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Analysis of Epigenetic Modification in Leber's Hereditary Optic Neuropathy (LHON) Cells

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ABSTRACT Leber's Hereditary Optic Neuropathy (LHON) is a mitochondrially inherited vision loss disease due to pathogenic mitochondrial mutations in complex 1 encoding genes which are incurable to date. Mitochondrial respiratory chain complex 1 consists of 45 subunits and *NDUFS4* is a nuclear-encoded accessory submit which has a significant role in mitochondrial complex 1 assembly. This preliminary study focuses on the histone modification changes in the *NDUFS4* gene. For this study chromatin immunoprecipitation (ChIP) assay was performed using LHON *ND4* mutant cell lines and PBMC from a healthy control. Five histone modification antibodies such as $H_3K_{18}Ac$, $H_3K_{27}Ac$, $H_3K_9Me_2$, $H_3K_4Me_3$, and $H_3K_{27}Me_3$ were used for the experiment. ChIP-qPCR was performed to determine the histone enrichment in the *NDUFS4* promoter region. ChIP-qPCR data showed that H3K18Ac histone enrichment has variation in LHON *ND4* mutant cells compared to PBMCs. As a primary step, this study has tried to figure out the histone modification changes in the *NDUFS4* gene in the LHON cells derived from *ND4* mutant patients and control PBMCs. Epigenetic studies in nuclear-encoded mitochondrial proteins in LHON may help researchers for a better understanding of disease pathology.

INTRODUCTION

Epigenetics means alterations in the environment and behaviour that affect the functioning of genes without literally modifying the DNA sequence. Simply, changes in the phenotype of an individual having an intact genotype. Despite possessing an unmodified genotype, epigenetic alterations are capable of changing the way the DNA sequences are read. Studies suggest reversibility in these alterations though they can potentially be inherited (Sun et al. 2013; Park et al. 2019). DNA methylation, histone modification and non-coding RNAs are epigenetic modifications that can induce alteration in gene expression. Methylation modifications are the addition of methyl groups to specific sites of DNA which generally turns off the gene while histone modifications mean the incorporation of various groups mainly methyl and acetyl groups to various amino acids of the histone core that determine DNA stability and control gene expression. Methylation and acetylation modification of histones activate or repress the gene expression according to the site of modification (Sun et al. 2013). studies related to epigenetic roles in disease progression are on the rise in various diseases. Epigenetic studies based on cancer (Jung et al. 2020; Zhao et al. 2019), type II diabetes (Ling and Rönn 2019), fatty liver (Stevanovic et al. 2020) and eye diseases (Mohana et al. 2021; Lanza et al. 2019) are burgeoning as they are responsible for most of the functional protein alterations. Despite surging epigenetic studies in familiar and recurrent diseases, there are a few rare yet lethal diseases which should be probed from the epigenetic point of view as they could aid in diagnostic or therapeutic means (Jha et al. 2021; Lopes 2020).

Leber's Hereditary Optic Neuropathy (LHON) is one such rare, mitochondrial, gender-biased, retinal ganglion cells (RGCs) degenerative eye disorder, which is manifested by vision loss (Hage and Vignal-Clermont 2021). Mainly three primary mitochondrial mutations (*MT-ND1, MT-ND4 and MT-ND6*) which encoded for subunits

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of complex 1 are responsible for the pathogenicity of LHON. The reason for the loss of RGCs is proposed to be the deprivation in ATP synthesis (Ito and di Polo 2017) which is normally produced in mitochondria by oxidative phosphorylation of nutrients. Oxidative phosphorylation relies on several enzyme complexes (complex I-IV) which are encoded by both mitochondrial and nuclear genomes. Respiratory complex 1 is the largest protein complex of the respiratory chain which consists of 45 subunits and the majority of them are encoded by nuclear genes. *NDUFS4* (NADH: Ubiquinone Oxidoreductase Subunit S4) is an essential accessory subunit for the respiratory complex1 assembly and function. The first nuclear-encoded pathogenic mutation in complex 1 was identified in the NDUFS4 subunit (Van et al. 1998). Mutations in the *NDUFS4* gene cause mitochondrial complex 1 deficiency such as Leigh syndrome (Hage and Vignal-Clermont 2021). In recent decades studies have discussed the effect of epigenetic alterations in the progression of neurodegenerative diseases (Mohana et al. 2021b). Studies have reported the involvement of environmental factors is inevitable in the progression of LHON. Environmental factors interact with genetic makeup which develops epigenetic variations for disease development. Epigenomic profiling offers perceptions of variations in epigenetic modifications in neurodegenerative diseases. Since LHON is a neurodegenerative disease and is caused by complex 1 dysfunction, this study was designed to experimentally decipher the histone modification variations in the *NDUFS4* gene in LHON.

Objectives

To study the histone modification changes in the *NDUFS4* gene in LHON mutant cells compared to control cells.

MATERIAL AND METHODS

Isolation of PBMCs

4 ml of blood were collected from individuals in EDTA tubes. Density gradient centrifugation was performed with an equal volume of Ficoll and blood at 1200 rpm for 30 min without break. After centrifugation, differential layers were formed and PBMCs were collected from the buffy coat layer which was characteristically white and cloudy. The Buffy coat layer was gently removed using a pipette and added the cells to PBS. Cells were repeatedly washed with PBS to completely remove erythrocytes and platelets. Written informed consent was obtained from the volunteers before collecting the blood. This study was approved by Institutional Review Board (IRB) and ethics committee and all the procedures were performed in accordance with institutional guidelines and the Declaration of Helsinki.

LHON *ND4* Mutant Fibroblast Cell Culture

LHON *ND4* mutant dermal fibroblast cells were cultured using 4.5g/ml high glucose DMEM with 10 percent FBS, 1 percent L-glutamine,1 percent non-essential amino acid and antibiotics in 5 percent CO_2 at 37^0C. Cells were passaged at the stage of 70 percent-80 percent confluency.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assay was performed with PBMCs of the control sample and LHON patient-derived *ND4* mutant cell lines using SimpleChIP® Sonication Chromatin IP Kit from Cell Signalling Technology (MA, US). LHON mutant cell confluency percentages were estimated at the time of cell harvest and 4×10^5 LHON mutant cells were used for the experiment. For the chromatin preparation from PBMC and cell lines, 37 percent formaldehyde was used to crosslink cells for 10 min at 37^0C. The crosslinking reaction was quenched by the addition of 10X glycine. Cells were lysed and chromatin was fragmented using the sonication method. Fragmented chromatins were subjected to immunoprecipitation using 5 different antibodies at various dilutions (Table 1) against specific histone modifications such as Histone 3 lysine 18 acetylation ($H_3K_{18}Ac$), Histone 3 lysine 27 acetylation ($H_3K_{27}Ac$), Histone 3 lysine9 di-methylation ($H_3K_9Me_2$), Histone 3 lysine 4 trimethylation ($H_3K_4Me_3$), and Histone 3 lysine 27 trimethylation ($H_3K_{27}Me_3$). Immunoprecipitated regions were pulled down using protein G magnetic beads and eluted the chromatin was followed by reversal of the crosslinking using NaCl and proteinase K.

Table 1: Antibody dilutions against different histone modifications for immunoprecipitation

Antibody	*Dilution concentration*
Acetyl-Histone H3 (Lys18) H3K18AC	1: 25
Acetyl-Histone H3 (Lys27) H3K27AC	1:100
Di-Methyl-Histone H3 (Lys9) H3K9ME2	1: 25
Tri-Methyl-Histone H3 (Lys4) H3K4ME3	1: 50
Tri-Methyl-Histone H3 (Lys27) H3K27ME3	1: 50

Dilution concentration of monoclonal antibodies used against the activation and repressive markers of histone modifications

Quantification of Histone Modification

DNA was purified from the eluted chromatin and qPCR was performed using Bio-Rad CFX Real-Time PCR system. The PCR reaction system contains 10 µl of SYBR Green Simple ChIP qPCR RT PCR mix (Cell Signalling Technology, US), 5 µM of forward and reverse primer of NDUFS4 gene and 2 µL of PCR template, with a total volume of 20 µL. PCR reaction program fixed as 95 °C for 3 min; 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. Immunoprecipitation efficiency is determined using the percent input method. Each immunoprecipitation signal is expressed as a percent of the total input sample in this method. The Ct value of 2 percent input was used to normalize the experimental sample.

RESULTS

Culturing of *ND4* Mutant LHON Cell Line

Skin-derived *ND4* mutant fibroblast cells from the LHON patient were grown in DMEM (4.5g) high glucose medium with 10 percent FBS, L-glutamine, non-essential amino acids and antibodies. 70 percent-80 percent of cell confluency reached around 5-7 days (Fig. 1). On day 7, cells fully attained their fibroblast morphology and expressed their cell surface markers.

Identification of Histone Modification Changes in *NDUFS4* Gene in *ND4* Mutant LHON Cell Lines

The researchers assessed levels of H3K4Me3, H3K18Ac, H3K27Ac, H3K9Me2 and H3K27Me3 histone modifications across the *NDUFS4* gene in the *ND4* mutant LHON cell lines and control PBMCs using the qRT-PCR assay (Fig. 2). The assessment showed a nominal increase in the level of H3K18Ac. The researchers observed no significant difference in H3K27Me3, H3K4me3, H3K27Ac, and H3K9Me2. The histone modifications of H3K27Ac, H3K9Me2, and H3K27Me3 in

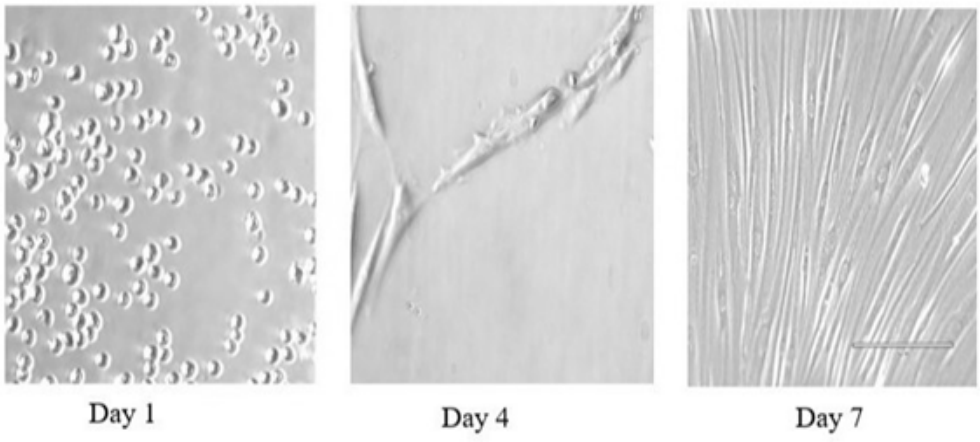

Fig. 1. Phase contrast microscopy imaging of ND4 mutant LHON cell lines at days 1, 4 and 7. The images were taken in magnifications 10X and 20X. At day 7 the cells are completely attached and attained their complete morphology

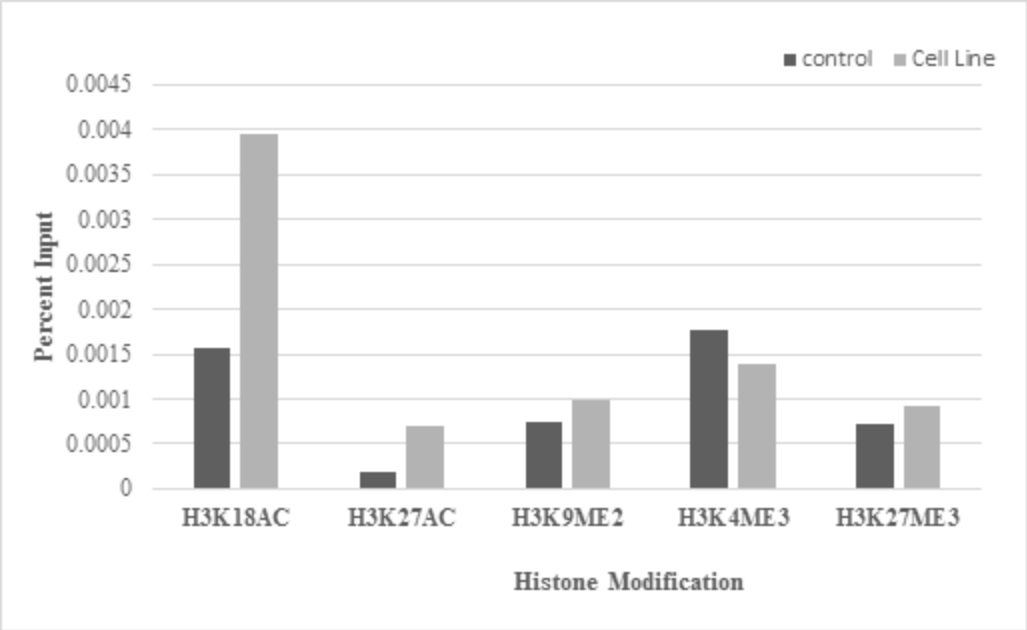

Fig. 2. qRT-PCR analysis of histone modifications in NDUFS4 gene in LHON mutant cell lines and PBMCs. The activation marker H3K18Ac shows nominal increase in LHON mutants when compared to PBMCs. There is no significant difference in other modifications

LHON mutant cell lines show a slight increase in their expression when compared to PBMCs whereas in H3K4Me3 modification in PBMCs shows increased expression. It is likely that histone modification differences in the *NDUFS4* gene would have functional implications on respiratory complex 1 thereby playing a key role in LHON pathogenesis.

DISCUSSION

Most of the mitochondrial proteins are nuclear encoding and epigenetic modifications are associated with the nuclear and mitochondrial genome regulation. Other than mt DNA mutations, these epigenetic modifications of nuclear genes are closely related to mitochondrial dysfunction and disease (Kahlhöfer et al. 2021; Smiraglia et al. 2008). The epigenetic modifications that affect the gene expression and epigenetic regulation of mitochondrial DNA by nuclear-encoded proteins are still uncertain. *NDUFS4* has a key role in mitochondrial complex1 assembly and it is considered a hot spot for pathologic mutations in the neurodegenerative disease called Leigh syndrome. Loss of function of *NDUFS4* causes detrimental complex1 dysfunction and this dysfunction could cause several neurodegenerative diseases (Kahlhöfer et al. 2021). Since LHON is a neurodegenerative disease, identification of epigenetic modification alterations in the *NDUFS4* gene provides a better understanding of the disease mechanism. The mechanism of disease expression in the case of LHON is still unclear. In addition to primary mutation, secondary mutations and epigenetic or environmental risk factors would have an impact on the pathogenesis of LHON (Mohana et al. 2021). The relative role of all potential factors like mt-DNA, nuclear DNA, and various epigenetic factors involved in developing visual loss has to be clearly identified.

In this immunoprecipitation study, the researchers observed significant enrichment of $H_3K_{18}Ac$ histone modification in LHON *ND4* mutant cell lines compared to control PBMCs.

Other modifications also have variations among these two groups. $H_3K_{18}Ac$ is an activation marker for gene expression and it might help for the stable gene expression of *NDUFS4* for proper complex 1 function. Since the complex 1 *ND4* subunit is mutated in LHON, these epigenetic modifications might endeavour consistent gene regulation of other nuclear-encoded complexes 1 subunit. Epigenetic mechanisms and regulations of nuclear genes in LHON have not yet been explored. This is a preliminary study to identify a few histone modification changes in the *NDUFS4* gene in LHON. More studies are required to validate the results and understanding of epigenetic regulation mechanisms in LHON.

CONCLUSION

Histone enrichment changes are observed in the *NDUFS4* gene in comparison to LHON *ND4* mutant cells and control PBMCs. This is the preliminary study on the identification of histone modifications of *NDUFS4* in LHON. An obvious histone enrichment difference has been observed in H_3K_{18} Acetylation modification on LHON mutant cells. More validation studies have to be conducted to find out the functional implications of epigenetic modifications in LHON.

RECOMMENDATIONS

This data has to be validated with more control samples. LHON patient PBMCs also can use for comparing the histone modification changes in the *NDUFS4* gene.

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AUTHORS CONTRIBUTION

APN and SM outlined and drafted the manuscript, SM reviewed the manuscript.

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ETHICS APPROVAL

This study was approved by Institutional Review Board (IRB) and ethics committee and all the procedures were performed in accordance with institutional guidelines and the Declaration of Helsinki.

CONSENT TO PARTICIPATE

Written informed consent was obtained from individual participants included in the study.

DECLARATION OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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