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The altered activity of complex III may contribute to the high penetrance of Leber's hereditary optic neuropathy in a Chinese family carrying the *ND4* G11778A mutation

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ABSTRACT

The *ND4* G11778A mutation is the most common mitochondrial DNA mutation leading to Leber's hereditary optic neuropathy (LHON). Despite considerable clinical evidences, the modifier role of nuclear background and mitochondrial haplotypes in phenotypic manifestation of LHON remains poorly understood. We investigated the effect of these modifiers on bioenergetics in lymphoblastoid cell lines derived from five affected subjects of one Chinese family carrying the G11778A mutation and five Chinese controls. Significant reductions in the activities of complexes I and III were observed in mutant cell lines from the Chinese family, whereas the mutant cell lines from other families carrying the same mutation exhibited only reduced activity of complex I. The reduced activities of complexes I and III caused remarkably higher reductions of ATP synthesis in mutant cell lines from the Chinese family than those from other families. The deficient respiration increased generation of reactive oxygen species. The defect in complex III activity, likely resulting from the mitochondrial haplotype or nuclear gene alteration, worsens mitochondrial dysfunction caused by the G11778A mutation, thereby causing extremely high penetrance and expressivity of optic neuropathy in this Chinese family. Our data provide the first experimental evidence that altered activity of complex III modulates the phenotypic manifestation of LHON-associated G11778A mutation. Thus, our findings may provide new insights into the pathophysiology of LHON.

1. Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited eye disease that generally affects children to young adults with the rapid, painless, bilateral loss of central vision (Newman, 1993; Nikoskelainen, 1994; Yu-Wai-Man et al., 2009). Mutations in mitochondrial DNA (mtDNA) are the molecular bases for this disorder (Howell, 2003; Wallace, 2005; Wallace et al., 1988). Of these, three primary mutations *ND1* G3460A, *ND4* G11778A and *ND6* T14484C mutations, which alter genes encoding the subunits of respiratory chain complex I (NADH dehydrogenase), account for approximately 90% of LHON pedigrees in some countries (Brandon et al., 2005; Brown et al., 1995; Carelli et al., 2009; Mackey et al., 1996; Mashima et al., 1998). These LHON-associated mtDNA mutations often occur nearly homoplasmy or homoplasmy. The primary defects in these mutations appeared to be a failure in the activity of NADH dehydrogenase (Brown et al., 2000; Hofhaus et al., 1996b), thereby leading to a deficient function of oxidative phosphorylation, a decrease in ATP synthesis and an increasing generation of reactive oxygen species (ROS). Subsequently, the energy failure and increasing oxidative stress may cause the degeneration of the retinal ganglion cells (Carelli et al., 2009; Yu-Wai-Man et al., 2009).

Typical features in LHON pedigrees are marked variations in penetrance and gender bias among affected subjects (Newman, 1993; Riordan-Eva et al., 1995). Matrilineal relatives within and among families, despite carrying the same LHON-associated mtDNA mutations such as G11778A mutation, exhibited a wide range of severity, age-of-onset and penetrance of optic neuropathy (Newman et al., 1991; Qu et al., 2009; Riordan-Eva et al., 1995; Zhou et al., 2010). Therefore, other modifier factors such as nuclear modifier genes, mitochondrial haplotypes and environmental factors are necessary for triggering optic neuropathy (Carelli et al., 2009; Hudson et al., 2007; Pello et al., 2008; Phasukkijwatana et al., 2010). However, these modifier factors remain poorly understood. To elucidate the pathophysiology of LHON, we have performed the clinical, genetic and molecular characterization of 23 Han Chinese families carrying the *ND4* G11778A mutation (Qian et al., 2005; Qu et al., 2005; 2006; 2009; 2010). Among these, the penetrance of optic neuropathy among 9 Chinese families ranged from 5.3% to 27.3% (Qu et al., 2009), while 25 of 28 matrilineal relatives in a large Han Chinese family exhibited the

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variable severity, and age-of-onset of optic neuropathy (Zhou et al., 2010). The lack of secondary LHON-associated mtDNA mutation in this family strongly indicated that the other modifiers contribute to the higher occurrence of visual loss in this family. To further understand the role of the modifier(s) in the manifestation of LHON-associated ND4 G11778A mutation, we have generated lymphoblastoid cell lines derived from five affected matrilineal relatives carrying the G11778A mutation from this family and five vision normal Chinese control subjects. These cell lines were examined for the endogenous respiration, substrate-dependent respiration, the rate of ATP production and the generation of reactive oxygen species.

2. Materials and methods

2.1. Cell cultures

Lymphoblastoid cell lines were immortalized by transformation with the Epstein–Barr virus, as described elsewhere (Miller and Lipman, 1973). Cell lines derived from five matrilineal relatives of the Chinese family (II-1, III-3, IV-3, IV-9 and IV-10) and from five genetically unrelated control individuals (A2, A4, A7, A8 and A30) were grown in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS). The bromodeoxyuridine (BrdU)-resistant 143B.TK$^-$ cell line was grown in DMEM supplemented with 5% FBS.

2.2. Mitochondrial DNA analysis

Genomic DNA was isolated from cell lines derived from mutant ant control subjects using the Puregene DNA Isolation Kits (Gentra Systems). An analysis for the presence and level of the G11778A mutation was carried out as detailed elsewhere (Qu et al., 2005). The quantification of mtDNA copy numbers from cell lines was performed by slot blot hybridization (Guan et al., 1996).

2.3. Oxygen consumption measurements

Rates of O_2 consumption in intact cells were determined with YSI 5300 Oxygraph (Yellow Springs Instruments) on samples of 1×10^7 cells in 1.8 ml of special DMEM-glucose lacking glucose medium, supplemented with 10% dialyzed FBS (King and Attardi, 1989). Polarographic analysis of digitonin-permeabilized cells, using different respiratory substrates and inhibitors, to test the activity of the various respiratory complexes, was carried out as detailed elsewhere (Hofhaus et al., 1996a).

2.4. ATP measurements

The levels of ATP in cells were measured using the ATP Bioluminescence Assay kit HS II (Roche Applied Science) according to the manufacturer's instructions and other study (McKenzie et al., 2007). In brief, samples of 2×10^6 cells were incubated for 2 h in the record solution (156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 20 mM HEPES, pH 7.35) with either 10 mM glucose, 10 mM glucose plus 2.5 μg/ml oligomycin (glycolytic ATP generation), or 5 mM 2-deoxy-d-glucose plus 5 mM pyruvate (oxidative ATP production). Cells were lysed and then incubated with the luciferin/luciferase reagents. Samples were measured using a SpectraMAX GEMINI XS microplate luminometer (MDS, Inc., Brandon, FL).

2.5. ROS measurements

ROS measurements were performed as detailed elsewhere (Mahfouz et al., 2008; Wang et al., 2011). Briefly, approximate 2×10^6 cells of each cell line were harvested, resuspended in PBS, supplemented with 100 uM of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and 2% FBS. After incubation at 37 °C for 20 min, cells were washed, resuspended in PBS in the presence of freshly prepared 2 mM H_2O_2 and 2% FBS and then incubated at room temperature for another 5 min. Finally, cells were resuspended with 1 ml of PBS with 0.5% paraformaldehyde. Samples with or without H_2O_2 stimulation were analyzed by the BD-LSR II flow cytometer system (Beckton Dickson, Inc), with an excitation at 488 nm and emission at 529 nm. 10,000 events were analyzed in each sample.

2.6. Statistical analysis

Statistical analysis was performed by the unpaired, two-tailed Student's *t*-test contained in Microsoft Office Excel (version 2003). Correlation analysis was performed using the curve fitting routine in the Graph Prism package (GraphPad Software, Inc). Differences were considered significant at a $p<0.05$.

3. Results

3.1. The Chinese pedigree and derived lymphoblastoid cell lines

A large Han Chinese pedigree with maternally inherited Leber's hereditary optic neuropathy, as shown in Fig. 1, has been previously described (Zhou et al., 2010). Immortalized lymphoblastoid cell lines used for this investigation were derived from 5 affected matrilineal relatives of the Chinese family and 5 genetically unrelated Chinese control subjects with normal vision. Of these, the ages of affected matrilineal relatives (II-1, III-3, IV-3, IV-9 and IV-10) varied from 14 to 75 years, respectively. Theses subjects suffered from visual impairment ranging from profound to mild vision loss at the age of 8 to 20 years (Zhou et al., 2010). Furthermore, the age of the control subjects (A2, A4, A7, A8 and A30) ranged from 25 to 38 years.

The presence and degree of the G11778A mutation in these cell lines were examined. In fact, the G11778A mutation was present in homoplasmy in these cell lines derived from 5 matrilineal relatives of this Chinese family but absent in the cell lines derived from five Chinese controls. An analysis of the mtDNA content of the individual cell lines was carried out by slot blot hybridization using a digoxin-labeled mitochondrial 12S rRNA probe, and by normalizing quantitative differences among DNA samples on the basis of hybridization with a nuclear 28S rRNA probe (Guan et al., 1996). The average relative levels of mtDNA content of the cell lines derived from controls and affected matrilineal relatives were 111% and 98% of those to 143B.TK$^-$ cells (9100 molecules per cell) (King and Attardi, 1989). There was no significant difference in the mtDNA copy numbers among the mutant and controls.

3.2. Oxygen respiration defects

The endogenous respiration rates of cell lines derived from five affected individuals carrying the G11778A mutation and from five Chinese control subjects lacking this mutation were measured by determining the O_2 consumption rate in intact cells, as described previously (King and Attardi, 1989). As shown in Fig. 2, the rate of total O_2 consumption in the lymphoblastoid cell lines derived from five affected individuals ranged between ~66.1% and 94%, with an average consumption of ~74.8% relative to the mean value measured in the control cell lines ($p=0.0002$).

In order to further investigate which enzyme complexes of the respiratory chain were affected in the mutant cell lines, O_2 consumption measurements were carried out on digitonin-permeabilized cells, using different substrates and inhibitors (Hofhaus et al., 1996a). As shown in Fig. 3A, in the cell lines derived from five affected individuals, the rate of malate/glutamate-driven respiration, which depends on the activities of NADH:ubiquinone oxidoreductase

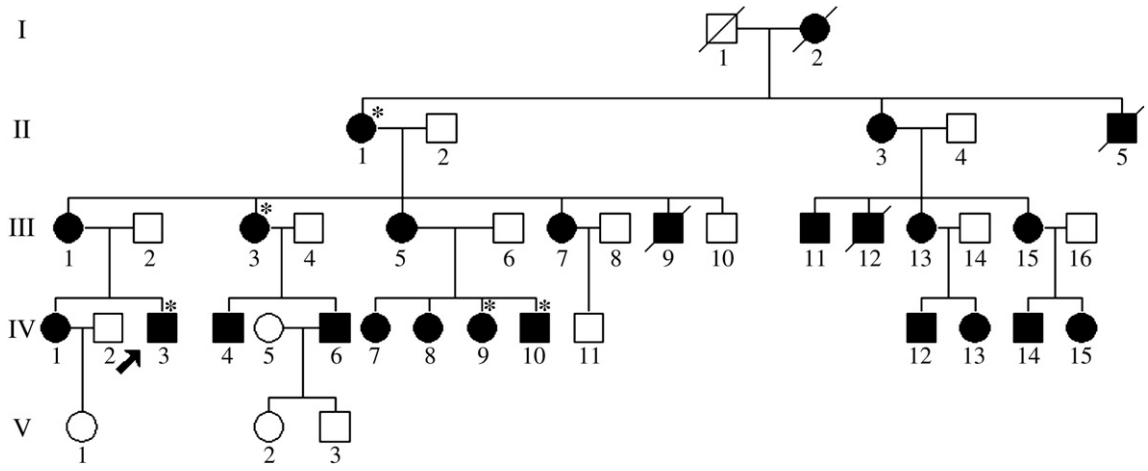

Fig. 1. The Chinese pedigree with Leber's hereditary optic neuropathy. Asterisks denote subjects used for this investigation.

(complex I), ubiquinol–cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV), but usually reflects the rate-limiting activity of complex I (21), was very significantly decreased, accounted for 60.4%–72.7% (~67.9% on the average; $p<0.001$) relative to the average rate in the control cell lines. Similarly, the rate of succinate/glycerol-3-phosphate (G-3-P)-driven respiration, which depends on the activities of complexes III and IV, but usually reflects the rate-limiting activity of complex III, was significantly affected in the mutant cell lines, accounted for 62.6%–86% (~73.8% on the average; $p=0.022$) relative to the average rate in the control cell lines. Furthermore, the rate of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene-diamine (TMPD)/ascorbate-driven respiration, which reflects the activity of complex IV, exhibited a 75.1% to 123.8% oxygen consumption in complex IV activity (~99.9% on the average; $p=0.51$) in the mutant cell lines relative to the average rate in the control cell lines.

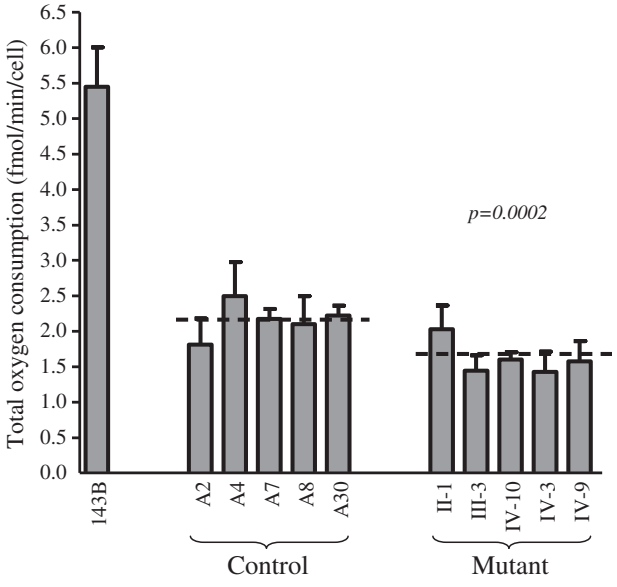

Fig. 2. Analysis of total oxygen respiration. Average rates of endogenous O_2 consumption per cell line are shown, with error bars representing doubled standard errors. Three to five determinations were made for each cell line. The horizontal dashed lines represent the average value for each group. *p* value refers to the statistical significance according to the student *t* test between the mutants and controls.

3.3. Marked decreases in ATP generation

The capacity of oxidative phosphorylation in mutant and wild type cells was examined by measuring the levels of cellular ATP using a luciferin/luciferase assay. Populations of cells were incubated in the media in the presence of glucose, glucose with oligomycin, and 2-deoxy-D-glucose with pyruvate (McKenzie et al., 2007). As shown in Fig. 4, the levels of ATP production in mutant cells in the presence of glucose (total cellular levels of ATP) or glucose with oligomycin to inhibit the ATP synthase (glycolysis) were comparable with those measured in the control cell lines. By contrast, the levels of ATP production in mutant cell lines, in the presence of pyruvate and 2-deoxy-D-glucose to inhibit the glycolysis (oxidative phosphorylation), ranged from 6.5% to 22.6%, with an average of 15.5%, relative to the mean value measured in the control cell lines ($p<0.0001$).

3.4. Increased ROS production

The increasing production of ROS in mutant cells has been suggested as one of the pathophysiologies in LHON (Carelli et al., 2009; Wong et al., 2002). The ROS production was measured by a quantitative flow cytometry assay. Cells were stained with a fluorescence dye 2′7′-dichlorofluorescin diacetate (DCFH-DA). Upon crossing cellular membrane, as shown in Fig. 5A, DCFH-DA was converted into non-fluorescent 2′,7′-dichlorofluorescin (DCFH) by intracellular esterase. DCFH, oxidized by intracellular ROS, became highly fluorescent 2′7′-dichlorofluorescein (DCF). The total levels of ROS (O_2^-, H_2O_2 and $OH^{\cdot}$) in mutant and control cells were measured by quantifying the fluorescence signals using a flow cytometry. As shown in Fig. 5B, the levels of ROS in the mutant cells were significantly higher than those in control cells, either stimulated or not stimulated by H_2O_2 stimulation. Table 1 showed that the levels of ROS in the mutant cells, not stimulated by H_2O_2, were not significantly increased, with an average of 118%, relative to that measured in the control cell lines. However, the levels of ROS generation in mutant cell lines stimulated by H_2O_2 varied from 151% to 269%, with an average of 194% ($p=0.001$) relative to the mean value measured in the control cell lines. To delineate the reaction upon increasing level of ROS under oxidative-stress, the ratios of geometric mean intensity between unstimulated and stimulated with H_2O_2 in each cell line were calculated. As shown in Fig. 6 and Table 1, the levels of ROS generation in mutant cell lines with the stimulation of H_2O_2 ranged from 118% to 214%, with an average of 165% ($p<0.001$), as compared with those measured in the control cell lines.

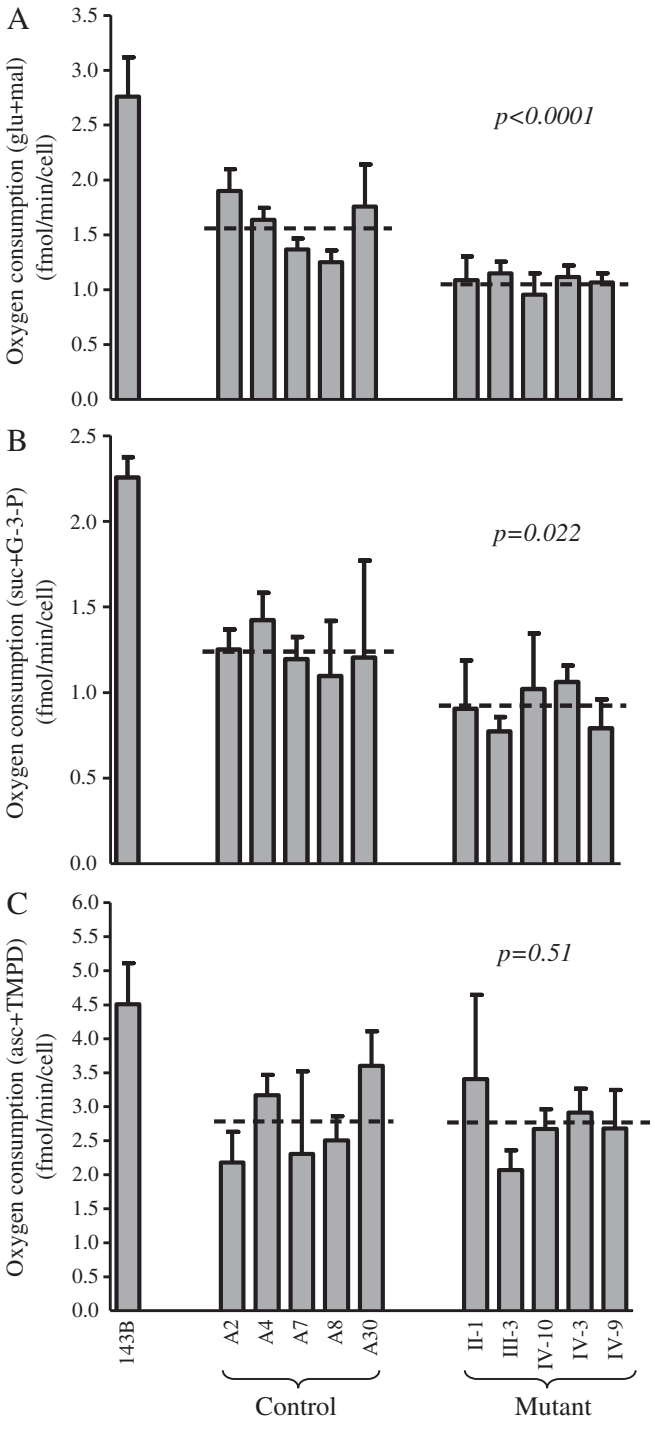

Fig. 3. Polarographic analysis of HO_2 consumption in digitonin-permeabilized cells of the various cell lines using different substrates and inhibitors. The activities of the various components of the respiratory chain were investigated by measuring on 1×10^7 digitonin-permeabilized cells the respiration dependent on malate/glutamate, on succinate/G3P and on TMPD/ascorbate. Four to six determinations were made on each of the lymphoblastoid cell lines. Graph details and symbols are explained in the legend to Fig. 1. mal/glu, malate/glutamate-dependent respiration; succ/G-3-P, succinate/G3P-dependent respiration; and asc/TMPD, TMPD/ascorbate-dependent respiration.

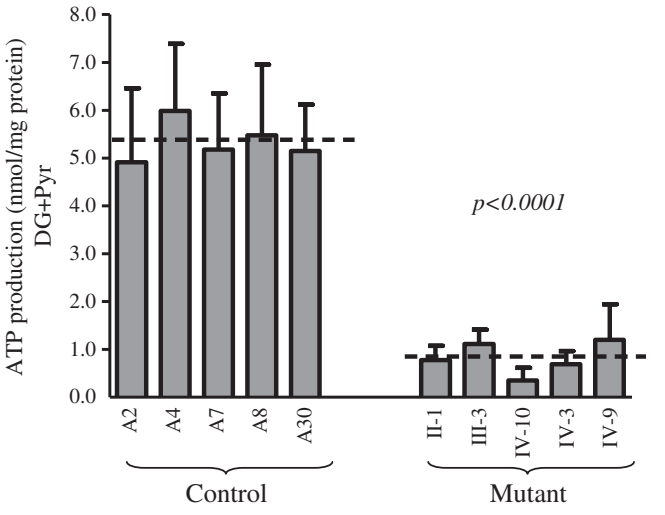

Fig. 4. Measurement of cellular ATP levels using bioluminescence assay. Cells were incubated with 5 mm 2-deoxy-d-glucose plus 5 mm pyruvate to determine ATP generation under mitochondrial ATP synthesis. Average rates of ATP level per cell line are shown, with error bars representing doubled standard errors. Six to seven determinations were made for each cell line. The horizontal dashed lines represent the average value for each group. *p* value refers to the statistical significance according to the student *t* test between the mutants and controls.

4. Discussion

In the present study, we have further investigated the molecular pathogenesis of the LHON-associated *ND4* G11778A mutation in a large Chinese pedigree with extremely high penetrance and expressivity of optic neuropathy. Strikingly, 25 of 28 matrilineal relatives of this Chinese family exhibited visual impairment, while the average penetrances of visual impairment in other 11 Chinese pedigrees carrying the G11778A mutation were 19.2% (Qian et al., 2005, Qu et al., 2005, 2009). Furthermore, the average age-at-onset for visual impairment in this Chinese family was 15 years. Conversely, the average age-at-onset of visual impairment in other 11 Chinese pedigrees (Qian et al., 2005, Qu et al., 2005, 2009), 66 and 49 Caucasian pedigrees carrying the G11778A mutation were 18, 24 and 28 years, respectively (Brown and Wallace, 1994; Nikoskelainen, 1994; Riordan-Eva et al., 1995). In addition, the ratio between affected male and female matrilineal relatives is 1:1.5 in the Chinese family, whereas this ratio was 4.5:1 and 3.7:1 from two large cohorts of Caucasian pedigrees carrying the G11778A mutation, respectively (Brown and Wallace, 1994; Nikoskelainen, 1994; Riordan-Eva et al., 1995). These data strongly support the notion that the G11778A mutation is necessary, but by itself is insufficient to induce a clinical expression of LHON (Brown et al., 2000; Hofhaus et al., 1996b). Therefore, the modifier factors may worsen mitochondrial dysfunctions caused by the G11778A mutation, thereby leading to high occurrence of optic neuropathy in this family.

In the present investigation, all lymphoblastoid cell lines carrying the G11778A mutation revealed significantly mitochondrial dysfunctions, which could be associated with the G11778A mutation and the contribution of nuclear modifier genes or mitochondrial haplotypes. In particular, these cells showed ~32% decrease in NADH dehydrogenase-dependent respiration after digitonin permeabilization. These data are in good agreement with the observations that there were 30% to 40% reductions in NADH dehydrogenase-dependent respiration in lymphoblastoid or transmitochondrial cell lines derived from Caucasian families carrying the G11778A mutation (Brown et al., 2000; Hofhaus et al., 1996b). These observations strongly indicated that the primary defect in the G11778A mutation was a failure in the activity of NADH dehydrogenase. Remarkably, 26% decrease in the rate of succinate/glycerol-3-phosphate (G-3-P)-driven respiration was observed in the mutant cell lines carrying the G11778A mutation in this Chinese family. This result was in contrast with the previous studies that the rates of succinate/glycerol-3-phosphate (G-3-P)-driven respiration in the cell lines carrying the G11778A mutation were comparable to those controls (Brown et al., 2000; Hofhaus et al., 1996b; Pello et al., 2008). Indeed, the mutations in *Cytb* gene have

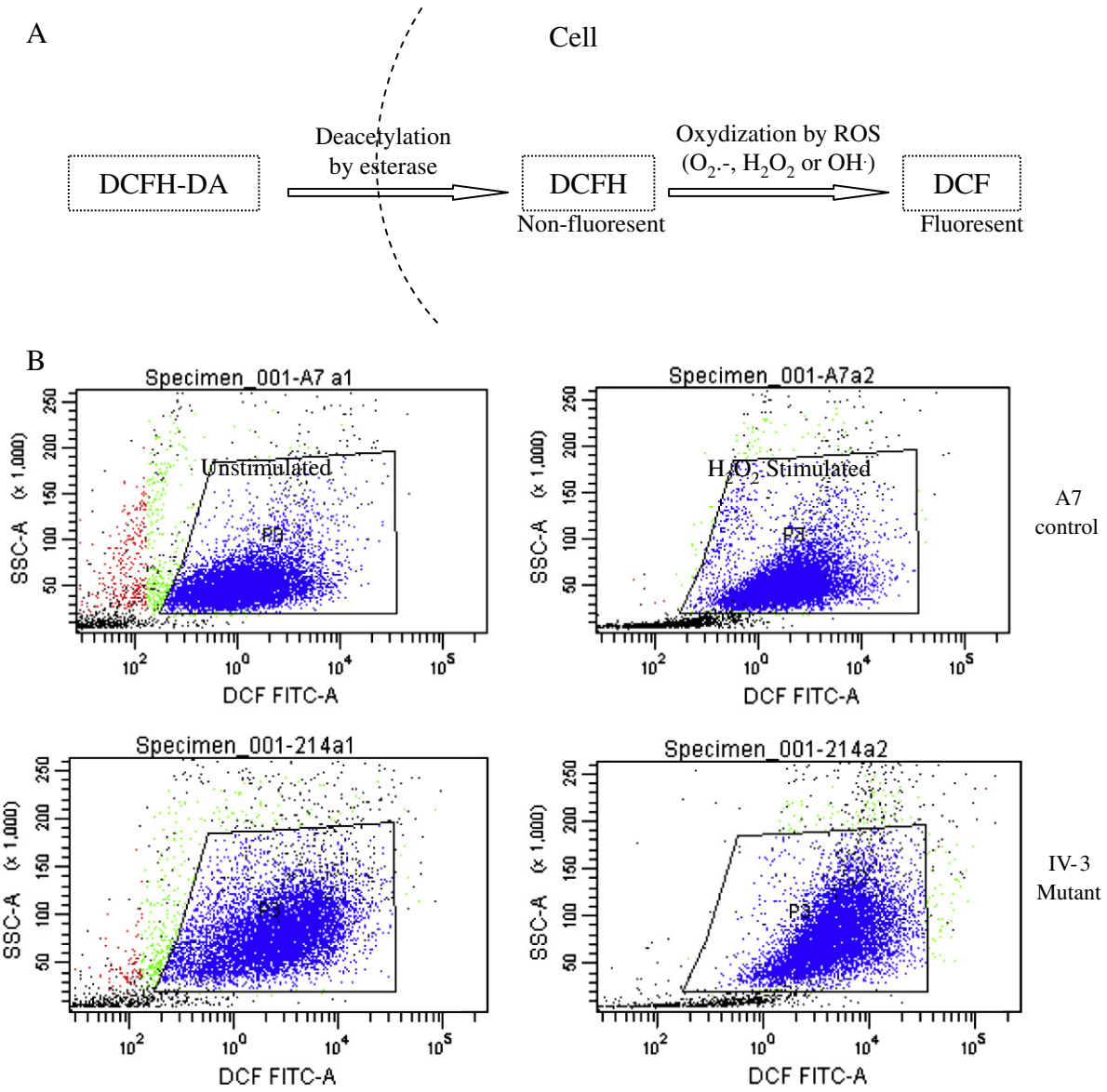

Fig. 5. The ROS production assays. (A) Schema: upon crossing cellular membrane, fluorescence dye 2′7′-dichlorofluorescin diacetate (DCFH-DA) is deacetylated by esterase and generates non-fluorescent 2′,7′-dichlorofluorescin (DCFH). DCFH is oxidized by intracellular ROS to generate highly fluorescent 2′7′-dichlorofluorescein (DCF). (B) ROS Assay by a flow cytometer, representative two-parameter dot-plots of the side light scatter and DCF FITC fluorescent intensity of control cell line A7 and mutant cell line IV-3 are shown. Left column shows results of those without H_2O_2 treatment, right column shows the results of those stimulated with H_2O_2.

been associated with the phenotypic manifestations of LHON. These are the putative LHON-associated mtDNA mutations such as G14831A (Fauser et al., 2002) and T15674C (Abu-Amero and Bosley, 2006), and the secondary LHON-associated mtDNA mutations including A14841G, G15297A, A15395G, G15773A and G15812A (Brandon et al., 2005). Ten variants in the *Cytb* gene were present in mitochondrial genome of this Chinese family (Zhou et al., 2010). The frequency of these variants was further analyzed among 2703 human mitochondrial genomes using the mtDB: Human Mitochondrial Genome Database (Ingman and Gyllensten, 2006). The T15109C and T15139C variants were found only one each among 2703 human mitochondrial genomes. The frequency of these variants was even less than the G11778A mutation (which is found 2 times). On the other hand, other eight variants were more frequent (from 4 to 83 times/2073) than these two variants in the *Cytb* gene. This suggests that these two variants may contribute to the decreased activity of complex III and high penetrance of optic neuropathy. Alternatively, mutation(s) in nuclear genes may contribute to the deficiency of complex III (cytochrome bc1 complex) activities. In fact, the nuclear genes encode 10 of 11 subunits in complex III (Wallace, 2005). Up to date, none of mutations in nuclear genes involved in complex III activities has been identified to be associated with LHON. Furthermore, similar to other studies, no differences in the activities of complex IV were detected between control and mutant cell lines derived from this Chinese family carrying the G11778A mutation.

There was a very significant correlation between the overall respiratory capacity or the rates of NADH dehydrogenase-dependent respiration or succinate/glycerol-3-phosphate (G-3-P)-driven respiration and the level of ATP production ($p<0.01$) or the level of ROS production ($p<0.01$) in the control and mutant cell lines. This correlation is clearly consistent with the importance that the marked decrease in the activities of complexes I and III in the mutant cell lines derived from this family carrying the G11778A mutation play a critical role in decreasing ATP synthesis and increasing ROS generation. In the

Table 1
Analysis of ROS generation.

Subjects	MFI[a] unstimulated	MFI stimulated	Ratio stm vs unstm[b] (%)
Control			
A2	3180 ± 132	3271 ± 439	103 ± 17
A4	3237 ± 196	3363 ± 250	104 ± 4
A7	1365 ± 198	1376 ± 348	101 ± 22
A8	1843 ± 169	2154 ± 168	118 ± 15
A30	4174 ± 977	5583 ± 679	136 ± 17
Mean	2760 ± 334	3149 ± 376	114 ± 15
Mutant			
II-1	2954 ± 383	7236 ± 245	245 ± 28
III-3	3809 ± 585	8459 ± 670	222 ± 45
IV-3	2399 ± 199	4669 ± 238	195 ± 13
IV-9	3067 ± 211	4748 ± 430	155 ± 36
IV-10	4058 ± 194	5482 ± 890	135 ± 21
Mean	3258 ± 314	6119 ± 495*	188 ± 29*

* $p<0.05$ according to the student t test between the mutant and control cell lines.
[a] MFI — mean fluorescence intensity. Each cell line was in parallel stimulated with or without H_2O_2. Geometric mean intensity was applied to determine the MFI values shown with doubled standard errors. Three to nine determinations were made for each treatment of cell lines.
[b] Stm — stimulated, unstm — unstimulated.

present investigation, 85% decrease of ATP synthesis in mutant lymphoblastoid cell lines from this Chinese family most likely resulted from the reduced activities of complexes I and III, while 40%–60% reduction of ATP synthesis in cybrid cell lines carrying the *ND1* G3460A, *ND4* G11778A, *ND5* A13528G, *ND6* T14484C or G14279A mutation may only be caused by the defective activity of complex I (Baracca et al., 2005; Beretta et al., 2004; Fauser et al., 2002). These results further support the role of mitochondrial haplotypes and nuclear modifier genes in the high occurrence of LHON in this Chinese family. The X-linked or autosomal recessive genes such as *PARL* were implicated to have a role in phenotypic expression of LHON (Hudson et al., 2005; Phasukkijwatana et al., 2010). Moreover, altered activities of complexes I and III can lead to more electron leakage from electron transport chain, and in turn, increase the generation of ROS (Lenaz et al., 2004; Yen et al., 2006). Here, a marked increase of ROS production was detected in cell lines derived from the Chinese family carrying the G11788A mutation. The observation is a good agreement with the significant increase of ROS in cybrids cell lines carrying the G11778A mutation (Beretta et al., 2004; Porcelli et al., 2009). However, 2.5 fold more cellular hydroperoxide were detected in neuronal NT2 cells carrying the G11778A mutation (Wong et al., 2002). This discrepancy is likely attributed to differentiation-specific effects (Wong et al., 2002). The increasing generation of ROS can damage DNA, lipids, proteins and membranes. As a result, these biochemical defects can lead to dysfunction or apoptosis in retinal ganglion cells, thereby producing the clinical phenotype (Carelli et al., 2009; Ghelli et al., 2003).

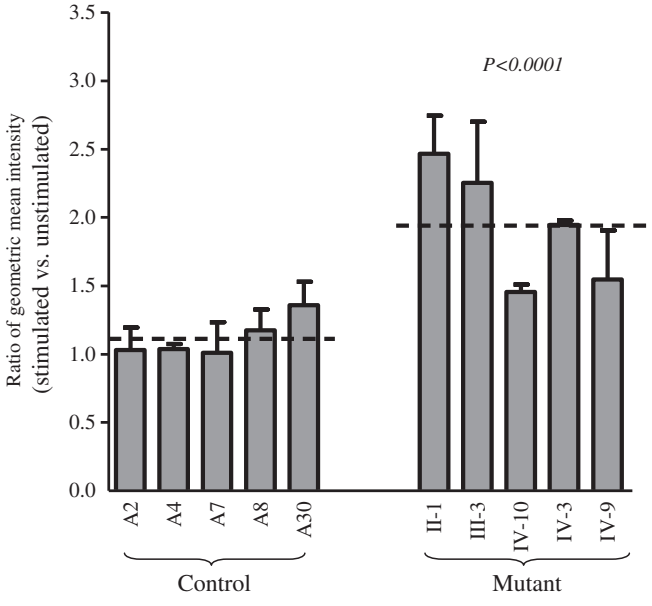

Fig. 6. Ratio of geometric mean intensity between with or without H2O2 stimulation. The rates of production in ROS from five affected matrilineal relatives and five control individuals were analyzed by BD-LSR II flow cytometer system with or without H_2O_2 stimulation. The relative ratio of intensity (stimulated vs unstimulated with H_2O_2) was calculated. The average of three determinations for each cell line is shown, with error bars representing two standard errors of the mean (SE).

In summary, these results provide the first experimental evidence that the altered activity of complex III modulates the phenotypic manifestation of LHON-associated G11778A mutation in this Chinese family. The deficient activity of complex I is the primary defect caused by the G11778A mutation. The complex III activity defect, resulting from alteration in the mitochondrial haplotype or nuclear modifier gene(s), worsens the mitochondrial dysfunction, thereby leading to high penetrance and occurrence of optic neuropathy in this Chinese family. Thus, our findings may provide new insights into pathophysiology of LHON.

Acknowledgments

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