the endocrine glands and others. Among neuropathies there are diseases of peripheral nerves, motive nerves and diseases of nerve-muscle joints.

Muscular dystrophies are claimed to be the most common and best known group of neuromuscular disorders in the world. The name "muscular dystrophy", though widely used, is not correct, if it refers only to one of about forty neuromuscular diseases.

Muscular dystrophies are classified into nine kinds: myotonic (MMD) [1], Duchenne muscular dystrophy (DMD) [2], Becker muscular dystrophy (BMD) [3], Limb-Gridle (LGMD) [4], Facioscapulohumeral (FSH, FSHD) [5], Congenital (CMD) [6], Oculopharyngeal (OPMD) [7], Distal

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(DMD) and Emery-Dreifuss (EDMD) [8, 9]. Additionally, there are several subtypes within some of the above mentioned kinds. Each type and subtype can have a different genetic background. Genetic defects can result in disorders, and lack of some proteins necessary for the proper functioning of muscles. Symptoms of a neuromuscular disease depend on the type of gene defect.

So far, no effective therapy has been found to stop or remove the development of Duchenne muscular dystrophy, though it is the oldest and the best-known kind of this disorder. It was Dr Guillaume Duchenne (1806-1875) who first described, classified and created a medical test for diagnosis of muscular dystrophy.

Nowadays there are many procedures that enable us to ease some symptoms of the disease or to slow down its development. Neuromuscular disorders attack at every stage of life: in infancy, pubescence, and adulthood as well as in senility.

Neuromuscular disorders occur with incidence of 1 per 1000 births, while incidence of muscular dystrophy amounts to 1 per 2000 births. Also, it is observed that the incidence of Duchenne muscular dystrophy (both most common and severe) may reach 1 per 3500 male births.

Despite the fact that neuromuscular disorders as a whole and muscular dystrophies in particular show higher incidence than other, more diagnosable, genetic diseases, patients suffering from neuromuscular disorders and muscular dystrophies were discriminated against in the past, especially in developing countries.

The available information concerning neuromuscular disorders is limited, insufficient and poorly propagated. That is the reason why there is so little knowledge about differences in various kinds of neuromuscular disorders and muscular dystrophies, their occurrence, development, average life span, hereditary patterns, and methods and means of medical care.

Many of those affected by the disease notice a dramatic decrease in the quality of their lives, and they often suffer from complications that may lead to death because of little knowledge and lack of information. Both society and the medical community should be blamed for the existing situation.

On the other hand, a great number of people affected by these disorders living in developing countries do not have access to advanced diagnostic methods which would let them recognize or exclude types of diseases they suffer from. Thanks to such knowledge such patients could obtain information about predictions and the risk of transferring the disease to their offspring. An additional advantage is recognition of the conditions of potential risk of transferring these diseases to the offspring.

Many patients affected by the aforementioned disorders notice that the quality of their lives decrease dramatically, and suffer from complications that may eventually be fatal due to unawareness and lack of information.

DMD GENE

Duchenne Muscular Dystrophy and the allelic Becker Muscular Dystrophy result from defects in the dystrophin gene [10]. The dystrophin gene was mapped to the short arm of X chromosome, in the p21 region. It is the largest known gene in the human genome, highly complex, with about 2.5 million base pairs (bp), which is 1% of X chromosome and about 0.1% of the total human genome [11, 12]. Although the total size of the DMD gene is large, the coding (translated) region is relatively small with the mRNA product of 14 kb long. DMD comprises 85 exons including seven unique first exons linked to the independent tissue-specific promoters, generating at least three full-length and four shorter dystrophin isoforms [13]. The DMD gene consists of 99.4% of non-coding sequences.

Not only is the DMD gene the largest human gene, but also the most complex. So far 7 promoter-sites of transcription initiation have been found. Activity of different promoters in cells leads to a synthesis of seven different dystrophin forms – protein products of the DMD gene. There are three types of 14 kb mRNAs transcribed from these promoters. Each of them consists of a specific first exon and common units of 78 exons processing a 427 kDa protein: Dp427m, Dp427c, and Dp427p, respectively. Dp427m is expressed (predominantly) in skeletal muscle, cardiac muscle, and smooth muscle. At very low concentrations it can be found in the brain. Dp427c and Dp427p are expressed in cortical neurons and in the cerebellar Purkinje cells.

The shorter dystrophin protein is an effect of four internal promoters that regulate it independently. The unique first exon is used by each of these promoters, and in consequence this procedure creates different protein isoforms, i.e. 71 kDa (Dp71), 116 kDa (Dp116), 140 kDa (Dp140), and 260 kDa (Dp260). However, these are not the only dystrophin isoforms. The DMD gene generates an even greater number of them by using an alternative poly(A)-addition site, localized in intron 70 of the dystrophin gene, and also by alternative splicing at the 3'-end of the dystrophin gene [14].

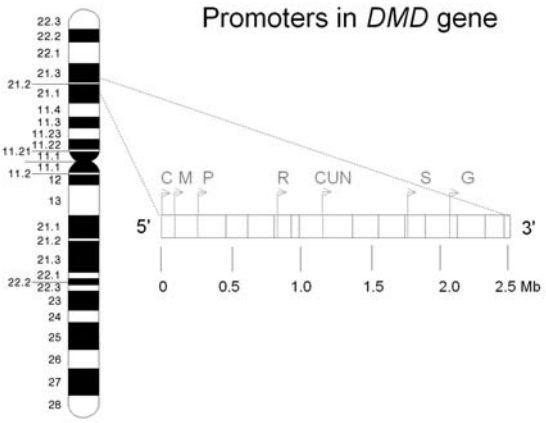

Figure 1. Promoters in DMD gene

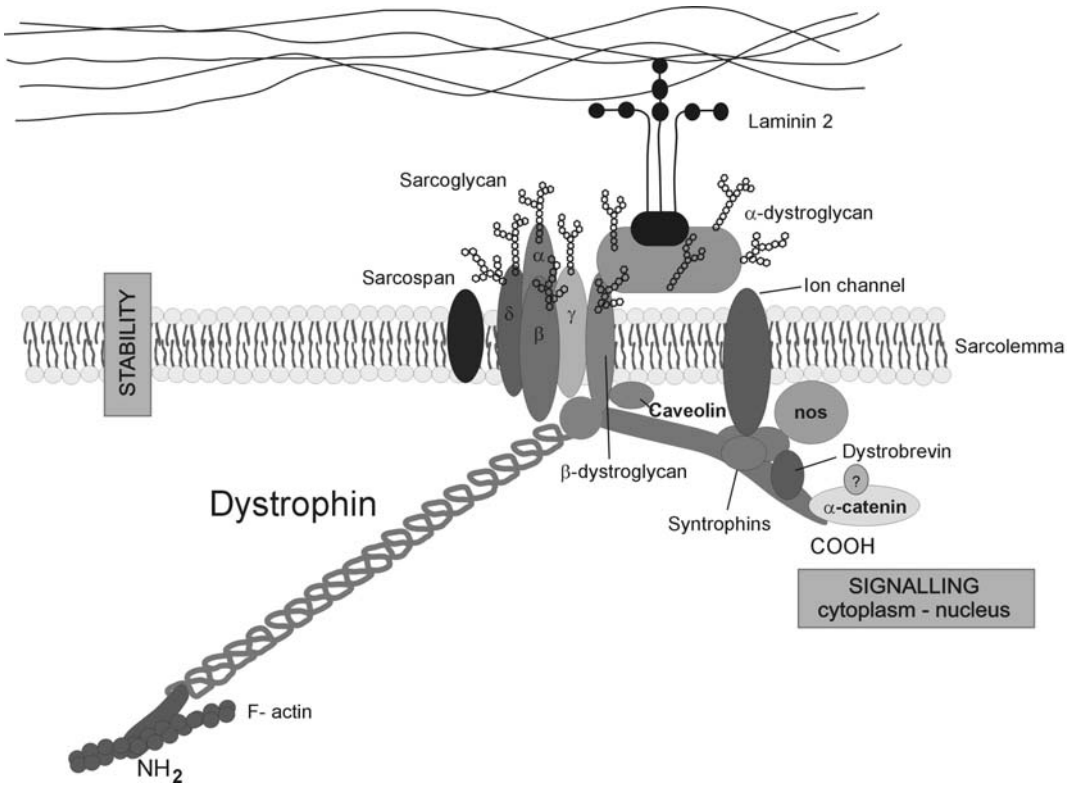

Figure 2. Dystrophin is a structural protein situated on the cytoplasmatic side of the membrane

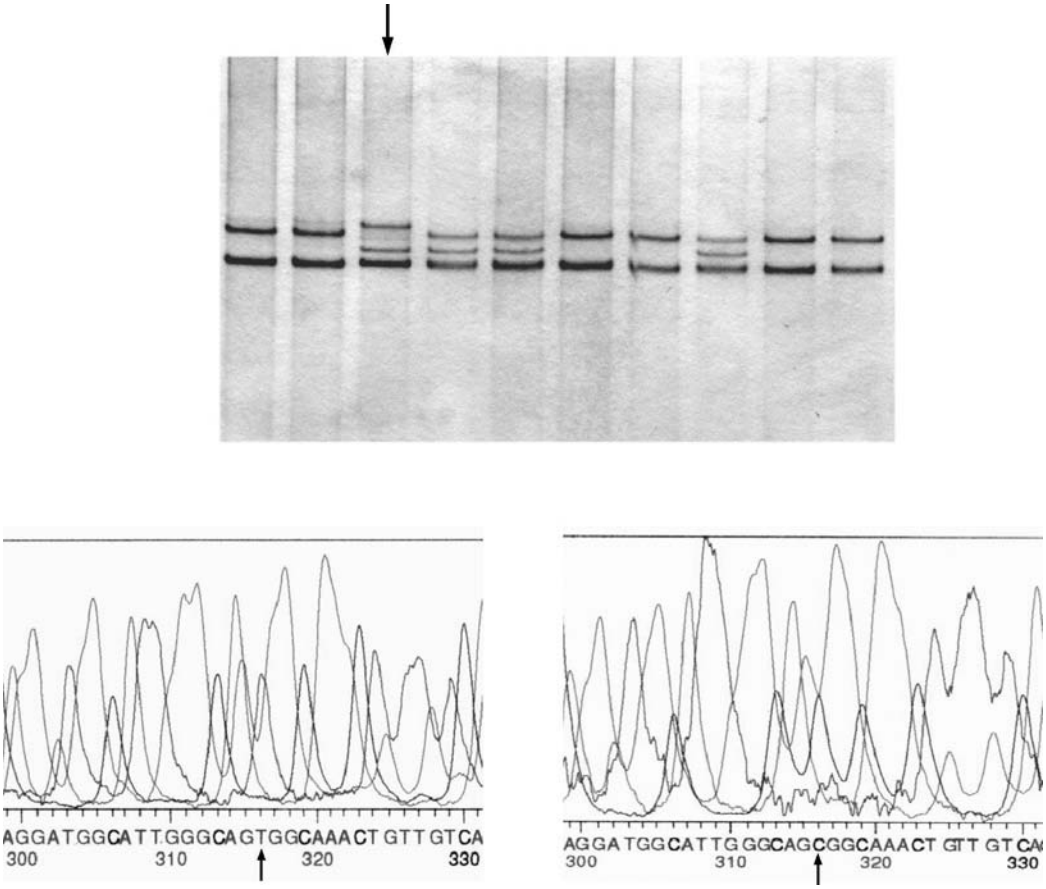

Figure 3. Substitution C>T in exon 45 in DMD gene

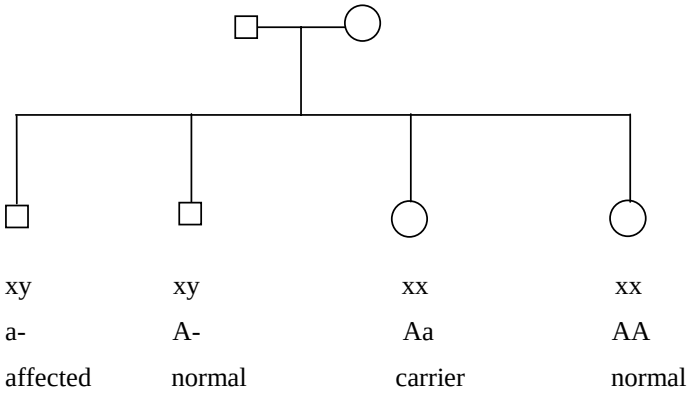

Figure 4. Linkage of DMD gene with X chromosome. 50% of male offspring of female carriers is affected by DMD, and 50% of female offspring are carriers

THE DYSTROPHIN COMPLEX

The full-length dystrophins (coded by the Dp427 transcripts) consist of four main domains: the cysteine-rich, rod, N-terminal, and C-terminal domains [15, 12]. The N-terminal domain binds cytoplasmic actin filaments and the cysteine-rich domain to the sarcolemmal dystrophin-associated protein (DAP) complex through which dystrophin is linked to extracellular matrix components. Absence of either dystrophin or any protein component constituting the DAP complex results in instability of muscle fibers, leading to various types of muscular dystrophy [16, 17].

The smaller dystrophin isoforms lack the NH2 (actin-binding) terminal domain but retain the cysteine-rich and COOH-terminal domains, preserving the binding sites for the DAP complex. Therefore, smaller dystrophin isoforms may have additional functions that are yet unknown.

Dystrophin is a structural protein situated on the cytoplasmatic side of the membrane (Fig. 2). Its C-terminus is bonded to a large complex of membrane glicoproteins, while its N-terminus is attached to cytoskeleton compounds [18].

Basically, women do not suffer from DMD because even if a mutation occurs in one of two homologous alleles on the X chromosome, there is still a correct one to mask the effect of allele with mutation. But they are mutation carries and can transfer the mutated gene to their offspring (next generation), which means severe disease in male children.

The high rate of mutation involves deletions (60%) comprising up to several exons. The remaining are duplications (6%) and point mutations (34%). The latter can change the mRNA reading frame. The principles of frame shift changes are given in Table 1.

The reading frame plays the key role in the process of information flow from DNA to protein via RNA. As a result of mutation disrupting the reading frame in the DMD gene a wrong variant of protein is produced, or dystrophin is not produced at all. According to Monaco et al. [19], mutations that do not change the reading frame cause a mild version of DMD, while mutations causing a frame shift are responsible for severe forms of DMD.

Table 1 shows that some mutations result in a change in the reading frame, which then leads to defective protein production (synthesis). Of course, there are many reasons why dystrophin is not produced or is produced in a wrong form. In the animal model of *mdx* mouse the lack of dystrophin is caused by point mutation [20].

GENE THERAPY FOR DMD

Effective gene therapy for DMD requires proper protein supplement to most of the striated muscle cells. The problem is the large size of dystrophin gene, about 2.4 Mb, necessitating minigene cassettes that can express therapeutic levels of functional protein. A vector has to carry these expression cassettes and transduce striated muscle myoblasts. Also, the therapeutic gene must not trigger toxic or immunological reactions that lead to muscle damage or pain in the patient. As it can be seen there are many areas of intensive research including gene cloning, preparing of exact genetic construct, administration of it, transduction to the muscle and immunological acceptance.

Introduction of such a large gene amounting to 2.4 Mb into the human body is not only technically difficult, but it also involves a strong immunological response. Therefore, the first step is to minimize the size of the gene as much as possible.

Table 1. The principles of frameshift changes

Type of mutation	Effect of mutation in 5 “triplets” coding 5 “amino acids” – words
Wild sequence	**THE** *CAT* was NOT *his*
Nucleotide addition (frameshift is changed)	**THE** **C***CA T*wa sNO T*his*
Nucleotide deletion (frameshift is changed)	**THE** *A*tw asN O*th is*
Nucleotide substitution (frameshift is not changed)	**THE** *CAR* was NOT *his*
Nucleotide inversion (frameshift is not changed)	**THE** *TAC* was NOT *his*
Nucleotide duplication (frameshift is changed)	**THE** *CAT T*wa sNO T*hi s*

As it was mentioned above, the muscle isoform of dystrophin is encoded on 14 Kb mRNA, and transgenic animal studies have shown that this cDNA prevents dystrophy in *mdx* muscles [21]. While considerably smaller than the natural dystrophin gene, a full-length dystrophin expression cassette is too long for most viral vectors. That is the reason why researchers are still working on the possibility of producing a truncated but functionally correct form of dystrophin. Numerous observations show that in some mildly affected Becker Muscular Dystrophy (BMD) patients, mutations caused deletions of a significant part of gene [22, 23].

The removal of some dystrophin domains does not result in a dramatic change of function, but only slightly modifies it without causing damage in the functioning of the body, for example, the central rod (Rod) and C-terminal (CT) domains. According to McCabe et al. [24] and Rafael et al. [25] the CT domain is composed of 277 amino acids and is minimally required for dystrophin function. The Rod domain contains 24 spectrin-like repeats and builds up about 80% of dystrophin. Microdystrophin called (ΔR-R23), which contains only the three first and the last of the 24 spectrin-like repeats, is proved to have the ability of repairing morphological dysfunctions, providing it is introduced into young entities. Such experiments were conducted on mice [26]. A mouse is believed to be a very good animal model [26]. However, one must remember that in the case of myopathies, it is biomechanics which plays a significant role and it greatly differs in bi-pedal and quadruped organisms. It can be clearly seen in the cases of spinal cord damage. In man spinal cord damage almost always results in paresis, while quadruped animals can move quite well even with a severely damaged spinal cord.

Supplying a therapeutic gene into the body is a very important phase. Studies on gene delivery have focused on adenoviruses (Ad), adeno-associated viruses (AAV), retroviruses and plasmids [27].

Adenoviruses cause a strong immunological response due to antibodies against viral proteins and some recombinant proteins. That is why the usage of adenoviruses is being restricted to research into the immuno-response of model organisms such as mice or rats.

In our study we have focused on plasmids as generating minimal immunogenicity and toxicity, being relatively easy and safe, and characterized by an extremely large cloning capacity. A disadvantage of adenoviruses is the relatively low transduction efficiency according to classical protocols. This efficiency can be increased by using high-pressure injection or/and electroporation [28, 29, 30]. First clinical treatment for DMD patients using plasmids was conducted in France, but no results have been published so far.

Some major problems facing gene therapy are inherited and inquired mechanisms of the immunological protection system, which limits the spread of the therapeutic gene in the body. In severe cases the body's response can be so strong that it can lead to death [31]. Because of that fact clinical experiments using gene therapy are preceded with numerous tests, on animals and utilize the least possible doses of viral vector carrying therapeutic gene.

Intracellular injection of plasmids creates a mild cytotoxic T-lymphocyte (CTL)-mediated immune response against human dystrophin when delivered to *mdx* muscles [32, 33].

Direct injection of the DNA plasmid into target muscle results in small gene expression. One of numerous reasons for the small expression is the capillary barrier, as capillaries intimately surround muscle fibers. All kinds of vectors have some problems to pass through vessels. To get around this obstacle vasodilators such as histamine and papaverine can be of help. However, these pharmaceutics are dangerous when used systematically. A related method is high-pressure injection or electroporation. In this method 200-800 μg of plasmid DNA is delivered to animals of rabbit size. Electroporation is performed for two different voltage values (180V and 900V).

Vector transduction under these conditions is about 1 million times higher than without electroporation. Gene expression depends also on target muscle and lasts about one month, after which it gradually dies off. It should be taken into consideration that electroporation causes small muscle damage and inflammatory response observed usually within 48 hours after injection (manipulation). Infiltration caused by an inflammatory response usually disappears about 2 to 4 weeks after manipulation.

REPAIR OF DMD GENE

Repairing damaged DMD gene seems to be one of the most important challenges for gene therapy. That is why many research groups all over the world are working on a method enabling effective therapy for DMD.

The following approaches have been investigated to date: (1) chimeroplasts; (2) antisense

oligo(ribo)nucleotides (AONs); and (3) delivery or upregulation of utrophin that can compensate for dystrophin deficiency [34, 35].

Chimeroplasts are RNA-DNA chimeric oligonucleotides to induce repair of the point mutations in the dystrophin gene in *mdx* mice. Bertoni and Rando [36] demonstrated that targeting chimeraplasts could repair the exon 23 point mutation in differentiated myofibers *in vivo*, following intramuscular injection. The aforementioned scientists proved that chimeraplast-mediated gene repairment might be effective as an approach to gene therapy for muscular dystrophies due to point mutations.

Another approach consists of antisense oligonucleotides. Genes are made of double-helical DNA. When a gene is switched on, the genetic code in that segment of DNA is copied out as a single strand of RNA, i.e. messenger RNA. It is called a "sense" sequence, because it can be translated into an amino acids helix to form a protein. The opposite strand in a DNA is called the "antisense" strand. This antisense coding sequence may act as drugs by binding to messenger RNAs from disease genes, so that the genetic code in the RNA cannot be read, ceasing the production of the disease-causing protein. First successes have been achieved also in DMD treatment, particularly in its mild variants [37].

CONCLUSIONS

It has been 15 years since the first gene therapy for severe combined immunodeficiency (SCID) was successfully performed. So far gene therapy for muscular dystrophy has been still a dream; however, it has entered now the phase of clinical experiment. Geneticists are able to make small genetic constructions with dystrophin 3.5kb large, which allows maintaining all functions of the enormous, 2.4Mb, dystrophin gene. It is a hope for us that soon we will witness the first effects of Duchenne Muscle Dystrophy gene therapy. However, it requires many experiments in the field of genetic engineering, and involves introduction of vectors into the patient, their transduction and avoidance of toxic effects as well as immunological rejection.

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