ORIGINAL ARTICLE Egypt. J. Med. Hum. Genet. Vol. 10, No. 2, Nov. 2009

Caveolin 3 gene and mitochondrial tRNA methionin gene in Duchenne muscular dystrophy

Iman E. Abdel-Meguid[1], Soheir S. Korraa[2] and Ekram Abdel-Salam[1]

[1]Department of Pediatrics, Genetics Unit, Faculty of Medicine, Cairo University, [2]Department of Radiation Health, National Centre for Radiation Research and Technology, Atomic Energy Authority

ABSTRACT

Background: It was recently reported that Duchene muscular dystrophy (DMD) patients and mdx mice have elevated levels of caveolin-3 expression in their skeletal muscles. However, it remains unknown whether this increased caveolin-3 levels contribute to the pathogenesis of DMD. Also mitochondrial DNA mutation in the tRNA methionin (tRNA Met) gene has been shown to be associated with muscle weakness, severe exercise intolerance, lactic acidosis and growth retardation. Since DMD is X-linked maternally inherited disease, mitochondrial mutation in tRNA (Met) gene can be suspected to be the cause for the inefficient splicing of dystrophin gene during its expression and can be implicated as the cause of dystrophin inactive protein.

Aim of the Work: The aim of the present study is to investigate whether mutations in caveolin gene leads to its increased expression and/or mutation in the tRNA (Met) gene can be associated with DMD pathogenesis.

Patients and Methods: Expression of caveolin mRNA by RT-PCR and mutations in caveolin gene and tRNA (Met) gene were measured in 28 patients presented with DMD symptoms using the single strand conformation polymorphism assay (SSCP).

Results: Results gave further proof to decreased expression of inducible nitric oxide synthase (iNOS) mRNA, which leads to increased expression in caveolin 3 mRNA in lymphocytes of DMD patients compared to controls. However using SSCP, there was no evidence for tRNA (Met) gene mutation among DMD patients and only one patient presented a mutation in the caveolin gene compared to controls.

Conclusion: There is an inverse relation between iNOS and Caveolin 3 in lymphocytes of DMD patients compared to controls. However, Caveolin 3 gene mutation is excluded as the main cause of increased caveolin gene expression. Also, there was no evidence for tRNA (Met) gene mutation among DMD patients.

Key Words:

mRNA, duchene muscular dystrophy (DMD), inducible nitric oxide synthase (iNOS) mRNA, mitochondrial DNA.

Corresponding Author:

Eman E. Abdel Meguid
E-mail: moni_ehsan@hotmail.com

INTRODUCTION

Duchene muscular dystrophy (DMD) is an X-linked (Xp21) recessive disease affecting 1 in 3,500 males, resulting in morbidity in childhood and early adulthood[1]. DMD is caused by a variety of mutations in the open reading frame of the dystrophin gene, resulting in a deficiency of the protein product and loss of function of the dystrophin-glycoprotein complex (DGC)[2]. DGC plays a primary role in mechanical stabilization of the plasma membrane in striated muscles and provide hints for secondary functions in organizing molecules involved in cellular signaling that allows the muscle to adapt to muscle activity by either atrophy or hypertrophy[3,4]. The DGC is composed of several protein components, which spans the muscle sarcolemma, linking the cortical cytoskeleton with the extracellular matrix[5,6]. Associated with the DGC complex is caveolin and nitric oxide synthase. Caveolin-3 is localized in the sarcolemma and co- fractionates with the original dystrophin complex (DC)[7], while neuronal nitric oxide synthase (nNOS) is normally localized to the membrane of muscle cells.[8]

Caveolin-3 protein is a product of the caveolin gene family and many signaling molecules are localized to the caveolae and interact with caveolins[9]. The interaction of caveolins with signaling molecules has been implicated in the activation and in the inactivation of signal transduction events. One of the best characterized examples of this interaction and its role in signal transduction is the binding of caveolins to inducible nitric oxide synthase (iNOS)[10]. Interaction of caveolins with NOS is thought to hold the enzymes in an inactive conformation and only release their inhibitory constraints through caveolin dissociation, allowing the signaling to take place[11]. Thus the decreased expression of nitric oxide synthases iNOS mRNA can lead to increase in caveolin mRNA.

Nitric oxide synthase (NOS), an enzyme that produces nitric oxide (NO), is another component of the DGC[8]. It has been implicated that dystrophin-deficient muscle is a consequence of neuronal NOS (nNOS) deficiency and not nNOS mislocalization[12]. Because NO is a versatile and rapid reactant with other free radicals, the extreme disruptions of its production in muscle could cause major shifts in the redox environment of the muscle. Furthermore, NO functions as a pluripotent signaling molecule in muscles and other tissues and perturbations in its production could have broad effects on muscle homeostasis[13]. Experimental evidence demonstrates the importance of neuronal NOS deficiency in the pathology of mdx dystrophy. Expression of a muscle-specific neuronal NOS transgene in dystrophic muscle prevented the majority of histologically discernible pathology in mdx mice, including reducing sarcolemmal lesions by about 80%.[14]

Mitochondria are implicated in oxidative stress and abundant evidence implicates oxidative stress as a potential regulator of proteolytic pathways leading to muscle atrophy during periods of prolonged disuse disease[15]. Mitochondrial DNA (mtDNA) is a short circular molecule of packed form of DNA. The rapid evolution and maternal inheritance of mtDNA make it a valuable marker

for progeny of a given mother[16,17]. Since inefficient splicing of dystrophin gene during its expression can be implicated as the cause of dystrophin inactive protein[18], mutation in tRNA can be expected to be the cause. It has been lately identified that mtDNA mutation in the tRNA (Met) gene presented by muscle weakness, severe exercise intolerance, lactic acidosis and growth retardation. Muscle biopsy showed unusually severe dystrophic features in muscle biopsies[19]. Accordingly, it is important to raise a sensitive method to detect mtDNA mutations.[20,21]

The aim of the present study is to find new biomarkers implicated in pathogenesis of DMD. Those biological markers are: Caveolin 3, nitric oxide synthetases expression and mitochondrial tRNA (Met) gene and caveolin 3 mutations.

PATIENTS AND METHODS

Subjects were 24 DMD diagnosed boys clinically and at the molecular level, mean age 8.1 ± 1.9 years versus 20 age and socioeconomic matching healthy boys, mean of age 8.2 ± 2.2. Patients and controls were chosen to be free from any infection and receiving no therapeutic treatment known to increase the oxidative stress. Blood samples were drawn after their parents' consent.

Reverse Transcriptase - polymerase Chain Reaction (RT-PCR) Analysis for Caveolin-3 and Nitric Oxide synthase Genes.

Total RNA was extracted from circulating mononuclear cells separated from the peripheral blood by Ficoll-Hypaque density gradient separation using Histopaque-1077 (Sigma), using QIAGEN RNeasy extraction Kit extraction kit (QIAGEN Inc, USA). The RNA samples were reverse transcribed using Superscript reverse transcriptase, using QIAGEN OneStep RT-PCR kit (QIAGEN) according to manufacturer's instructions. 10 µl of RT reaction were mixed with different primers together with 25 µl of Ready Mix RedTaq PCR. Primer pairs for caveolin-3 (sense primer 5'-GGGCAACATCTAGAAGCCCAACAA-3'; anti-sense primer 5'-CTGATGCACTGAATTCCAATCAGGAA-3'). The iNOS primer pair used was as follows: forward: 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' reverse: 5'-GGCTGTCAGAGCCTCGTGGCT-TTGG-3' and β-actin samples (forward: 5'-GTG GGG CGC CCC AGG CAC CA-3'; reverse: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'). iNOS was amplified with the following cycle parameters: 95°C for 3 minutes; 94°C for 45 seconds; 59°C for 45 seconds, 72°C for 2 minutes for 35 cycles, then 72°C, for 7 minutes and 4°C for 24 hours. PCR for caveolin initial denaturation was for 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with final extension at 72°C for 10 min. PCR for β-actin were run in the following procedures: at 94°C for 7 min, 1 circle; at 94°C for 1 min, at 72°C for 1 min, 30 cycles; at 72°C for 7 min. Reaction products were then separated on a 1.2% agarose gel, ethidium stained and photographed. The size of the PCR fragments representing iNOS was 557 bp, β-actin 540 and caveolin 345 bp.

Genomic and mtDNA extraction:

Total DNA (including mtDNA) was extracted from peripheral blood lym-

phocytes by Takara DNA extraction kit. After DNA extraction, about 50 µg DNA at 150-500 ng/µL concentration was obtained.

SSCP Analysis:

SSCP analyses were used to screen for CAV gene and tRNA(Met) gene mutations. Cav 3 primers were GAGTTGAGGCTTCCCTTGCC and reverse CGAACAGGAAGCCCCAGAGCA. Mitochondrial met was mtDNA primer pairs were (sense primer 5'-CAAATTCCTCCCTGTACGAAAGG-3'; antisense primer 5'-AATGAGGAGTAGG AGGTTGGCC-3'). PCR was carried out. PCR amplification was carried out in a Perkin-Elmer System thermocycler using the following profile: 1 cycle of denaturation at 94°C for 5 minutes; 5 cycles at 94°C for 20 seconds, 64°C for 20 seconds, 72°C for 30 seconds and 25 cycles of 94°C for 20 seconds, 62°C for 20 seconds, 72°C for 30 seconds; followed by a 5-minute extension at 72°C.

Amplified samples were diluted with 50 µl of formamide buffer and 50 µl of 0.1% SDS/10 mmol/L EDTA. The mixture was denatured at 94°C for 5 minutes, then cooled rapidly on ice and held for 5 minutes. For each sample, 3 to 5 µl was loaded onto 10% nondenaturing polyacrylamide and run at 8 W overnight at room temperature, stained and photographed.

RESULTS

Results showed significantly increased expression in caveolin 3 mRNA relative expression (1.5 ± 0.4 vs.1 ± 0.3) among DMD patients compared to controls (Fig. 1 and Table 1), whose expression was measured as a relative expression to β-actin mRNA expression (Fig. 2). Relative expression of iNOS was significantly lower among DMD patients compared to controls (1.2 ± 0.6 vs. 1.9 ± 2.2) as demonstrated in (Table 1 and Fig. 3).

Applying the SSCP, there was only one patient presented a mutation in the caveolin gene compared to controls (Fig. 4) and no evidence for tRNA (Met) gene mutation among DMD patients (Fig. 5).

Table 1: Relative mRNA expression in iducible nitric oxide synthase and caveolin 3.

	DMD	Controls	t & p
Caveolin 3 mRNA	1.5 ± 0.4	1 ± 0.3	t= 8.5, p < 0.0000001
Nitric Oxide mRNA	1.2 ± 0.6	1.9 ± 2.2	t = 11.2, p < 0.0001

Fig. 1: Caveolin 3 mRNA relative expression in DMD patients compared to controls. Lanes 13- are controls and lanes 46- are DMD patients. It is evident that DMD patients have a higher expression of Caveolin-3 mRNA compared to controls.

Fig. 2: β-actin mRNA expression in DMD patients compared to controls. No significant differences in. β-actin mRNA between DMD and Controls.

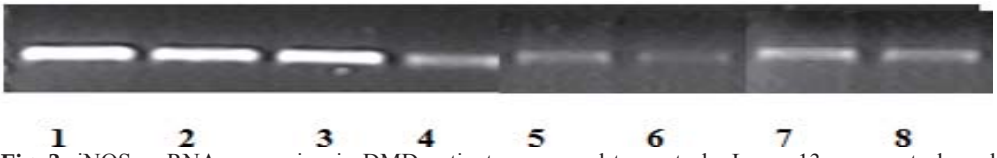

Fig. 3: iNOS mRNA expression in DMD patients compared to controls. Lanes 13- are controls and lanes 48- are DMD patients. It is evident that DMD patients have a lower expression of iNOS mRNA compared to controls.

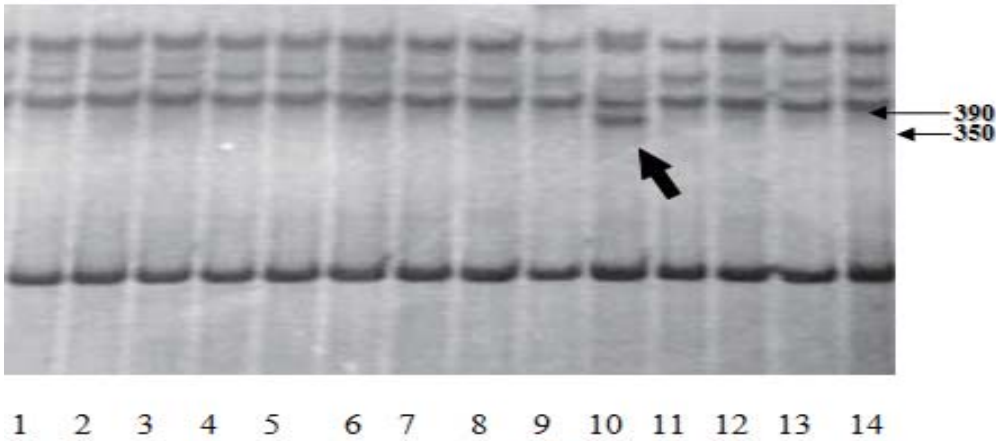

Fig. 4: SSCP chart for Caveolin 3. Lanes 15- denotes controls and 614- denotes DMD cases. Mutation is observed in case 10.

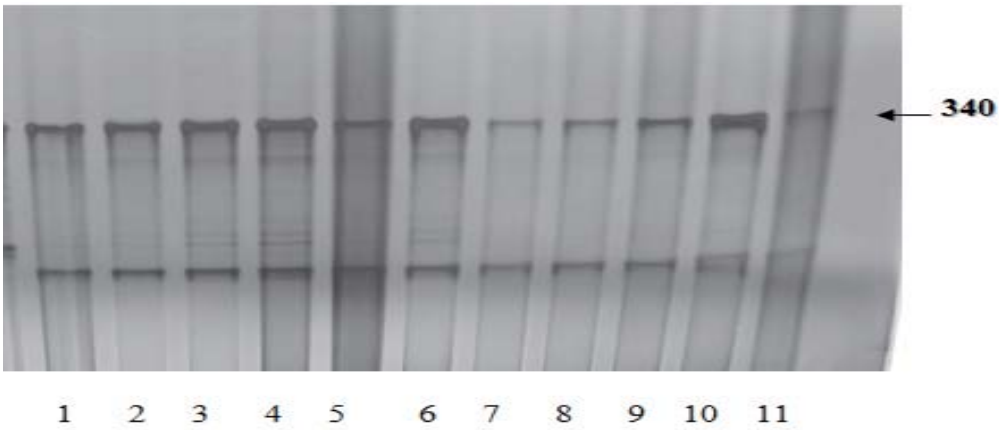

Fig. 5: SSCP chart for tRNA (Met) gene. Lanes 15- denotes controls and 611- denotes DMD cases. No mutations are observed neither in cases nor controls.

DISCUSSION

iNOS is a nitric oxide (NO) producing enzyme. NO has been shown to play an important role in the regulation of blood flow in exercising skeletal muscle by modulating the vasoconstrictor response to activation of α-adrenergic receptors[22,23]. In healthy muscles, NO has been implicated in myofiber differentiation[24], modulation of contractile force (and exercise-induced glucose uptake[25]. Also, NO has a potential role in protecting cells from damage oxygen intermediate superoxide, when there is an imbalance in the production of either NO or superoxide because of the altered chemistry for the production of highly reactive free radical peroxynitrate[26]. Previous studies performed to evaluate alterations and functions of nitric oxide synthase (NOS) in DMD depend on studies carried out on animal models of dystrophic (mdx) mice. There is a controversy regarding the expression of iNOS mRNA in DMD models. While some studies indicated that iNOS mRNA is increasingly expressed in (mdx) mice compared to controls[27]. Others indicated by immunohistochemical and Western blot analysis, that iNOS is expressed and active in the smooth muscle cells of normal mouse and defective in young adult (2-month-old) mdx mice[28]. Another study demonstrated the presence of protein inhibitor of nNOS (PIN) mRNA, which is significantly higher in PIN mRNA in dystrophic muscles compared with normal muscles of mdx mouse[29]. However the negative correlation between caveolin mRNA expression and iNOS expression supports that increased expression of caveolin 3 could be the cause for decreased expression of iNOS mRNA.[30]

Results of the present study showed increased expression of caveolin 3 mRNA expression in lymphocytes of DMD patients compared to controls. This provides confirmation to previous findings that showed up-regulation of caveolin-3 expression in humans with DMD[31]. Increased number of caveolae in the skeletal muscles of DMD patients has been observed by immo-electron microscopy techniques, but not in other forms of neuronally based muscular dystrophies[32]. In accordance with an increased number of caveolae in DMD patients, it was recently reported that mdx mice (an animal model of DMD, with a dystrophin deficiency) have increased levels of caveolin-3 expression in their skeletal muscles (by ≈2- to 3-fold).[33]

Results of the present study showed only one case with mutation in caveolin 3 gene. This excludes caveolin gene mutation from being associated with all cases of DMD pathogenesis. A recent study showed that overexpressed wild-type caveolin-3 as a transgene in mice revealed:

i- A dramatic increase in sarcolemmal caveolae.

ii- Hypertrophic, necrotic and regenerating skeletal muscle fibers with central nuclei.

iii- Down-regulation of dystrophin and β-dystroglycan protein expression. This implicates the possibility that overexpression of caveolin-3 disrupts the normal processing or structure of the dystrophin complex, leading to its degradation. Our current results support this hypothesis. In addition, these mice showed elevated serum creatine kinase

levels, consistent with the myo-necrosis observed morphologically.[34]

Mitochondrial DNA (mtDNA), which exhibits a maternal inheritance and a high rate of evolution, has been widely used as a genetic marker when analyzing maternally inherited diseases[35]. The mammalian mitochondrial genome (mtDNA) is a 16–17 kb double-stranded circular DNA molecule of characteristic low-molecular mass, simple structure, high evolution rate and maternal inheritance, as well as absence of tissue specificity. On average, each somatic cell has 100–500 mitochondria and each mitochondrion has 1–15 mtDNA molecule (s).[35]

Results of the present study did not detected mitochondrial mutation in DMD patients. Interpretation of the mtDNA sequence data can be extremely difficult because mtDNA is highly polymorphic and it is often difficult and time consuming to establish whether a mutation is pathogenic or not, particularly if the base change has not been reported before Deleterious mutations, which segregate with the disease clinically, are usually (but not exclusively) heteroplasmic[36]. Heteroplasmic mutations means that affected individuals have a high percentage of mutated mtDNA, while unaffected individuals have a lower percentage.[36]

CONCLUSION

There is an inverse relation between iNOS and Caveolin 3. A significant decrease in iNOS mRNA expression in neutrophils results in significant increased expression in caveolin 3 mRNA in lymphocytes of DMD patients compared to controls. This may be due the interaction of caveolins with iNOS[10]. However using SSCP, there was no evidence for tRNA (Met) gene mutation among DMD patients and only one patient presented a mutation in the caveolin gene compared to controls, which excludes gene mutation for increased caveolin overexpression.

REFERENCES

1. Emery AE. The muscular dystrophies. Lancet 2002, 23; 359 (9307):687-95.

2. Hoffman EP, Brown RH,Jr, Kunkel LM. Dystrophin: The protein product of the Duchenne muscular dystrophy locus. Cell 1987, 24;51(6):919-28.

3. Ervasti JM, Sonnemann KJ. Biology of the striated muscle dystrophin-glycoprotein complex. Int. Rev. Cytol. 2008;265:191-225.

4. Chockalingam PS, Cholera R, Oak SA, Zheng Y, Jarrett HW, Thomason DB. Dystrophin-glycoprotein complex and Ras and Rho GTPase signaling are altered in muscle atrophy. Am. J. Physiol. Cell. Physiol. 2002; 283(2):C500-11.

5. Sciandra F, Bozzi M, Bianchi M, Pavoni E, Giardina B, Brancaccio A. Dystroglycan and muscular dystrophies related to the dystrophin-glycoprotein complex. Ann. Ist. Super. Sanita 2003;39(2):173-81.

6. North AJ, Galazkiewicz B, Byers TJ, Glenney JR,Jr, Small JV. Complementary distributions of vinculin and dystrophin define two distinct sarcolemma domains in smooth muscle. J. Cell Biol. 1993; 120(5):1159-67.

7. Rando TA. The dystrophin-glycoprotein complex, cellular signaling and the regulation of cell survival in the muscular dystrophies. Muscle Nerve 2001; 24(12):1575-94.

8. Williams JC, Armesilla AL, Mohamed TM, Hagarty CL, McIntyre FH, Schomburg S, et al. The sarcolemmal calcium pump, alpha-1 syntrophin and neuronal nitric-oxide synthase are parts of a macromolecular protein complex. J. Biol. Chem. 2006, 18; 281(33):23341-8.

9. Okamoto T, Schlegel A, Scherer PE, Lisanti MP. Caveolins, a family of scaffolding proteins for organizing «preassembled signaling complexes» at the plasma membrane. J. Biol. Chem. 1998, 6;273(10):5419-22.

10. Venema VJ, Ju H, Zou R, Venema RC. Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. J. Biol. Chem. 19, 7; 272(45):28187-90.

11. Kurzchalia TV, Parton RG. Membrane microdomains and caveolae. Curr. Opin. Cell Biol. 1999; 11(4):424-31.

12. Tidball JG, Wehling-Henricks M. The role of free radicals in the pathophysiology of muscular dystrophy. J. Appl. Physiol. 2007;102(4):1677-86.

13. Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. Physiol. Rev. 2001;81(1):209-37.

14. Wehling M, Spencer MJ, Tidball JG. A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. J. Cell Biol. 2001, 1;155(1):123-31.

15. Powers SK, Kavazis AN, DeRuisseau KC. Mechanisms of disuse muscle atrophy: Role of oxidative stress. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2005; 288(2):R337-44.

16. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature 1981, 9;290(5806):457-65.

17. Finnila S, Hassinen IE, Ala Kokko L, Majamaa K. Phylogenetic network of the mtDNA haplogroup U in Northern Finland based on sequence analysis of the complete coding region by conformation-sensitive gel electrophoresis. Am. J. Hum. Genet. 2000; 66(3):1017-26.

18. Grossman LI, Shoubridge EA. Mitochondrial genetics and human disease. Bioessays 1996; 18(12):983-91.

19. Vissing J, Salamon MB, Arlien Soborg P, Norby S, Manta P, DiMauro S, et al. A new mitochondrial tRNA(Met) gene mutation in a patient with dystrophic muscle and exercise intolerance. Neurology 1998;50(6):1875-8.

20. Horvath R, Lochmuller H, Scharfe C, Do BH, Oefner PJ, Muller Hocker J, et al. A tRNA(Ala) mutation causing mitochondrial myopathy clinically resembling myotonic dystrophy. J. Med. Genet. 2003; 40(10):752-7.

21. Wong LJ, Wladyka C, Mardach Verdon R. A mitochondrial DNA mutation in a

patient with an extensive family history of Duchenne muscular dystrophy. Muscle Nerve 2004; 30(1):118-22.

22. Lee KH, Baek MY, Moon KY, Song WK, Chung CH, Ha DB, et al. Nitric oxide as a messenger molecule for myoblast fusion. J. Biol. Chem. 1994, 20; 269(20):14371-4.

23. Reid MB. Nitric oxide, reactive oxygen species and skeletal muscle contraction. Med. Sci. Sports Exerc. 2001; 33(3): 371-6.

24. Miles AM, Bohle DS, Glassbrenner PA, Hansert B, Wink DA, Grisham MB. Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. J. Biol. Chem. 1996, 5; 271(1):40-7.

25. Buchwalow IB, Minin EA, Müller FU, Lewin G, Samoilova VE, Schmitz W, et al. Nitric oxide synthase in muscular dystrophies: A re-evaluation. Acta Neuropathol. 2006; 111(6):579-88.

26. Mule F, Vannucchi MG, Corsani L, Serio R, Faussone Pellegrini MS. Myogenic NOS and endogenous NO production are defective in colon from dystrophic (mdx) mice. Am. J. Physiol. Gastrointest.Liver Physiol. 2001; 281 (5): G1264-70.

27. Guo Y, Petrof BJ, Hussain SN. Expression and localization of protein inhibitor of neuronal nitric oxide synthase in Duchenne muscular dystrophy. Muscle Nerve 2001; 24 (11): 1468-75.

28. Gath I, Ebert J, Godtel Armbrust U, Ross R, Reske Kunz AB, Forstermann U. NO synthase II in mouse skeletal muscle is associated with caveolin 3. Biochem. J. 1999, 15;340(Pt 3):723-8.

29. Repetto S, Bado M, Broda P, Lucania G, Masetti E, Sotgia F, et al. Increased number of caveolae and caveolin-3 overexpression in Duchenne muscular dystrophy. Biochem. Biophys. Res. Commun. 1999, 11;261(3):547-50.

30. Bonilla E, Fischbeck K, Schotland DL. Freeze-fracture studies of muscle caveolae in human muscular dystrophy. Am. J. Pathol. 1981; 104(2): 167-73.

31. Song KS, Scherer PE, Tang Z, Okamoto T, Li S, Chafel M, et al. Expression of caveolin-3 in skeletal, cardiac and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. J. Biol. Chem. 1996, 21; 271(25):15160-5.

32. Vatta M, Ackerman MJ, Ye B, Makielski JC, Ughanze EE, Taylor EW, et al. Mutant caveolin-3 induces persistent late sodium current and is associated with long-QT syndrome. Circulation 2006, 14; 114(20):2104-12.

33. Vaghy PL, Fang J, Wu W, Vaghy LP. Increased caveolin-3 levels in mdx mouse muscles. FEBS Lett. 1998 10; 431 (1): 125-7.

34. Satoh M, Kuroiwa T. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. Exp. Cell Res. 1991;196(1):137-40.

35. Fearnley IM, Walker JE. Conservation of sequences of subunits of

mitochondrial complex I and their relationships with other proteins. Biochim. Biophys. Acta 1992, 7; 1140(2):105-34.

36. Legros F, Malka F, Frachon P, Lombes A, Rojo M. Organization and dynamics of human mitochondrial DNA. J.Cell. Sci. 2004, 1;117(Pt 13):2653-62.