การแสดงออกของโปรตีน Heat Shock 60 (Hsp60) ในไมโทคอนเดรียของผู้ป่วยโรค Leber Hereditary Optic Neuropathy ชาวไทย

The Expression of Mitochondrial Heat Shock Protein 60 (Hsp60) in Thai Leber Hereditary Optic Neuropathy Patients

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บทคัดย่อ

โรค Leber Hereditary Optic Neuropathy (LHON) เป็นโรคไมโทคอนเดรียที่พบบ่อยผู้ป่วยจะมีอาการตาบอดทั้ง 2 ข้าง อาจจะพร้อมกันหรือไม่พร้อมกันก็ได้ มักจะพบในผู้ป่วยชายและเริ่มเป็นเมื่ออายุเข้าสู่วัยรุ่น โรค LHON นี้เกิดจากการกลายพันธุ์ของยีนไมโทคอนเดรียที่มักพบที่ตำแหน่ง G3460A หรือ G11778A หรือ T14484C อย่างไรก็ดี การกลายพันธุ์ของยีนไมโทคอนเดรียดังกล่าวไม่ครอบคลุมลักษณะปรากฏบางอย่างของโรค เชื่อกันว่ายีนนิวเคลียสมีผลต่อลักษณะปรากฏของโรคนี้ ผลการศึกษาพบว่าโปรตีนในไมโทคอนเดรียที่ถูกถอดรหัสจากนิวเคลียสจำนวน 29 ตัว มีการแสดงออกแตกต่างกันในระหว่างกลุ่มผู้ป่วยและญาติผู้ป่วยที่มีการกลายพันธ์ของยีนไมโทคอนเดรียแต่ไม่มีอาการ นการศึกษานี้คณะผู้วิจัยทำการศึกษาโปรตีน heat shock 60 (Hsp60) ซึ่งเป็นโปรตีนในไมโทคอนเดรียและเป็นโปรตีนแชพเพอรอนที่สำคัญ จากการศึกษาวิจัยพบว่าปริมาณของโปรตีนชนิดนี้ลดลงอย่างมีนัยสำคัญในเซลล์ไฟโบรบลาสต์ของผู้ป่วย LHON ที่มีการกลายที่ตำแหน่ง G11778A เมื่อเปรียบเทียบกับกลุ่มญาติในครอบครัวผู้ป่วยที่มีการกลาย G11778A แต่ไม่ปรากฏอาการ และกลุ่มควบคุม การลดลงของปริมาณโปรตีน Hsp60 ในไฟโบรบลาสต์เซลล์ของผู้ป่วย LHON นี้อาจแสดงถึงความผิดปกติในการหมุนเวียนของโปรตีนที่ใช้ในไมโทคอนเดรียซึ่งอาจมีผลต่อการก่อโรค LHON

ABSTRACT

Leber Hereditary Optic Neuropathy (LHON) is one of the most common mitochondrial diseases that can cause complete blindness, particularly in young men. The majority of LHON cases are caused by one of the three prevalent mitochondrial DNA (mtDNA) mutations - G3460A/ND1, G11778A/ND4, and T14484C/ND6. However these mutations cannot explain the several remarkable features of LHON, therefore, the nuclear factors is suggested to be involved in the phenotypic expression of LHON. Our Previous findings demonstrated 29 nuclear-encoded mitochondrial proteins with altered expression pattern between affected and unaffected groups among Thai LHON patients. In the present study, heat shock protein 60 (Hsp60) a vital mitochondrial chaperone was investigated and found to be significantly down regulated in 11778G>A LHON affected fibroblast compared to the fibroblasts from either control individuals or from unaffected relatives. Therefore, the reduced expression level of Hsp60 in the fibroblast of affected LHON groups may suggest the defect in the protein turnover of the mitochondria, which might contribute to the pathogenesis of LHON.

คำสำคัญ: LHON, ไมโตคอนเดรียโปรตีน, Hsp60

Keywords: LHON, mitochondrial protein, Hsp60

INTRODUCTION

Leber's hereditary optic neuropathy (LHON, OMIM 535000) is a maternally inherited mitochondrial genetic disease which can cause the degeneration of retinal ganglion cells and the visual loss (1-2). The age of onset of the disease is between 15 to 35 years but the individuals can be affected at any age ranging 8 to 60 years (2). LHON is caused by the mutation in mitochondrial genome. Three most common mitochondrial DNA (mtDNA) mutations are found at nucleotide position 3460, 11778 and 14484 causing the amino acid substitution in NADH dehydrogenase (ND)-1, ND-4 and ND-6 subunits of complex I of the respiratory chain (3). Although the LHON mutation is necessary to cause the disease, it can not explain some phenotypic expression of LHON, such as male preference, incomplete penetrance and larger age range of onset (3). This complexity suggested the involvement of the mitochondrial and/or nuclear genetic background, and/or the environmental factors (3).

Several studies have been done to gain a better understanding in the pathogenesis of LHON, particular about nuclear modifiers (4-5). From our previous study, the differential mitochondrial proteomic profiles of LHON-affected, unaffected and control cases were investigated (6) and showed the possible involvement of 29 proteins in the pathophysiology of LHON. Majority of them were found with altered expression levels between unaffected, affected and control groups. Out of which, heat shock protein (Hsp) 60 was observed to be significantly altered in the affected LHON patients as compared to the controls, suggesting its possible functional involvement in the pathogenesis of LHON. Hsp60, encoded by HSPD1 gene, is a nuclear encoded mitochondrial chaperone located inside the mitochondrial matrix, which assists polypeptide folding and refolding of the denatured proteins in the cells in an ATP dependent manner. The present study aimed to analyse the mitochondrial expression level of Hsp60 protein in order to assess its contribution to LHON phenotypic expression.

MATERIALS AND METHODS

1. Samples and study design

Samples used in this study were the cultured skin fibroblast taken from the skin biopsy of the individuals belonging to the affected LHON and the unaffected LHON from 3 families and the control cases. Three affected LHON individuals and 3 unaffected individuals from these 3 families were selected. Affected individuals were defined by ophthalmologist according to clinical phenotypes such as pale optical disc and total loss of colour vision at the age of onset. The control samples were taken from unrelated normal subjects with no history of eye diseases. The conduction of this study was approved by the Ethics Committee of the Mahidol University, Faculty of Medicine Siriraj Hospital (Ethical clearance number 161/2551)

2. Cell Culture

The fibroblast cells were cultivated at 37oC under 5% CO2 atmosphere in complete Dulbecco's modified Eagles' medium containing 12% heat-inactivated fetal bovine serum, amphotericin B (1 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), uridine (50 µg/ml).

3. Mitochondrial protein extraction

Differential centrifugation method was used to isolate the mitochondria from fibroblasts (7). The cultured fibroblast was collected in ice cold PBS, pH 7.4 and sonicated using a probe sonicator. The cells were then centrifuged at 1,000g for 10 minutes to remove the cell debris and the supernatant were collected, followed by centrifugation at 20,000g for 30 minutes. The pellet obtained was washed with the fresh buffer containing 0.25 M sucrose and 10 mM HEPES (pH 7.4) and then centrifuged at 20,000g for 20 minutes. Thereafter the pellet was lysed using laemmli buffer and stored at -80^{o}C until further use.

4. Western blot Analysis

The mitochondrial protein (30 µg) of each samples was loaded and separated on 12% polyacrylamide gel and then electro-transferred to PVDF membrane. Non- specific binding sites in the membrane were blocked with 5% skim milk at room temperature for 1 hr. Then the membrane was probed with specific Hsp60 primary antibody and voltage dependent anion channel (VDAC) primary antibody in 5% skimmed milk for overnight at 4°C. The VDAC protein in the sample was referred as mitochondrial internal loading control. The membrane was incubated with secondary antibody conjugated with horse radish peroxidise in 5% skimmed milk for 2 hr at room temperature. The protein bands were visualized by enhanced chemiluminescence method. The detected band intensities were analysed using Image J software.

5. Statistical Analysis

All the experiments in this study were conducted at least three times. The band intensities values of Hsp60 protein were measured in percentage for each three group and were normalized with the band intensity values of VDAC. The unpaired two-tailed non-parametric t-test (with Welch-correction) was used to determine a significant difference in the mean band intensity value among either of two groups. *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

In the present study, the expression of Hsp60 protein was observed to be significantly down regulated in the fibroblasts derived from LHON-affected patients as compared with that from unaffected and control individuals (Fig 1A and 1B). Hsp60 protein level was observed to be 3 fold lower in affected cells than in control and unaffected cells (Figure 1A and 1B.). No association between the age onset of the affected LHON individuals and the low level of Hsp60 was detected since similar pattern of down regulation in the cells derived from the patients of lower age of onset (9 years) and higher age of onset (36 years) (Fig. 1A, lane A7 and A8) was observed.

Hsp60 is an important chaperone involving in the cellular maintenance and stress response (8). The important role of mitochondrial chaperone, like Hsp60, is increasingly recognized in neurodegenerative diseases (9). For example, it has been demonstrated that the inactivation of Hsp60 expression by an amino acid change in human Hsp60 protein due to the 292G>A mutation is responsible for a neurodegenerative disorder, hereditary spastic paraplegia (10). The down regulation of Hsp60 in LHON affected cells may suggest the lack of neuroprotective effect in the neuronal cells of affected people, which might lead to the optic neuropathy in LHON. Further detail studies are needed to elucidate the functional role of Hsp60 in the manifestation of LHON.

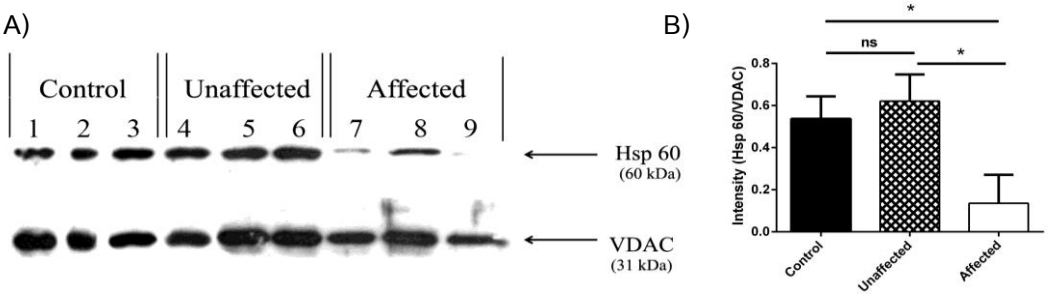

Figure 1 Cultured skin fibroblasts derived from three different controls, unaffected and affected LHON individuals were analyzed for mitochondrial Hsp60 protein determination by western blot analysis. VDAC protein was used as internal loading control (A). The visualized band intensities of Hsp60 was measured by ImageJ software and normalized with VDAC band intensity. The difference in the mean of Hsp60/VDAC band intensity was analyzed by non-parametric t-test ($p<0.05$, * ; $p>0.05$, ns) (B).

CONCLUSION

The expression of the Hsp60 protein has been found to be significantly lower in the fibroblasts from affected LHON patients than the fibroblasts from unaffected patients and control individuals. Our findings suggest the possible involvement of mitochondrial chaperone Hsp60 in the expression of LHON.

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