Alternative splicing of ndufs2 and cox10 OXPHOS genes during aging

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Abstract

Age-related macular degeneration (AMD) is associated with mitochondrial disorders, and abnormalities in the oxidative phosphorylation system (OXPHOS) contributed to mitochondrial dysfunction. Alternative RNA splicing of two genes, ndufs2 from complex I and cox10 from complex IV, were studied in retina and eyecups of young and middle-aged mice. Two novo splice variants of COX10,1098 bp and 1287 bp were detected in retina and eyecup in the young mice. RT-PCR products were found to have 45 bp and 234 bp spliced out. The 45 bp deleted variant, has not been reported in any database.

Keyword: AMD, OXPHOS genes, alternative splicing

Introduction

AMD is a major cause of blindness which lead to the deterioration of the center of the retina, among the elderly period. Nearly 40% of people over 75 years old, have some pathologic signs of AMD [1]. Many lines of evidence suggest that mitochondrial disorders in eyes contribute to AMD, and mitochondrial disorders are common in other neurodegeneration diseases, such as Parkinson's disease and Alzheimer disease [2,3]. Mitochondrial disorders are clinical phenotypes associated with mitochondrial dysfunction, and abnormalities of OXPHOS.

OXPHOS is responsible for 90% of ATP production in a respiring cell. It has five multi-subunit complexes, the respiratory chain complexes, and two additional electron carriers. It is controlled on the genetic level by two distinct genomes: the mitochondrial genome and the nuclear genome. The mitochondrial genome of nearly 16.6 kb, encodes 13 subunits of complex I, III, IV and V. 7 mitochondrial DNA(mtDNA) and 38 nuclear DNA(nDNA) encodes subunits for complex I; and complex III contains one subunits encoded by mtDNA and 10 subunits by nDNA. Complex IV is made of 3 mtDNA encoded and 10 nDNA encoded subunits, and complex V contains 17 subunits, 2 are mtDNA encoded. The mitochondrial genome also contains 22 tRNA and two ribosomal RNA genes which are used to translate. nDNA are required for mitochondrial fundamental functions, such as mtDNA replication, transcription, translation and post-translation modification and mitochondrial membrane channels [4,5].

Deficiencies of OXPHOS are able to lead direct and indirect changes in metabolic homeostasis, which includes concentrations of ROS, Ca^{2+}, and ratios of ADP/ATP, and NAD/NADH [6]. Transcriptional response of OXPHOS genes, both nDNA and mtDNA, is often used to adapt these metabolic changes. Besides, OXPHOS genes have a feature of co-expression. And it is evident from studies of transcriptome analysis across different species. Particularly, in mice, co-expression of OXPHOS genes are studied among various tissues [7]. What is more, it is found that OXPHOS genes are likely to have co-regulations. There may be the likely core promoters for most OXPHOS genes and assembly factors [8].

Complex I deficiency is the most common cause of the mitochondrial disease. NDUFS2 is a constitutive component of the core of complex I, considered to be essential for electron transfers. It

is encoded by nDNA and has thirteen exons. The NDUFS2 mutation may lead to dysfunction of OXPHOS complex I where the mutation caused a catalytic defect .This mutation may have relation with Leigh syndrome [9]. Leigh syndrome is a severe neurological disorder disease with features of progressive degeneration of mental and movement abilities.

Cytochrome c oxidase(COX), embedded in the inner mitochondrial membrane, is in the terminal enzyme of electron transport chain. It catalyzes transfer of electrons from cytochrome c to oxygen [10]. Deficiency of COX is one of the most common metabolic reasons associated with respiratory chain defects and it is associated with various mitochondrial and neurodegenerative diseases [11,12]. COX10, one of subunits of COX, is heme-O-farnesyl transferase and also an assembly factor for COX in complex IV. It is encoded by nDNA and has seven exons. It is reported that mice lacking COX10 in skeletal muscle exhibited a progressive mitochondrial disease phenotype [13]. Mutations of COX10 have previously been found to be related with tubulopathy and leukodystrophy [14].

In this experiment, mice eyes are used instead of human eyes which are impeded with low availability. Eyeballs are isolated from young mice, middle-aged mice and β5 integrin knockout(ko) middle-aged mice. Retina and eyecups, which contain retinal pigment epithelium cells and choroid, are dissected separately from those eyeballs. Eyecups in ko middle-aged mice will experience more oxidative stress than wild type mice and extra oxidative stress is more likely to cause mutations on OXPHOS, while retina are supposed to have similar oxidative stress in ko and wild type middle-aged mice [15]. What is more, with aging, damage in mtDNA and oxidative stress increase with high probabilities and accumulations of both factor will contribute to AMD [16].

Materials and Methods

Animal

Mice were provided by Finnemann's lab, Department of Biological Sciences, Fordham University.Young, middle-aged and ko middle-aged mice were three and six months old, respectively. Mice were sacrificed by CO2 and eyeballs were isolated immediately and immersed in Davidson's fixative (32% ethanol, 11.5% acetic acid, 8% formaldehyde). Retina and eyecups were separated and then were grind.

RNA extraction

RNA was extracted from young mice retina, eyecups and middle-aged mice retina and eyecups using RNeasy® Plus Mini Kit (QIAGEN), according to the manufacturer's instructions.

Primers

Seven pairs of primers were designed for this experiment (Table 1). 4 pairs of primers were used to cover gene ndufs2 and the other primers were used to cover gene cox10. Most expected size of product is about 500 bp.

RT-PCR

RT-PCR was performed using QIAGEN® One-Step RT－PCR Kit following instructions.Ten nanograms of RNA was amplified in 20 μl RT-PCRs (4 μl 5×RT buffer, 0.8 μl 10 mM dNTPs, 0.8 μl 10 pmol/μl forward primer, 0.8 μl 10 pmol/μl reverse primer, 0.8 μl enzyme mix, 2 μl 10 ng/μl RNA and 10.8 μl ddH_2O). Temperature cycles as follow: one cycle of 50 ℃ for 30min and 95 ℃ for 15min, 94 ℃ for 30 s, 57 ℃ for 30s, and 72℃ for 30s, and a final extension of 72 ℃for 10 min followed by a final hold at 4°C. Cycle number was 50.

Electrophoresis

5 μl of loading dye was added to each RT-PCR product. 5 μl of each product was then added to a 1% agarose gel, and electrophoresis was performed at 160 V. Band intensities were visualized by ethidium bromide in a UV trans-illuminator (BioRad). 100 bp marker was used to to measure the size of bands.

Gel extraction and sequencing

The target products was extracted by QIAquick Gel Extraction Kit(QIAGEN) following the manufacturer's instructions and subsequently sequenced by GENEWIZ® in order to identify PCR products.

Tabel 1. The summary of primers and size of expected products

Name	Sequence of primers	Expected size of products
Ndf 1-3 F	TGCAGCCCGGTAAAGATG	491 bp
Ndf 1-3 R	AGCAACTTCTCCACAGCTATC	
Ndf 3-7 F	CACCGAGGCACGGAGAAG	503 bp
Ndf 3-7 R	GTCGACTGTCCTATTTCGCCAG	
Ndf 6-10 F	CTACCTCTTGGGCTTCTGGAT	498 bp
Ndf 6-10 R	TTCAATGGCAGTATATGTGGCTC	
Ndf 9-13 F	ACCGAAGCGAGCAGAGATG	391 bp
Ndf 9-13 R	ACACACACAGAGACACACAGC	
Cox 1-3 F	TTCCTCGCGCCTCCTCAC	279 bp
Cox 1-3 R	GACGGCAGCTCATCCTCG	
Cox 2-4 F	AGAGCTATACAGGGATTGCCAC	452 bp
Cox 2-4 R	GCAAAGCCTGCTGAAGTGG	
Cox 3-7 F	GCTGTCCAAGATCAAACTCACA	491 bp
Cox 3-7 R	ATTGCCAGGAGTACAGGATC	
GAPDH-MF	ACCAGGGCTGCCATTTGC	179 bp
GAPDH-MR	TGGAAGATGGTGATGGGCTTCC	
ND5-F	AGTCCGATTCCACCCACTCAC	485 bp
ND5-R	CTCAGGCGTTGGTGTTGC	

Results

No alternative variants were found in NDUFS2

In order to detect alternative splicing of NDUFS2, four pairs of primers were used for ndfus2, from exon1 to exon3 and from exon3 to exon7, from exon6 to exon10, and from exon9 to exon13. They overlapped all exons of ndufs2. If alternative splicing existed, two or more bands were observed from electrophoresis gel. But no alternative splicing was observed in ndufs2 (Fig. 1). Only expected bands were obtained. And expression level of ndufs2 were also constant during aging (Fig. S1). The result indicated that alternative splicing of NDUFS2 might not be affected by aging.

Figure 1. RT-PCR results for ndufs2 using four pairs of primers. C is negative control.YE is young mouse eyecups; and YR is young mouse retina. ME is middle age mouse eyecups; and MR is middle age mouse retina.All expected size of bands is about 500 bp. No alternative splicing was found.

Spliced variants of COX10 were observed

In order to detect alternative splicing of COX10, three pairs of primers were used for cox10, from exon1 to exon3 and from exon2 to exon4, and from exon3 to exon7. No alternative splicing were detected with first two pairs of primers(Fig. S2). However, it was surprising that three bands in young mice and two bands in middle-age mice were detected within primers from exon3 to exon7 (Fig. 2).Variant C was only found to be expressed strongly in young mice. Three bands were purified and sent out for sequencing and were confirmed that they were three variants of COX10 by blast (Fig. 3). The sequences of three bands were aligned and analyzed by blast and clustalW. It was found that variant A was expected results, in which no exons were spliced out and variant B resulted from part of exon6, 45 bp was spliced out. No reading frame shift happened. Conserved region for alternative splicing acceptor site were also analyzed and compared with variant A (Fig. 4). Polypyrimidine tract, T and C rich region, and 3' end AG were necessary for acceptor site, and they both found spliced end region on variant B. In variant C, exon6 was entirely spliced out, without read frame shift. Variant B and variant C were not reported in mouse database, but variant C matched a homologous variant in human database. Variant B has not been reported in any database.

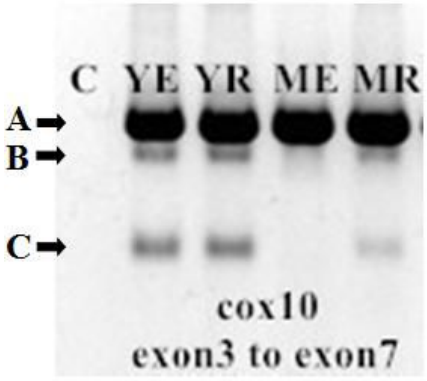

Figure 2. RT-PCR results of cox10 from exon3 to exon7.
Size of band A and band B was near 500 bp. Size of band C was near 200 bp. MR also had band C, but it was very weak.

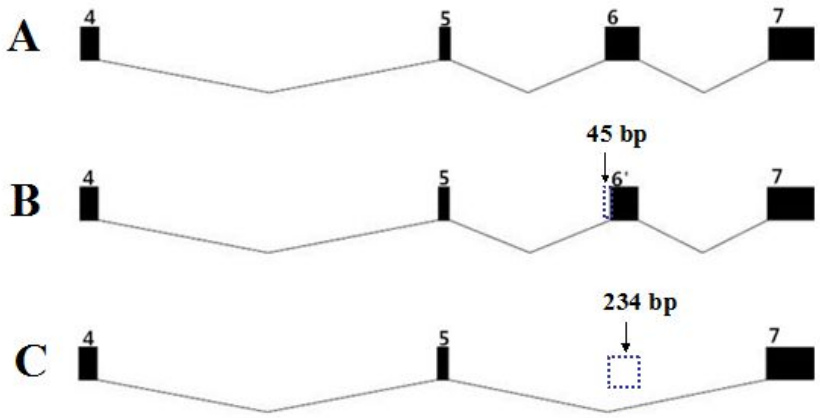

Figure 3. Alternatively spliced transcripts of COX10 observed in this study.
Variant A was the expected transcript. In variant B, 45 bp of exon6 were spliced out, deleting 15 amino acids, and not causing frame shift mutation. In variant C, all 234 bp of exon6 were spliced out.

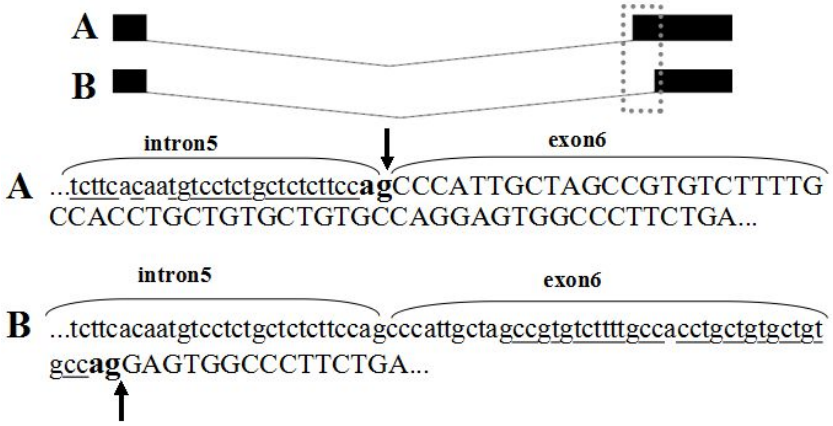

Figure 4. Comparison of acceptor sites of variant A and variant B.
Arrows indicate end of acceoptor sites. Lower case letters were spliced out sequences; and upper case letters were sequences contained in variants. Both variants had polypyrimide tract, underline letters and

Topology prediction

COX10 consists of 443 amino acids and enzymatic region is located from 159th amino acid to 414th amino acid. Transmembrane domains are predicted by TMHMM and it reveals that variant A has nine putative transmembrane domains. Major part of COX are embedded in inner mitochondrial membrane. In variant B, 15 amino acids are spliced out and it lacks 2 transmembrane domains. Variant C, without exon6, just have 4 or 5 transmembrane domains.

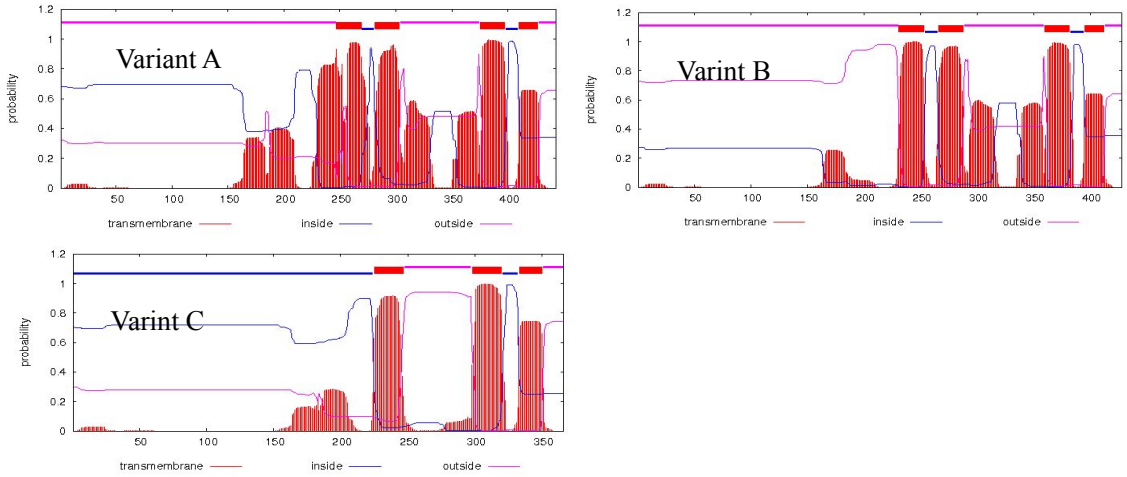

Figure 5. Predicted topology of three spliced variants of COX10.Transmembrane-spanning domains and the extracellular and intracellular loops are depicted. Red squire represents transmembrane domain. The x axis represents the amino acid number, and y axis is probabilities. This model is predicted by the TMHMM Server v2.0 (www.cbs.dtu.dk)

Discussion

This project found expressions of three variants of COX10, in mice retina and eyecups, changed with aging. The spliced region in variant B is located in two transmembrane domains; and variant C lacks four transmembrane domains. It is surprising that variant C is expressed strongly in young mice eyes. The biological meaning of these spliced variants is not understood clearly and the linkage between COX10 alternative splicing machinery and aging is also unknown. Variant B and C are not found in mouse genomic plus transcript database in NCBI. Variant C matched a homologous variant in the human database, variant B, however, has not been reported in any database. Western blot of variant B and variant C are still required, because those two variants may not be translated into proteins. Results of alternative splicing of COX10 and NDUFS2 in ko middle-aged mice are the same with the wild type mice. The eyecups in ko middle-aged mice have more oxidative stress than wild type, so it was supposed to influence OXPHOS genes' transcriptional response. It is possible that middle-aged mice is not old to accumulate enough oxidative stress in eyecups.

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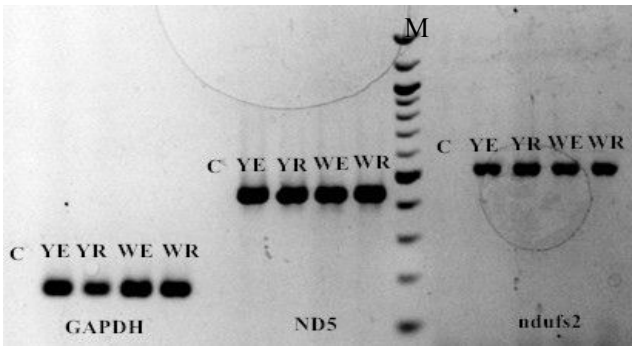
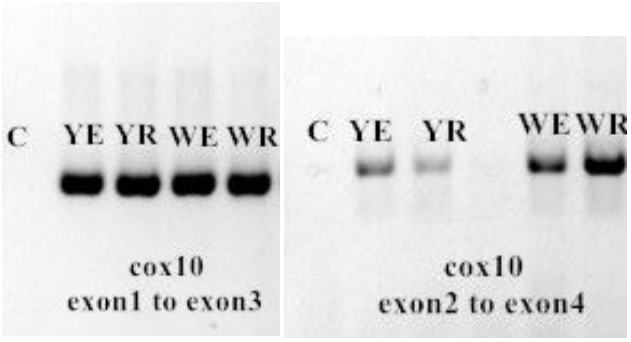


Supplemental Figure S1. RT-PCR results of ndufs2 for gene expression. The cycle number is 30. M is 100 bp marker. GAPDH is used as control. ND5 is form mitochondrial genome and it is also construct for complex I. Expression levels were similar with different samples.



Supplemental Figure S2. RT-PCR results of COX10, with two pairs of primers. Only expected bands were observed and no alternative splicing.