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

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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.

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Duchenne muscular dystrophy (DMD) is an X-linked (Xp21) recessive disease affecting 1 in 3,500 males, resulting in morbidity in childhood and early adulthood. 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). 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. The DGC is composed of several protein components, which span the muscle sarcolemma, linking the cortical cytoskeleton with the extracellular matrix. 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), while neuronal nitric oxide synthase (nNOS) is normally localized to the membrane of muscle cells.

Caveolin-3 protein is a product of the caveolin gene family and many signaling molecules are localized to the caveolae and interact with caveolins. 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). 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. 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. It has been implicated that dystrophin-deficient muscle is a consequence of neuronal NOS (nNOS) deficiency and not nNOS mislocalization. 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. 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%.

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. 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 evolutionary studies.
for progeny of a given mother. Since inefficient splicing of dystrophin gene during its expression can be implicated as the cause of dystrophin inactive protein, 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. Accordingly, it is important to raise a sensitive method to detect mtDNA mutations.

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'-GGGCAA-CATCTAGAGAAGCCTCAACCA-3', anti-sense primer 5'-CTGATGCAT-GAATTTGAAATGAAA-3'). The iNOS primer pair used was as follows: forward: 5'-CCCTCTCGAAGTTTCT-GGCAGCAGC-3' reverse: 5'-GGCT-GTCAGAGGCTGCTGTGCTGGC-3' and β-actin samples (forward: 5'-GTG GGCGCCACGCACTTACTGATGTTC-3'; reverse: 5'-CTC TTT TTT GAC TGT 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 CGAACAGGAAGCCCAAGAGCA. Mitochondrial met was mtDNA primer pairs were (sense primer 5’-CAAATTCCCTCCCTGTAAC-3’; antisense primer 5’-AATGAGGAGTAGGGAGTTGAGAGGCC-3’). Mitochondrial met was mtDNA primer pairs were (sense primer 5’-CAAATTCCCTCCCTGTAAC-3’; antisense primer 5’-AATGAGGAGTAGGGAGTTGAGAGGCC-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 inducible nitric oxide synthase and caveolin 3.

<table>
<thead>
<tr>
<th></th>
<th>DMD</th>
<th>Controls</th>
<th>t &amp; p</th>
</tr>
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<tbody>
<tr>
<td>Caveolin 3 mRNA</td>
<td>1.5 ± 0.4</td>
<td>1 ± 0.3</td>
<td>t= 8.5, p &lt; 0.0000001</td>
</tr>
<tr>
<td>Nitric Oxide mRNA</td>
<td>1.2 ± 0.6</td>
<td>1.9 ± 2.2</td>
<td>t = 11.2, p &lt; 0.0001</td>
</tr>
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**Fig. 1:** Caveolin 3 mRNA relative expression in DMD patients compared to controls. Lanes 1-3 are controls and lanes 4-6 are DMD patients. It is evident that DMD patients have a higher expression of Caveolin-3 mRNA compared to controls.
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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 1-3 are controls and lanes 4-8 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 1-5 denote controls and 6-10 denote DMD cases. Mutation is observed in case 10.

Fig. 5: SSCP chart for tRNA (Met) gene. Lanes 1-5 denote controls and 6-10 denote 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 \( \alpha \)-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 peroxynitrite\(^{26} \). 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 \( \approx \)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 \( \beta \)-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.\(^\text{34}\)

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\(^\text{35}\). 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).\(^\text{35}\)

Results of the present study did not detect 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\(^\text{36}\). Heteroplasmic mutations mean that affected individuals have a high percentage of mutated mtDNA, while unaffected individuals have a lower percentage.\(^\text{36}\)

**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\(^\text{10}\)

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**


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