Transcriptome and whole genome sequencing profiles in Leber's Hereditary Optic Neuropathy 14484T>C mutation carrying monozygotic twins reveal that prostanoid receptor is a possible modifier for LHON manifestation

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Abstract

Background

Leber's inherited optic neuropathy (LHON) is well known for incomplete penetrance. A pair of monozygotic twins carrying 14484T > C LHON mutation: one displayed LHON characteristics (affected LHON) and the other twin was an unaffected LHON carrier, were studied to identify possible modifier(s) for LHON manifestation.

Methods

Primary fibroblasts from affected and unaffected monozygotic twins with 14484T > C LHON mutation were treated with different insults to differentiate cellular phenotype between the two fibroblasts. RNA sequencing of the fibroblasts indicated differentially expressed genes and whole genome sequencing was used to identify candidates for disease modifier.

Results

Our results suggested that fibroblast from unaffected carrier was able to adapt to galactose and hydrogen peroxide insult, while affected fibroblasts were not. We found reduced expression of total SOD2 with high proportion of inactive SOD2 (acetylated SOD2) in affected LHON fibroblast, while decreased expression of SIRT3 was detected in affected LHON fibroblasts treated with combination of insults. Differential expression indicated enrichment of a pathway relating to negative regulator of cell death pathway in unaffected carrier fibroblast. Expression of receptor for prostaglandin E receptor (PTGER3) was found to be affected by two SNPs. Unaffected LHON fibroblast possessed rs75523942 that indicates a positive effect on PTGER3 expression, while affected LHON fibroblast possessed rs496483 that indicates a negative effect on PTGER3 expression. Discordant SNPs on prostaglandin E receptor 3 (PTGER3) were identified as eQTL.

Conclusions

This study indicates that prostanoid receptor could be a possible modifier for LHON manifestation of these twins.

Introduction

Leber's hereditary optic neuropathy (LHON), an inherited mitochondrial disease, typically manifests as painless central visual loss (central scotoma). The visual loss can occur in just one eye or both. Mitochondrial mutations result in death of retinal ganglionic cells (RGCs). LHON is the most common mitochondrial diseases with an estimated prevalence from 1:30,000 to 1:54,000 worldwide.¹⁻³ LHON is

characterized by its incomplete penetrance with gender bias, affecting about 50% of male mutation carriers and about 10% of female carriers.⁴ Nearly 95% of LHON cases are caused by one of the three primary mitochondrial DNA (mtDNA) mutations: 3460G > A in MT-*ND1*, 11778G > A in MT-*ND4* or 14484T > C in MT-*ND6* subunit of complex I⁵⁻⁷. Recovery of the eyesight in LHON is minimal and clinical severity may depend on type of mutation. Prognosis of patients with 11778G > A mutation is the worst while 3460G > A and 14484T > C patients display a milder phenotype^{8,9}.

In earlier studies, a mild to moderate reduction in complex I activity, and complex I dependent ATP synthesis was reported in LHON cybrids carrying either of the above three primary mutations¹⁰⁻¹³. However, LHON cybrids displayed severe ATP depletion and underwent apoptosis in galactose medium indicating bioenergetics insufficiency under OXPHOS stress condition^{14,15}. Antioxidant defenses such as glutathione (GSH) content, activities of glutathione reductase (GR), glutathione peroxidase (GPX) or mitochondrial, superoxide dismutase 2 (SOD2) were reduced in LHON cybrids when growing in galactose medium or exposed to hydrogen peroxide (H₂O₂). These evidences show that cells carrying LHON mutation are vulnerable to oxidative and metabolic stress condition¹⁴⁻¹⁶.

Therefore, increased reactive oxygen species (ROS) production and insufficient ATP production due to complex I defect have been regarded as major contributing factors to RGCs death in LHON. Like other neurons, RGCs demand the highest energy supply for firing action potential along the axon, especially in unmyelinated part¹⁷. Competent bioenergetics, along with functional antioxidant system are important to supply adequate ATP as well as prevent oxidative damage to mitochondrial DNAs, lipids and proteins under such conditions.

Compensatory increase in mitochondrial biogenesis of unaffected LHON carriers from European populations was proposed as an underlying factor for incomplete penetrance in 3460G > A and 11778G > A^{18,19}. Additionally, mtDNA haplogroup backgrounds,^{20,21} X-linked loci,^{22,23} and potential nuclear modifier gene(s) explored in genome wide studies²⁴⁻²⁶ were reported to influence LHON penetrance though findings varied across populations probably owing to diverse nuclear background in different ethnicities. In addition, environmental factors, such as smoking²⁷ and exposure to certain drugs²⁸⁻³⁰ may also trigger LHON expression.

With advances in high-throughput sequencing technologies, comparison of affected LHON patients and unaffected carriers using omics-wide approaches are of great help to identify disease modifying variants³¹. Monozygotic twins supposedly have identical genetic profiles but discrepancy between disease phenotypes may stem from small errors of DNA replication or variation in epigenetic predisposition^{32,33}. Consequences of genetic or epigenetic variations could eventually reflect in gene expression, thus comparing transcriptomics of such discordant monozygotic twins will provide invaluable clues to exploration of disease modifying gene(s), associated signaling pathways and regulatory biological processes. We exploited an oxidative and metabolic insult culture in order to demonstrate a difference in cellular phenotype in 14484T > C mutation carrying monozygotic twins, one

unaffected and one affected by LHON. We then created transcriptomic profiles using next-generation sequencing (NGS) based RNA sequencing (RNA-Seq) on the Illumina platform. Differentially expressed genes were identified and explored to find potentially interplaying signaling pathways and biological processes, which might be beneficial in understanding LHON pathogenesis and application for therapeutic interventions.

Results

Effect of hydrogen peroxide or galactose medium treatment on wild type and 14484T > C mitochondrial mutation carrying fibroblasts

Primary dermal fibroblasts from normal, unaffected LHON and affected LHON individuals were used to determine cellular phenotype. Treatment of hydrogen peroxide in affected LHON fibroblasts lead to decreased survival compared to normal fibroblasts (Fig. 1A and B). Increased production of reactive oxygen species (ROS) with decreased ATP production were found in H₂O₂ treated affected LHON fibroblasts compared to unaffected LHON fibroblasts (Fig. 1C and F). We subsequently treated all three fibroblasts with galactose medium; however, no differences in cell survival and ROS production were found (Fig. 1B and D). However, both unaffected and affected LHON fibroblasts significantly produced less ATP compared to normal fibroblast when cultured in galactose medium (Fig. 1E). Interestingly, combination of hydrogen peroxide and galactose medium treatment on affected LHON fibroblasts led to remarkably reduced survival compared to unaffected LHON and normal fibroblasts, and this finding was consistent with significant increase in ROS production compared to either unaffected LHON or wild type fibroblasts (Fig. 1B and D).

Combination of hydrogen peroxide and galactose medium treatment on affected LHON fibroblasts led to reduced Sirtuin 3 (SIRT3) expression and its functionality on superoxide dismutase 2 (SOD2)

Lower expression of SOD2 in affected LHON fibroblast was noted among culture of three fibroblasts with basal cultured condition (Fig. 2A and B). Treatment with either H₂O₂ or galactose medium on affected LHON fibroblasts led to reduced SOD2 expression in affected group (Fig. 2A and B). Combination of hydrogen peroxide and galactose medium treatment on affected LHON fibroblasts further reduced the expression of SOD2 expression compared to either hydrogen peroxide or galactose medium insult; however, a substantial surge of an inactive SOD2 form (lysine acetylation on the 68th amino acid of SOD2) was observed (Fig. 2C). As this Lysine acetylation of SOD2 is known to be regulated by Sirtuin 3 enzyme (SIRT3), we investigated the expression of SIRT3 and found that SIRT3 was marginally reduced in affected LHON fibroblasts treated with combination of the two insults (Fig. 3A and B).

RNA-Seq of monozygotic twins with 14484T > C LHON mutation

Total RNA extracted from unaffected and affected LHON fibroblasts with four different conditions, i.e. untreated, H₂O₂, galactose and a combination of both, were included in the RNAseq study. There were 40,112 transcripts detected from differential expression analysis, as shown in Fig. 4A and B. Five hundred and seventy-seven transcripts were upregulated, while 120 transcripts were downregulated in unaffected LHON fibroblast. Four gene ontologies, relating to negative regulator controlling cell death, were enriched in this gene list at the FDR < 0.05 (Fig. 4C and D).

Utilisation of whole genome sequencing and whole transcriptome sequencing of 14484T > C mutation carrying fibroblasts to identify a potential discordant SNPs causing disease phenotype

From DNA samples obtained from buffy coat, we identified 4,913,254 SNVs and small indels presented in unaffected carrier and 4,868,543 SNPs and small indels in affected LHON. In total, there were 263,668 variants present only in unaffected carrier and 219,957 variants present only in affected LHON.

The top twenty differentially expressed gene transcripts, based on adjusted *p*-value, were used to identify discordant SNPs from whole genome sequencing data of the twins. Eighty-three discordant SNPs were identified on 13 different genes in unaffected LHON fibroblast, while 63 discordant SNPs were identified on 13 different genes in affected LHON fibroblast. (Table 1)

The 146 discordant SNPs were investigated against expression quantitative trait loci database, but only four SNPs were present in the database with normalised effect size information (Table 1). Among these four SNPs, three were present in unaffected LHON while one SNP was present in affected LHON fibroblast (Table 2). However, the effect size of only two SNPs, i.e. rs75523942 in unaffected LHON and rs496483 in affected LHON, were in keeping with expression data from RNAseq, and those two were located on prostaglandin E receptor 3 (*PTGER3*).

Discussion

Culturing cells with galactose medium forces cells to rely on solely OXPHOS bioenergetics, thereby allowing us to exaggerate cellular phenotypes in diseases with mitochondrial dysfunction³⁴. We found that cell viability of 14484T > C mitochondrial mutation carrying LHON affected patient fibroblast was significantly lower than that of unaffected carrier in galactose culture supplemented with hydrogen peroxide. This combination of metabolic insult induced significantly higher level of ROS in affected LHON. This finding was in keeping with the previous report of mitochondrial mutations induced cybrids cells, in which viability of LHON cybrids were significantly reduced as the cells underwent apoptosis in galactose culture after 48 hours of incubation¹⁵. Increased cellular ROS level along with reduction in cell viability of affected patient fibroblasts in galactose culture under oxidative stress prompted us to determine functionality of the antioxidant system.

We looked into protein expression of the antioxidant enzyme mitochondrial Manganese superoxide dismutase 2 (SOD2) and also lysine (K)-68 acetylated SOD2, an inactive dismutase form. This K68-AcSOD2 was also previously reported to sensitise cells to peroxide stress³⁵. Our results indicated that

LHON affected fibroblasts expressed lower level of total SOD2 and that either peroxide, galactose medium or a combination aggravated reduced level of SOD2 expression, while combination of metabolic insults promoted expression of K68-AcSOD2 form, exclusively in affected LHON fibroblast. These findings suggested that aberrant regulation of acetylation switch on SOD2 may occur.

Sirtuin3 (SIRT3) is key regulator of mitochondrial protein acetylation, to curb production of mitochondrial ROS. Several studies showed that SIRT3 reduces ROS levels in order to prevent oxidative stress induced neuronal cell death via increasing activities of antioxidant enzymes activities^{36–39}. However, expression of SIRT3 protein was lower in affected, albeit a marginal change. SIRT3 depends on nicotinamide adenine dinucleotide (NAD⁺) metabolite to exert its activity. In diseases with mitochondrial dysfunction, conversion of NADH to NAD⁺ is defective, and it can potentially mitigate activity of NAD⁺ dependent enzyme. In fact, repletion of NAD⁺ through nicotinamide riboside (NR) supplement can improve mitochondrial biogenesis in mice carrying a dominant pathogenic mutation in the major mitochondrial DNA⁴⁰.

Our RNA sequencing data demonstrated that quinolate phosphoribosyl transferase (qPRT) gene transcript was significantly higher in unaffected LHON with adjusted p-value of 0.0071, and this may perhaps indicate that unaffected LHON fibroblasts had a better response to replete the pool of NAD⁺ compared to affected LHON fibroblasts. However, the discordant SNPs located in *SOD2*, *STAT3* and *QPRT* were not associated with eQTL in the GTEx database (<https://gtexportal.org>)⁴¹.

Differential expression between unaffected and affected LHON fibroblasts yielded an up and down regulated group of genes enriched on cell death and negative regulator of apoptosis. We subsequently utilised the top twenty of the differentially expressed gene transcript list to search for discordant SNPs as a mean to identify a potential cause of disease phenotype.

Our whole genome sequencing data from twins indicates that discordant SNPs located on *CLIC6* and *PTGER3* were associated with its expression in GTEx database. *CLIC6* is a non-classical ion channel that is structurally homologous to the Glutathione S- transferases Superfamily⁴², and was reported to be highly expressed in human retina⁴³. The discordant SNP of *CLIC6* present in unaffected twin is negatively associated with its expression. Expression of receptor for prostaglandin E receptor (*PTGER3*) was found to be affected by two SNPs. Unaffected LHON fibroblast possessed rs75523942 that indicates a positive effect on *PTGER3* expression, while affected LHON fibroblast possessed rs496483 that indicates a negative effect on *PTGER3* expression. *PTGER3* is a prostanoid receptor for a lipidic molecule, so called prostaglandin E2 (PGE₂)⁴⁴. Knockdown of *PTGER3* led to apoptosis, while overexpression of *PTGER3* protected myocardium against ischemic reperfusion injury⁴⁵, in which ROS play pivotal role in pathomechanism^{46,47}.

Conclusions

In conclusion, we utilised the preconditioned stress culture to differentiate cellular phenotype of patient with mitochondrial disease from the carrier. Discrepancy of the disease phenotype possibly stem from antioxidant mechanism. Cellular capability of the cells to replete pool of NAD⁺ may also contribute to this cellular phenotype.

Methods

Sample collection

Dermal fibroblasts were cultured from a pair of monozygotic twins carrying homoplasmic 14484T > C mutation, and one control individual. One of these twins is an affected LHON patient while the other one does not have a clinical phenotype. The twins were examined by a neuro-ophthalmologist and the control were an independent individual who visited Siriraj Hospital, Thailand for reasons unrelated to eye disease or any other chronic disease conditions. Determination of LHON mutations were done using Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique according to Sudoyo *et al.*⁴⁸. Informed consent was obtained for all the samples recruited and the study was designed to the adherence of Declaration of Helsinki and approved by Siriraj Institutional Review Board (COA: si710/2015), Mahidol University, Bangkok, Thailand.

Primary fibroblast culture

Fibroblast samples were proliferated in high glucose Dulbecco's Modified Eagle Medium (12800-058, Gibco, Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100U/ml), streptomycin (100ug/ml) and amphotericin B (5ug/ml), and the fibroblasts were cultured in humidified incubator at 37°C with 5% CO₂ supply. Passage number of fibroblasts used for all experiments were between 4–10 in order to avoid aging phenotype. For basal and galactose medium culture condition, glucose free DMEM (D5030, Sigma Aldrich) was supplemented with either 25mM of glucose or galactose, with supplements used in medium for proliferation. Galactose preconditioning was incubated for 48 hours prior to sample collection. For oxidative stress treatment, fibroblasts were treated with 100uM of H₂O₂ for 90 minutes prior to sample collection.

Determination of cell viability

Cell viability was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole) assay (Sigma Aldrich) according to manufacturer's protocol. Briefly, fibroblasts were seeded in triplicate, and cultured in preconditioned high glucose DMEM or 25mM galactose media for 48 hours. The medium was replaced by fresh medium supplemented with and without H₂O₂ treatment for 90 minutes. Number of viable cells was determined by Synergy H1 Hybrid Multiplate Reader (BioTek) at the 570nm absorbance. Three independent experiments were performed. Percentage of cell viability was expressed in a bar graph format.

Determination of cellular ROS content

Cellular ROS content was determined using hydroethidine (aka. dihydroethidium or DHE) assay (Invitrogen) according to manufacturer's protocol. Briefly, fibroblasts were seeded and treated with different culture condition in the same fashion as the treatment protocol for cell viability assay. Fibroblasts were subsequently stained with 10 μ M of DHE for 30 min at 37°C in the dark. DHE oxidation was determined by Synergy H1 Hybrid Multiplate Reader (BioTek) at the excitation/emission of 518nm/606nm. Cellular ROS content is expressed as percentage of the DHE oxidation of untreated controls in glucose or galactose medium respectively.

Determination of whole cell ATP content

Whole cell ATP content was determined by using ATP bioluminescence assay kit (Sigma) according to manufacturer's protocol. Fibroblasts were seeded in precondition of either high glucose DMEM or 25mM galactose media for 48hours. The medium was replaced by fresh medium supplemented with/without H₂O₂ treatment for 90 minutes. Fibroblasts were washed in cold PBS for twice, ATP was extracted in 0.1% TritonX-100 ATP extraction buffer, as described by Koszegi *et al.*⁴⁹. Supernatant were kept on ice, stored at -20°C and used for quantification of ATP and protein content. Protein content was determined by Bradford assay (Bio-Rad). Whole cell ATP level was expressed by μ mol/mg protein and percentage of ATP content in galactose was expressed by ratio of whole cell ATP level in galactose medium to that of glucose medium. Percentage of ATP content under oxidative stress was expressed by ratio of whole cell ATP level in H₂O₂ exposed cells to that of untreated cells.

Whole cell protein extraction and western blotting

Fibroblasts cultured in their respective experimental conditions were trypsinised and washed in PBS. Cell pellets were dissociated in RIPA lysis buffer with freshly added protease inhibitors (Sigma) and 1mM of phenylmethylsulfonyl fluoride (PMSF). Extracted protein concentration were measured using Bradford's method and stored at -80°C until use. Whole cell protein extracts were resolved in 10% SDS-PAGE. The PVDF membrane was blocked in 5% skimmed milk in 0.1% Tween-PBS (PBST) buffer for 1 hour to reduce non-specific binding and the blotted membranes were incubated with respective primary antibodies at 4°C overnight. The membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibody Chemiluminescence HRP solution (Millipore) was applied to visualize target protein bands on the Image Quant LAS 4000 (GE healthcare). Band intensities of proteins were determined using Image J software (NCBI). Primary and secondary antibodies used were SIRT3 (Cell Signaling Technologies); β -actin (Sigma); SOD2, Lys-68 acetylated SOD2, goat anti-rabbit and anti-mouse IgG HRP antibodies (Abcam).

RNA extraction and transcriptome profiling

Total RNA was extracted from fibroblasts grown in basal glucose culture, and culture with hydrogen peroxide, galactose or combination using RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. RNA Sequencing (RNA-Seq) was performed by Illumina using next generation sequencing. FASTQ files were uploaded to a web-based RNA-seq analysis pipeline by Basepair (<https://www.basepairtech.com/>).

Short-read data were aligned to the reference genome from UCSC (GRCh37) using STAR aligner⁵⁰ and differential counts obtained from HT-seq count were determined by DESeq2⁵¹. List of differentially expressed genes from twins with different conditions were pre-ranked based on p-value and log-2 fold change prior to Gene set enrichment analysis (GSEA). GSEA was performed using GSEA software v 4.0.3⁵².

DNA extraction and Whole Genome Sequencing (WGS)

DNA was extracted from whole blood samples and sent for WGS on Illumina's HiSeq-X platform (Macrogen Inc, Korea). The average depth of coverage was 42.2 (90.8% of the genome > 30x). GATKv4-0-3 best-practice workflow⁵³ was used for data processing and variant discovery. Structural variants were checked using Lumpy. We used Variant Effect Predictor (ensembl-VEP version 90.1)⁵⁴ to annotate these discovered variants. The data was analyzed on Firecloud-Terra (<https://firecloud.terra.bio/>) using GRCh38 for alignment and dbNSFP.v3.5a⁵⁵ as the main annotation resource. First, all high impact variants as predicted from VEP's gene model with known clinical significance from ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) were retained. Then, we kept the moderate impact variants predicted to be deleterious by metaSVM⁵⁶ and Combined Annotation Dependent Depletion (CADD)⁵⁷ score > 15. Furthermore, we kept only variants with minor allele frequency (MAF) under 1% from GnomAD⁵⁸, 1000 Genomes⁵⁹, ESP6500, and ExAC databases⁶⁰.

Expression quantitative trait loci (eQTL)

Transcripts, which were upregulated in unaffected LHON fibroblasts were listed based on adjusted p-value. Top twenty transcripts were checked against whole genome sequencing data of unaffected and affected LHON samples to identify discordant SNPs. Discordant SNPs in either unaffected or affected LHON fibroblasts were subsequently checked for their expression quantitative trait loci (eQTL) on the GTEx database⁴¹.

Statistical analysis

Data obtained from different experiments among study groups were compared between 2 groups by two-tailed Student's t-test or Wilcoxon Rank-Sum Test; among study groups by Kruskal Wallis Test or one-way ANOVA test followed by post-hoc Tukey's Test.

Abbreviations

Leber's hereditary optic neuropathy (LHON), Reactive oxygen species (ROS), expression quantitative trait loci (eQTL), whole genome sequencing (WGS), Adenosine triphosphate (ATP)

Declarations

Ethical approval and consent to participate

Informed consent was obtained for all the samples recruited and the study was designed to the adherence of Declaration of Helsinki and approved by Siriraj Institutional Review Board (COA: si710/2015), Mahidol University, Bangkok, Thailand.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Authors have declared no conflict of interest.

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Authors' contributions

KZT did experiment and data analysis. JT did experiment and data analysis. SK did characterize mitochondrial DNA mutation. HG did whole genome sequencing and transcriptomic data analysis. SR did transcriptomic analysis. WS critically read manuscript. WC diagnosed LHON patients, collected dermal fibroblasts. PL critically read manuscript. BS planned experiment, analysed data and wrote manuscript. CM planned experiment, analysed data and wrote manuscript.

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Tables

Due to technical limitations, table 1, 2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Bar graph demonstrates cell viability (A and B), level of ROS (C and D) or ATP content (E and F) of normal, unaffected LHON and affected LHON fibroblasts, which were treated with galactose, hydrogen peroxide or combination of both metabolic insults.

Figure 2

Western blot analysis of SOD2, acetylated SOD2 at the 68th amino acid of lysine (AcK68-SOD2) and β -actin (A) on protein extract from normal, unaffected LHON and affected LHON fibroblasts, which were treated with galactose, hydrogen peroxide or combination of both metabolic insults. Densitometric analysis of the protein band of SOD2 that was normalized against β -actin (B) and AcK68-SOD2 that was normalized against total SOD2 (C).

Figure 3

Western blot analysis of SIRT3 and β -actin (A) on protein extract from normal, unaffected LHON and affected LHON fibroblasts, which were treated with galactose, hydrogen peroxide or combination of both metabolic insults. Densitometric analysis of the protein band of SIRT3 (B) that was normalized against β -actin.

Figure 4

Volcano plot (A) and Heatmap (B) demonstrates a differential expression of gene transcripts between unaffected and affected fibroblasts. Four Gene Ontology pathways were enriched at the false discovery rate <0.05. (C and D)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1andTable2.pdf](#)