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Analysis of reactive oxygen species and antioxidant defenses in complex I deficient patients revealed a specific increase in superoxide dismutase activity

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Abstract
The mechanism of free radical production by complex I deficiency is ill-defined, although it is of significant contemporary interest. This study studied the ROS production and antioxidant defenses in children with mitochondrial NADH dehydrogenase deficiency. ROS production has remained significantly elevated in patients compared to controls. The expression of all antioxidant enzymes significantly increased at mRNA level. However, the enzyme activities did not correlate with high mRNA or protein expression. Only the activity of superoxide dismutase (SOD) was found to correlate with higher mRNA expression in patient derived cell lines. The activities of the enzymes such as glutathione peroxidase (GPx), Catalase (CAT) and glutathione-S-transferase (GST) were significantly reduced in patients ($p < 0.05$ or $p < 0.01$). Glutathione reductase (GR) activity and intracellular glutathione (GSH) levels were not changed. Decreased enzyme activities could be due to post-translational or oxidative modification of ROS scavenging enzymes. The information on the status of ROS and marking the alteration of ROS scavenging enzymes in peripheral lymphocytes or lymphoblast cell lines will provide a better way to design antioxidant therapies for such disorders.

Keywords: *Mitochondrial myopathies, reactive oxygen species (ROS), antioxidant enzymes, mitochondrial complex I*

Introduction

Mitochondrial encephalomyopathies form a group of heterogeneous disorders that are caused by defects in respiration and the oxidative phosphorylation system [1]. Dysfunction of the respiratory chain may lead to increased electron leak and over-production of reactive oxygen species (ROS) in mitochondria [2–4]. Thus, both the impairment of respiratory chain and the increased ROS production are associated with the pathophysiology of mitochondrial disorders [2]. The secondary consequences of elevated ROS levels are the formation of lipid peroxides, increase in hydroxyl radicals and aldehydic lipid peroxidation products in skin fibroblasts of patients with mitochondrial complex I deficiency [4,5]. Several studies have shown that the increase of endogenous oxidative stress elicited by impairment of respiratory chain function plays an important role in the pathogenesis and progression of mitochondrial diseases [6,7]. ROS levels are controlled by a set of enzymes within the cell known as the antioxidant enzyme system. Mitochondrial pathogenesis and related neurodegenerative disorders have been correlated with alteration in these enzymes. An antioxidant defense system is impaired and resultantly oxidative damage of DNA

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is enhanced in skeletal muscle of patients with mitochondrial disease [5,7,8]. It has been shown that the activities of free radical scavenging enzymes are impaired in the affected tissues of patients with mitochondrial disorders (PhD thesis, Chig yu lu, Yakes FM, et al. 1997). Besides, a lot of information is available regarding ROS and its causative presence in various disorders.

Numerous studies have described *in vitro* generation of free radicals by isolated mitochondria under controlled conditions [9–11]. Pitkanen and Robinson [12] have studied the superoxide production in isolated mitochondria of skin fibroblasts from patients with complex I deficiency and have shown its correlation with function of mitochondrial enzyme MnSOD. To gain a broader perspective of the consequences of respiratory chain deficiency upon the status of ROS production and the status of ROS scavenging enzymes, we investigated transcriptional, translational and activity responses of major antioxidant enzymes in complex I deficient lymphoblast cell lines established from paediatric patients with mitochondrial encephalomyopathies. The complex I deficiency in these patients was confirmed by spectrophotometry [13] and by in-gel activity staining. Further, we measured the basal levels of ROS, glutathione and antioxidant defensive enzymes, e.g. superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST). We also measured the same parameters for all cell lines in the presence of tert-butyl hydroperoxide (tBHP) added in the culture medium. Tert-butyl hydroperoxide is one of the most common pro-oxidant agents used to evaluate the effects of oxidative stress [14] and sensitivity of various enzymes/cell membrane structures to oxidative damage. The cell lines used were from six patients who displayed complex I defect. These cell lines were genotyped based on the variants previously identified by us in their mitochondrial genomes designated as G4812C, T11916A, T4216C, T3866C, C4640A and T3394C [13].

Materials and methods

Materials

All reagents (NADPH, βNADH, pyrogallol, ficoll, tBHP, Diethylene triamine pentaacetic acid (DTPA), reduced and oxidized glutathione); enzymes (Glutathione reductase, Superoxide dismutase (SOD)), CAT, Glutathione S transferase and kits (AnnexinV-FITC apoptosis detection kit) were purchased from Sigma (USA). Superscript II reverse transcriptase enzyme, 2′, 7′ Dichlorofluorescin-diacetate (H_2-DCFDA), Dihydroethidium (DHE) and Dihydrorhodamine (DHR) were from Molecular Probes (Invitrogen, USA).

Complex I deficient cell lines

Lymphoblast cell lines established from three controls and six paediatric patients with mitochondrial encephalomyopathies showing complex I deficiency were taken for analysis. Lymphocytes were separated by ficoll-percoll gradients as described earlier [13]. Cells were transfected with EBV and cultured until transformed and established. The cell lines were maintained in RPMI 1640 medium containing 10% heat inactivated foetal bovine serum.

Isolation of mitochondria and BN-PAGE

Mitochondria were isolated from at least 1×10^8 cells using mitochondria isolation kit (Pierce) as per manufacturer's protocol. Blue native gel electrophoresis (BN-PAGE) of mitochondrial fractions (100 μg protein/lane) and mitochondrial complex-I in-gel activity staining was performed as previously described [15]. Mitochondrial fractions isolated from cultured lymphoblast cell lines were electrophoresed through a 5–12% BN-PAGE gel and the activity of complex-I in the gel was defined according to the intensity of the colour reaction resulting from the reduction of nitrotetrazolium blue by the dehydrogenase portion of the enzyme. Deamino-NADH was used as an electron donor in the reaction. Transferring the proteins from BN-PAGE gel to polyvinylidine difluoride (PVDF) membranes and using complex I specific 39kDa antibody for immunoblotting assessed mitochondrial complex I assembly.

Tert-butyl hydroperoxide treatment

The cells were centrifuged at 300 g for 5 min and resuspended in fresh medium. To this 20 μm of tBHP was added in control and patient cells, incubated at 37°C for 30 min. Cells were washed twice with PBS and used for either protein extraction or stained with ROS specific dyes for FACS analysis.

Measurement of reactive oxygen species (ROS)

Intracellular steady state levels of ROS were carried out on living cells using three different ROS specific probes: Dichlorofluorescin-diacetate (H_2-DCFDA), dihydro rhodamine (DHR) and Dihydroethidium (DHE) [16]. DCFDA is specific for hydrogen peroxide (H_2O_2) and is de-acetylated inside the cell. The subsequent oxidation by intracellular oxidants yields a fluorescent product, 2′, 7′-dichlorofluorescin (DCF). Dihydro-ethidium (DHE) is specific for superoxide free radical ($O_2^{\bullet -}$), whereas dihydrorhodamine (DHR) is used to detect hydroxyl ($OH^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and peroxynitrite ($ONOO^-$) free radicals. Cells were collected and centrifuged for 5 min at 300 g. The pellet was washed once with PBS and then resuspended again in 1 ml

PBS, incubated with 10 μM DCFDA, 1 μM DHE or 10 μM DHR in separate experiments for 30 min at 37°C. The cells were washed and then suspended (1×10^6/ml PBS) and analysed immediately by flow cytometry. DCFDA and DHR were excited at 488 nm and emitted fluorescence was analysed at 525 nm using FACS vantage SE system (BD Biosciences). DHE was excited at 535 nm and emission was measured at 610 nm.

Dead cells were excluded by electronically gating the data on the basis of forward- vs side-scatter profiles; a minimum of 1×10^4 cells of interest were analysed further. Mean log of fluorescence intensity (MFI) values were obtained by the CellQuest Pro-software program (BD Biosciences).

Reverse transcription and quantitative PCR

RNA (5 μg) was reverse transcribed to cDNA using random hexamer primers and the Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using 7300 Real Time PCR System (Applied Biosystems) and Power SYBR Green PCR master mix supplied by Applied Biosystems. The conditions for the amplification of copper zinc superoxide dismutase, manganese superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase and the normalization gene, 18S ribosomal RNA, were as follows: denaturation step at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 15 s, a common step for annealing and elongation at 60°C for 60 s. At the end of the PCR, the samples were subjected to melting curve analysis. All reactions were performed thrice in triplicate. The primer sequences for all antioxidant enzymes were designed based on information from previous publications [17].

Western blot analysis

Cells were lysed in radioimmunoprecipitation (RIPA) buffer and sonicated for 1 min to shear the DNA. An equal amount of protein (40 μg/lane) for each sample was separated by SDS-PAGE (12% acrylamide) and transferred to PVDF membrane. The membrane was blocked in 5% (w/v) BSA in 0.02 M Tris-buffered saline with 0.1% (v/v) Tween-20 for 2 h at room temperature (RT). After incubation for 2 h at RT using 1:2000 dilution of primary antibody to MnSOD (Sigma, USA), membranes were probed with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, USA). Bound antibody was visualized using an ECL reagent (Amersham Biosciences, USA). Densitometry analysis of Western blot signal was performed using Quantity-One software (Bio-Rad).

Enzyme activity assays

SOD enzyme assay. Cells (10^7–10^8) were sonicated on ice for 4 min (20 s × 4 strokes at amplitude of 40 and 1 min delay between each stroke) in ice-cold 50 mm potassium phosphate buffer (pH 7.4) and centrifuged for 10 min at 12 000 g at 4°C. The supernatant obtained was used for activity analyses. Total superoxide dismutase (SOD) activity was examined according to the method described earlier [18]. This assay is based on the ability of SOD to scavenge superoxide anion radical ($O_2^{\bullet -}$), which decreases the overall rate of pyrogallol autoxidation. In brief, a 50 μl cell extract (300 μg protein) was added to 937 μl of 50 mM Tris-HCL buffer (pH 8.2) containing 1 mM DTPA. The reaction was initiated by the addition of 13 μl of 10 mM pyrogallol and the change in absorbance at 420 nm was recorded for 3 min. SOD activity was calculated as units per milligram of protein, with 1U of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%.

Glutathione peroxidase (GPx) assay. Cell extract was prepared as described for the SOD assay. GPx activity was examined in the supernatants by tert-butyl hydroperoxide dependent method described earlier [19]. The assay is based on the oxidation of reduced glutathione (GSH) by GPx coupled to the disappearance of NADPH by glutathione reductase (GR). In brief, the following solutions were pipetted into 1 ml cuvette: 500 μl 0.1 M phosphate buffer (pH 7.0), 100 μl enzyme sample (cell extract), 100 μl GR (0.24 U) and exactly 100 μl of 10 mM GSH. The mixture was pre-incubated at 37°C for 10 min. Thereafter, 100 μl NADPH solution was added and hydroperoxide-independent consumption of NADPH was monitored for 3 min. Adding 100 μl of pre-warmed tBHP solution (12 mM) started the overall reaction and the decrease in absorbance at 340 nm was monitored for 5 min. The non-enzymatic reaction rate was estimated by replacing the enzyme sample by buffer. The difference in final and initial absorbance was used to calculate enzyme activity. The rate of decrease in A_{340} was converted to the activity using the following formula:

$$1 \text{ milliunit/ml} = 1 \text{ nmol of NADPH/min/ml}$$
$$= \Delta A340/\text{min}/0.00622$$

whereas 0.00622 is the extinction coefficient of NADPH. Specific activity was calculated as activity per mg or per μg protein.

Glutathione S-transferase (GST) activity. GST activity was assayed in the cell lysates as described earlier [20]. Briefly, 25 μg protein was incubated in 50 mM KH_2PO_4 buffer pH 6.5, 1 mM GSH and 0.25 mM 1-chloro-2,4-dinitrobenzene. The reaction was followed for 2 min at 37°C at 340 nm and GST activity

was calculated using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ [21].

Other enzyme assays. GR and CAT activities were measured by using sigma kits as per the manufacturer's instructions. The activity calculations were done as per the formulae given in the kit manuals.

CuZnSOD ELISA

An ELISA was used for the detection of protein levels of CuZnSOD in cell homogenates. Antibody was obtained from Sigma (USA). Measurements were performed in triplicate.

Glutathione estimation

Glutathione estimation was performed using a glutathione assay kit available from Sigma. The sample preparation and calculations were carried out as per manufacturer's instructions provided in the kit manual.

Protein estimation

Protein concentrations were estimated by the Bradford method using Bio-Rad protein assay kit (Bio-Rad, USA).

Statistical analysis

All experimental values represent the mean of at least three independent determinations. Data are expressed as $\pm$SD and statistical significance was measured with a parametric student's *t*-test. Statistical significance is given as asterisks; $^{**}p < 0.05$ and $^{*}p < 0.01$.

Results

Investigation of respiratory chain complex I defect in cultured cells

A total of six complex I deficient lymphoblast cell lines established from patients with mitochondrial encephalomyopathies were investigated for ROS production and status of antioxidant defensive enzymes. The clinical features and laboratory findings for all the patients are described in Table I. Blue native PAGE and spectrophotometric analysis of respiratory chain enzymes showed a decreased activity of rotenone sensitive NADH dehydrogenase enzyme in these cell lines (Figure 1). From Figure 1A, it is clear that the complex I defect is severe in cell lines with mutations G4812C, T4216C, T11916A and T3394C, whereas it is partial in remaining two cell lines having T3866C and C4640A mutations, respectively, which was also confirmed by western blot analysis (Figure 1B). Cytochrome C oxidase and succinate-cytochrome *c* reductase activities were either normal or in some cases slightly elevated [13].

ROS production

FACS analysis of cells for ROS production showed a $\sim$1.3-fold increase in H_2O_2 and reactive nitrogen species ($NO_2^{\bullet}$, $ONOO^{-}$) in patient cells determined by DCF and DHR staining. Patient cells in comparison to controls observed an $\sim$1.5-fold increase in $O_2^{\bullet -}$ production, which was calculated by DHE fluorescence. Cell line T3394C and T3866C showed the maximum ROS production for all three species tested compared to C4640A, T4216C and G4812C cells. Cells treated with tBHP had a $\sim$1.5-fold increase in H_2O_2 and 2-fold increase in O_2^{-} production.

Table I. Clinical symptoms and laboratory findings of six patients with childhood mitochondrial encephalopathies.

Mutation	Clinical symptoms	Blood lactate*	CSF lactate*	Age (years)	Sex	Expected disorder
G4812C	Progressive loss of vision since 7–8 months, bilateral optic atrophy, in fundus also	ND	28.8	7	Male	Encephalopathy
T3866C	Loss of vision, MRI showing a focal lesion displaying, restricted diffusion in the splenium of corpus callosum, high signal in the cerebellar cortex, demyelination and ischemic aetiology	26.2	ND	7	Female	Encephalopathy
T3394C	Loss of peripheral vision in both eyes. MRI showed a focal lesion displaying, and restricted diffusion in the splenium of corpus callosum. High signal in the cerebellar cortex on DW images. Differential diagnosis: demyelination and ischemic aetiology	26.2	ND	4	Female	Encephalopathy
T11916A	Muscle weakness, regression of motor and mental milestones, Abnormal eye movements. MRI spectroscopy shows lactate peak in the basal ganglia and adjacent white matter	47.5	46.7	2.5	Female	Leigh's like
C4640A	Abnormal symmetrical signal intensity in areas bilateral caudate nuclei and lentiform nuclei. Similar areas in cerebral peduncles aqueductal region and medullary pyramid	ND	36.1	4	Male	Leigh's disease
T4216C	Seizures, leukodystrophy, MRI showing white matter abnormalities in cerebral hemispheres, frontal, parietal and temporal lobes. EEG suggestive of epileptiform activity over right cerebral hemisphere	ND	22	0.3	Female	Encephalopathy

ND = not determined, CSF = cerebrospinal fluid. *Normal level for CSF and blood lactate is 10.8–18.9 mg/dL.

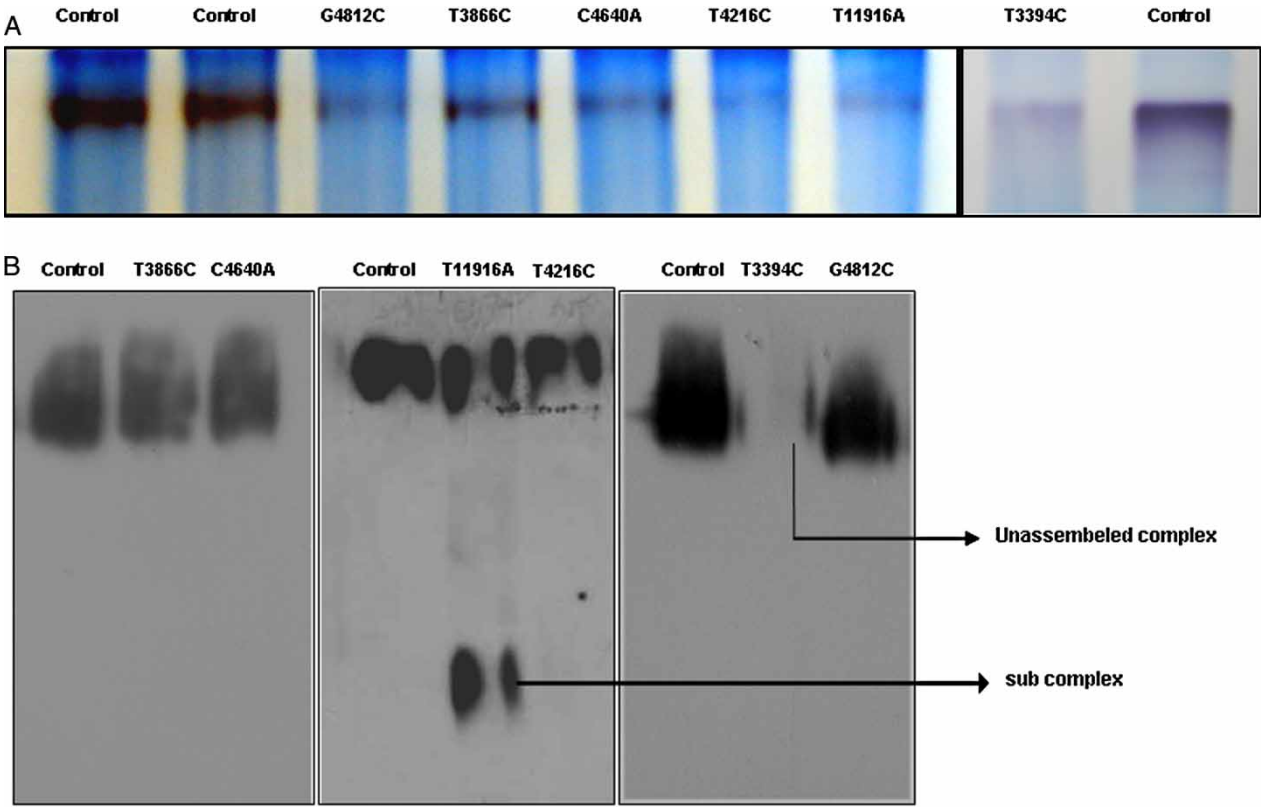

Figure 1. BN-PAGE in-gel activity assays performed using 100 μg of lymphoblast mitochondrial protein loaded on to the gel for both controls and patients. (A) BN-PAGE results showing severe complex I defect in four patients and partial complex I defect in two cell lines. (B) Western blot showing impaired complex I assembly in patients with mutation T3394C and T11916A. However, the assembly was not hampered much in other patients.

Tert-butyl hydroperoxide treatment resulted in an elevation of ROS in both control as well as in patient cell lines. Figure 2 shows that ROS production increased significantly in the patient cell lines ($p < 0.01$) than controls. We confirmed the cytotoxic effects of tBHP by annexin V and propidium iodide staining by FACS analysis and we found that tBHP did not cause any cell death at a final concentration of 20 μm in the medium.

Expression of antioxidant enzymes

The steady-state level of intracellular ROS depends upon the balance between rates of ROS generation and detoxification. A crucial role in determining ROS cellular homeostasis is played by the antioxidant defense system within the cell. Therefore, detailed analysis of soluble glutathione levels and enzymatic defenses (SOD, CAT, GPx, GR and GST) were performed for six patient derived (complex I deficient) and three control lymphoblast cell lines. Expression of antioxidant enzymes at mRNA level showed an increase in almost all antioxidant enzymes. A 1.2–1.5-fold increase in MnSOD and 1.1–1.5-fold increase in CAT, GST and GPx expression were observed in patient derived cell lines (Table II). However, CuZnSOD mRNA levels were increased 1.2–1.4-fold in only two patient cell lines with mtDNA genotypes T3394C and T11916A, whereas the mRNA expression for the other four patient derived cell lines was similar to that of the control cells (Table II).

Because mRNA levels may not necessarily reflect the corresponding protein levels, we have also investigated the protein contents of MnSOD by western blot and CuZnSOD by ELISA. Densitometry analysis of the blots showed higher protein concentrations of MnSOD in patient cells with respect to controls, the difference is quite significant ($p < 0.01$) in two cell lines with mtDNA genotypes T3394C and T11916A (Figure 3). The antibody used specifically detected MnSOD protein of molecular size 21 kDa. The protein contents of CuZnSOD as determined by ELISA represent absolute values and are given in nanograms per milligram of total protein. However, there were no significant differences in the protein levels for CuZnSOD between control and patient group (Figure 4). These results were in congruence with the mRNA expression data. Determination of protein expression for other antioxidant enzymes was not performed due to technical problems.

Activities of antioxidant enzymes

The activities were not correlating with higher mRNA and protein expression for antioxidant enzymes

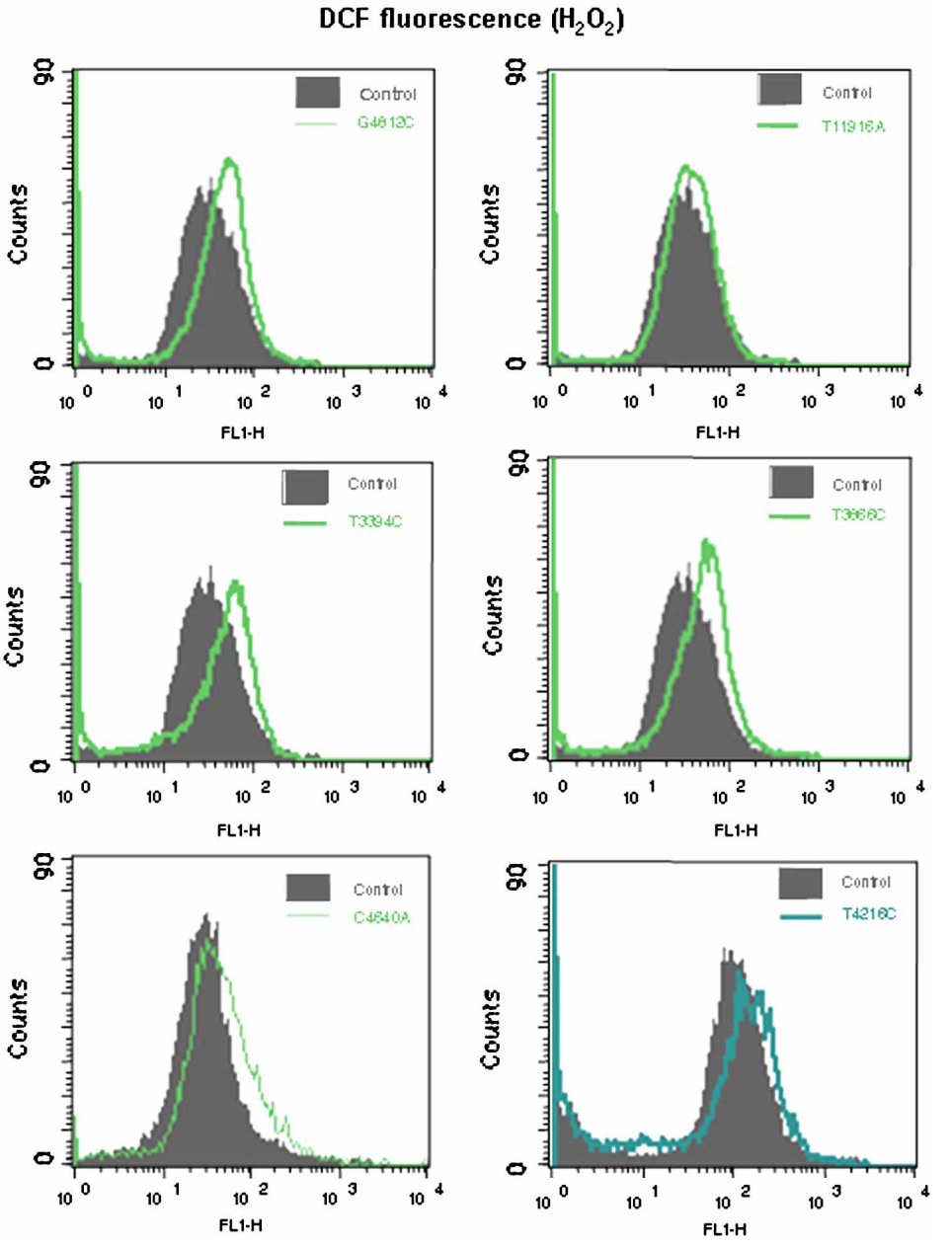

Figure 2 (*Continued*)

other than SOD. Only SOD activity was in agreement with mRNA or protein levels and significantly elevated in complex I defective cell lines (Table III). Intracellular GSH levels were not changed significantly and were slightly elevated in tBHP treated conditions (Figure 5).

The activities of the glutathione-related enzymes, GPx and GR are shown in Table III. GPx and GR are crucial antioxidant defenses as GPx transforms H_2O_2 to H_2O by coupling the oxidation of GSH to GSSG and GR mediates the reduction of GSSG to GSH. Total GPx activity measured in complex I defective cells was less than half of the activity present in controls. In contrast, GR activity was not significantly different from controls in both untreated and tBHP treated conditions. When we assessed the activity of CAT, a significant decrease ($p < 0.05$) of this enzyme was observed in complex I defective cells (Table III). GST enzymes metabolize xenobiotics as well as aldehydes, endogenously produced during lipid peroxidation by conjugation with GSH. Moreover, some GSTs also show glutathione peroxidase like activity [22]. GST activity was decreased to a similar extent as that of GPx in patient cells.

Incubation in the presence of tBHP caused a dramatic loss in CAT, GPx and GST activities. This decrease ranged from 50–60% in patients compared to that of controls. However, as seen from Table III, the SOD activity in patient cell lines was not affected by tBHP incubation and the activity

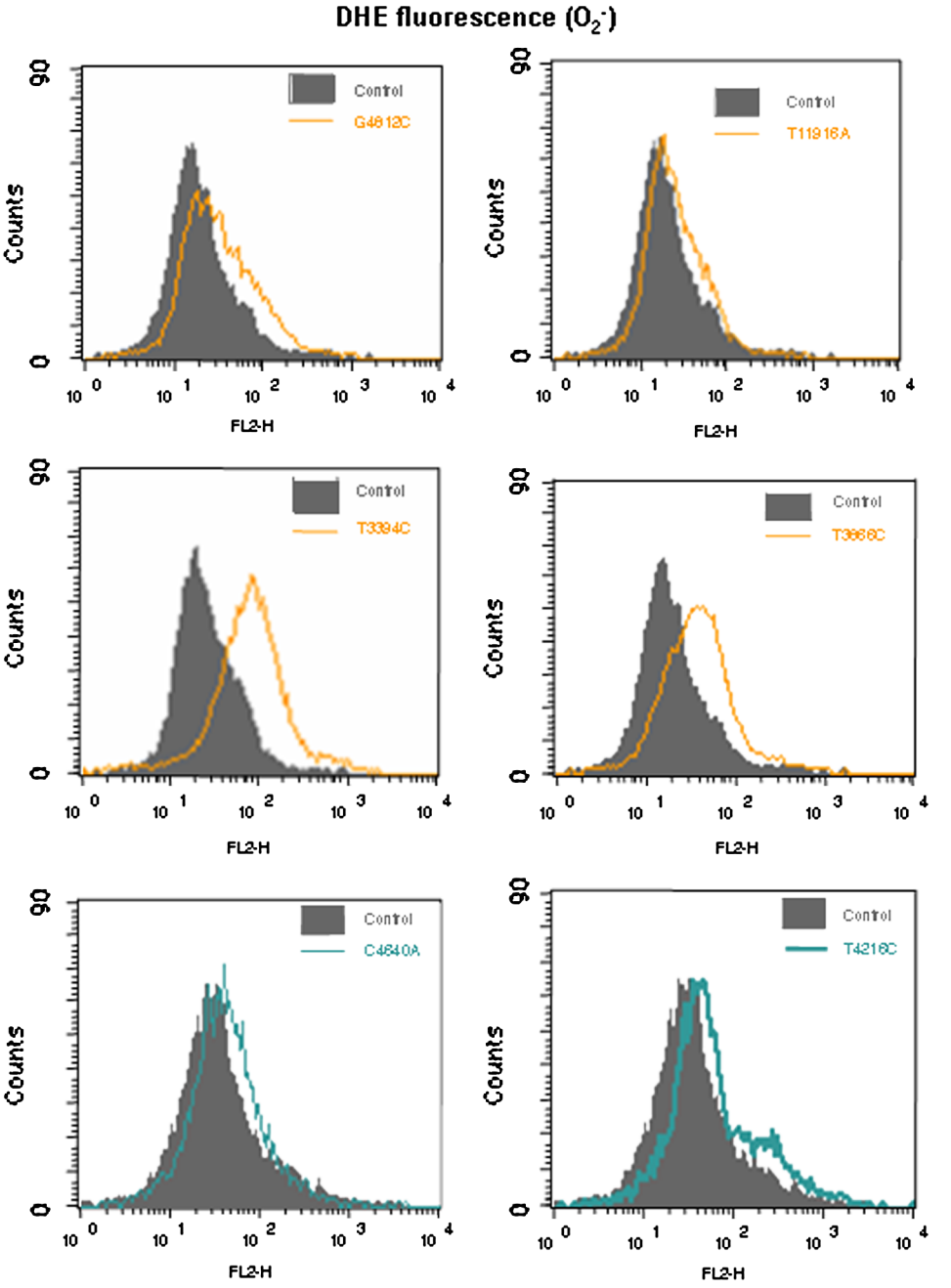

Figure 2 (*Continued*)

still remained significantly higher (~1.2–1.3-fold) than controls.

Discussion

The clinical data shown in Table I indicated that the above patients with mtDNA mutations previously reported by us had a typical mitochondrial disease. All these patients had either a severe or partial complex I deficiency, but whether this deficiency was because of these mtDNA mutations remains to be determined. As respiratory chain is an important source of ROS production, a defective respiratory chain may lead to an alteration in cellular ROS levels and thereby play an important role in disease progression and varied clinical phenotypes.

In this study, we demonstrated that ROS production is invariably high in complex I deficient lymphoblast cells established from these patients. Previous studies have also reported that complex I alone or along with complex III is the major source of ROS in cells [23–25]. Mutations in individual sub-units of complex I or III can also lead to a similar situation. The rate of $O_2^{\cdot-}$ formation by the respiratory chain is controlled by mass action and increases when the electron flow slows down [24]. Defectively assembled

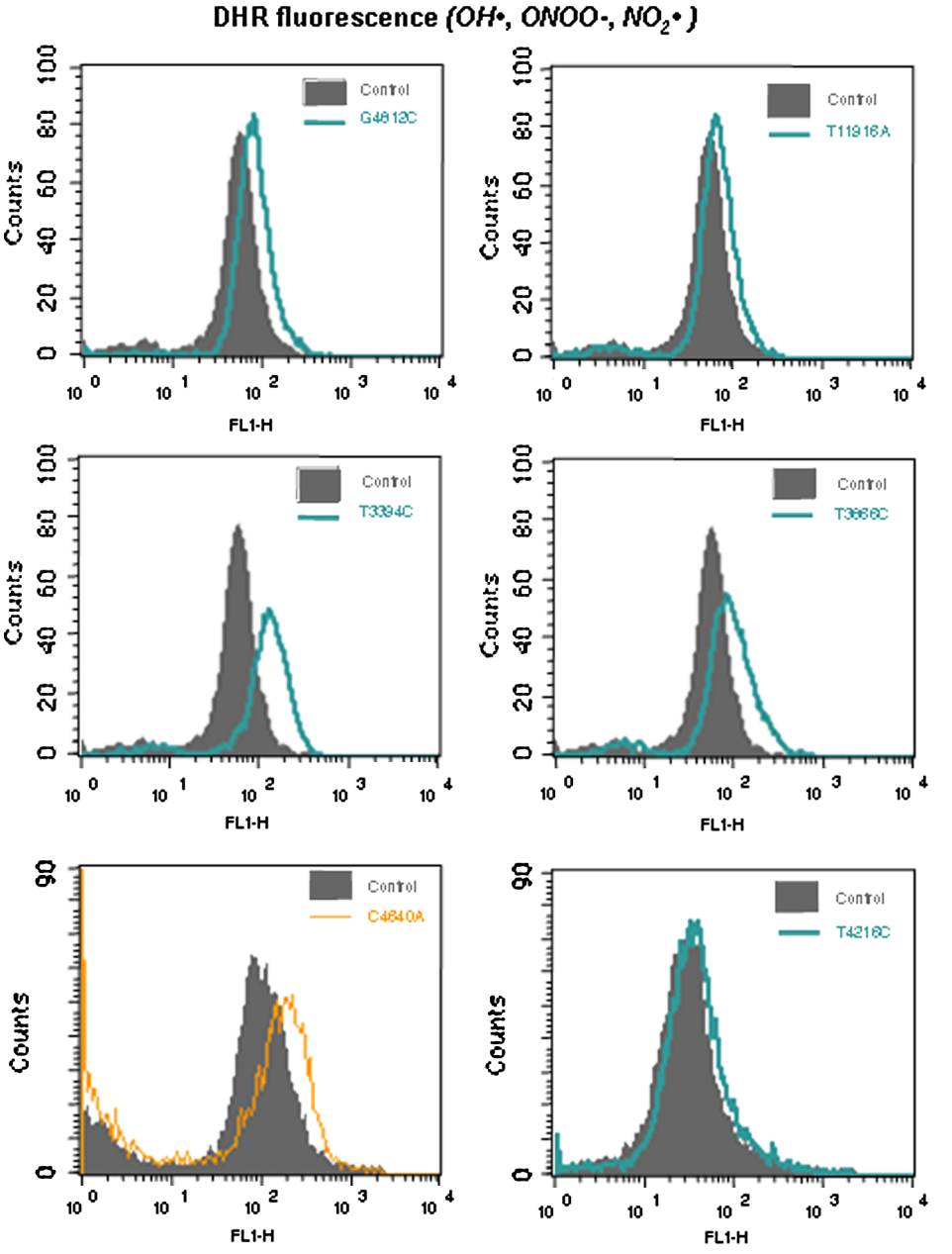

Figure 2. ROS production measured by flow cytometry in control and complex I deficient cell lines using the ROS-sensitive probes DCFDA, DHE and DHR (A) H_2O_2 production measured by DCF fluorescence (B) $O_2^{\cdot -}$ production (C) $OH^{\cdot}$, $NO_2^{\cdot}$ or $ONOO^-$ production.

complexes of the electron transport chain will have same adverse effects. Therefore, in complex I deficiencies, high ROS production is not only due to complex I defect but also due to complex III involvement, as complexes I and III are functionally related. This hypothesis has been recently reinforced by the demonstration that complex I assembly is dependent on the assembly of complex III [26].

There is ample evidence to show that both respiratory chain dysfunction and increased production of oxygen free radicals cause pathogenesis and results in progression of mitochondrial disease [27]. Most of the earlier studies regarding ROS and antioxidant defenses have been performed in patients with classical mitochondrial disorders such as MELAS/MERRF [28], NARP [29] and LHON [30–32]. In these studies, the ROS production and status of antioxidant defenses has been well correlated with disease-related mtDNA mutations. Recently, Chowdhury et al. [33] have also shown that the levels of antioxidant enzymes, CAT, MnSOD, CuZnSOD were upregulated in prostate cancer cell lines.

In a study on cybrids harbouring mitochondrial tRNA gene mutations (A3243G, A8344G), an increase in antioxidant enzyme activities was reported in response to an over-production of ROS in MELAS

Table II. mRNA expression profiling of the antioxidant enzymes in complex I deficient lymphoblast cell lines established from patients with mitochondrial encephalopathies.

	Catalase		GST		GPx		CuZnSOD		MnSOD	
	Untreated	tBHP treated	Untreated	tBHP treated	Untreated	tBHP treated	Untreated	tBHP treated	Untreated	tBHP treated
Control ($n=3$)	0.89±0.02	0.94±0.07	0.80±0.06	0.81±0.07	1.00±0.06	1.02±0.05	1.00±0.22	0.95±0.14	1.07±0.16	1.07±0.25
G4812C	1.12±0.09	1.15±0.09	0.94±0.12	0.93±0.10	1.31±0.17	1.32±0.10	1.14±0.10	1.15±0.10	1.10±0.14	1.16±0.20
T3866C	0.92±0.08	0.95±0.08	1.03±0.11	1.10±0.13	1.10±0.18	1.12±0.20	1.09±0.08	1.05±0.02	1.13±0.11	1.14±0.21
T3394C	1.44±0.09	1.43±0.01	1.25±0.13	1.27±0.08	1.40±0.21	1.43±0.20	1.27±0.12	1.31±0.22	1.51±0.22	1.50±0.30
T11916A	1.00±0.05	1.10±0.08	1.10±0.11	1.13±0.10	1.10±0.08	1.10±0.15	1.48±0.22	1.50±0.31	1.50±0.21	1.49±0.25
C4640A	1.10±0.05	1.00±0.03	1.08±0.05	1.09±0.09	1.10±0.14	1.09±0.07	1.06±0.30	1.06±0.32	1.17±0.12	1.18±0.20
T4216C	1.15±0.09	1.11±0.05	1.17±0.07	1.18±0.07	1.24±0.18	1.22±0.21	1.16±0.15	1.17±0.27	1.14±0.14	1.24±0.20

Figure 3. Western blotting analysis of MnSOD (25 kDa) protein in control and patient cells. 40 μg of cell protein extract was loaded in each lane on SDS/PAGE and blotted on PVDF. β-actin was used as a reference protein. The experiment was performed with only four patient cell lines with mtDNA genotypes as G4812C, T3394C, T3866C and T11916A. MnSOD protein was significantly increased in two patient cell lines as seen from lanes 3 and 5.

and MERRF cybrids, thus preventing oxidative damage [34]. Similarly, Floreani et al. [31] showed an increased protein expression while decreased enzyme activities of CAT, MnSOD, CuZnSOD and GPx in cybrids harbouring the LHON specific mutations. Further, Luo et al. [5], using fibroblast cultures with complex I deficiency, demonstrated low levels of MnSOD in cells with high ROS production, whereas the experiments of Esposito et al. [3] did not find any MnSOD increase in heart mitochondria of Ant1-deficient mice. Some hypotheses have been posed to explain this phenomenon, postulating a different tissue regulation in the expression of antioxidant enzymes, influence of redox state of the cell in enzyme activation [12] or decrease in antioxidant defenses due to mutations which produce high levels of ROS [35]. It seems that these earlier studies related to ROS production and antioxidant defenses

Figure 4. Bar graphs showing CuZnSOD protein levels in control and complex I deficient cell lines, as assessed by ELISA. Protein levels of CuZnSOD were slightly increased in patients but the increase was not statistically significant.

Table III. Activities of the antioxidant enzymes of complex I deficient lymphoblast cell lines established patients with mitochondrial encephalopathies.

	Catalase (Units/μg protein)		GPx (nmol/min/μg protein)		GR (Units/μg protein)		GST (μmol/min/ml)		SOD (μnits/0.1 mg protein)	
	Untreated	tBHP treated	Untreated	tBHP treated	Untreated	tBHP treated	Untreated	tBHP treated	Untreated	tBHP treated
Controls ($n=3$)	5.32±0.52	4.91±0.84	0.41±0.05	0.23±0.03	0.08±0.01	0.07±0.01	0.10±0.01	0.09±0.01	34.11±5.40	30.23±2.97
G4812C	3.38±0.29	3.01±0.46	0.20±0.02	0.05±0.01	0.07±0.01	0.06±0.01	0.09±0.01	0.04±0.00	39.80±2.85	32.45±2.03
T3866C	3.36±0.60	2.90±0.34	0.22±0.03	0.06±0.02	0.07±0.01	0.07±0.01	0.08±0.01	0.05±0.02	37.45±1.00	33.46±1.00
T3394C	4.08±0.34	2.62±0.52	0.24±0.04	0.08±0.05	0.07±0.01	0.06±0.01	0.07±0.02	0.04±0.01	44.53±5.90	39.82±3.33
T11916A	5.10±0.52	4.15±0.58	0.26±0.03	0.12±0.03	0.09±0.00	0.08±0.01	0.11±0.01	0.06±0.02	41.15±4.61	37.41±2.37
C4640A	4.15±0.32	3.00±0.37	0.38±0.03	0.15±0.01	0.08±0.01	0.08±0.01	0.10±0.01	0.06±0.01	43.00±3.85	38.12±2.99
T4216C	4.70±0.21	3.33±0.27	0.32±0.03	0.19±0.01	0.08±0.01	0.08±0.01	0.09±0.01	0.06±0.01	42.21±3.32	35.5±3.10

Figure 5. GSH concentrations in control and patient lymphoblasts before and after treatment with tBHP. No significant differences were observed between control and patient group in their intracellular glutathione levels.

in mitochondrial diseases are controversial. Hence, more thorough investigations are needed to correlate respiratory chain dysfunction with status of ROS and antioxidant defenses in different tissues accessible from patients with mitochondriopathies.

In the present study, based on our results with expression and activity analysis of antioxidant enzymes using lymphoblast cell lines from patients with complex I deficiency; we found an increased expression of almost all antioxidant enzymes at mRNA level but a significant decline in activities of three major cytosolic and peoxisomal enzymes such as GPx, CAT and GST. Any significant increase in SOD activity must be accompanied by a comparable increase in CAT and/or GPx activity to prevent the excessive accumulation of H_2O_2 in the cell and the failure of which can cause catastrophe [28]. A high SOD in comparison with GPx and/or CAT leads to increased production of H_2O_2 whereas low SOD relative to GPx and/or CAT could accumulate superoxide anions [28]. Several studies have shown that the cells over-expressing CuZnSOD were more susceptible to DNA strand breaks, growth retardation and related effects due to over-production and accumulation of $O_2^{\cdot -}$ and H_2O_2 [36,37]. However, it was also observed that some cells adapt themselves by an increase in GPx or CAT activity. In our study even though the expression of GPx and CAT was found to be upregulated in patients, the low enzyme activity may be attributed to post-translational events. It is well known that GPx activities are significantly decreased in the presence of ROS or in a condition of drug-induced ROS generation [38]. CAT inactivation has been ascribed to the presence of H_2O_2 [39]. We believe that the decrease in GPx activities displayed by patient cells may reflect the higher ROS concentrations in these cell lines. When an external ROS signal was applied in the medium in terms of tert-butyl hydroperoxide, we observed a

drastic decline in the GPx, GST and CAT activities; the decrease was significantly higher in patient cells than controls.

Interestingly, we found an increased SOD activity in patient cells that was well correlated with a high RNA and protein expression. As shown by ELISA and western blot analysis, the expression of MnSOD and CuZnSOD protein seems to be slightly increased in patients.

Complex I impairment in a variety of human diseases has been previously reported in association with MnSOD induction; although not all patients defective in complex I displayed an increase in MnSOD activity [12]. Furthermore, not all tissues have the same ability to upregulate MnSOD, as suggested by the different behaviour of skeletal and cardiac muscles observed in the ANT1-knockout animal model of oxidative stress [3]. In this context it is worth noting that MnSOD and CuZnSOD expression increases several fold in *Saccharomyces cerevisiae* during menadione-induced oxidative stress, without a parallel increase in their activities [40]. These results and our results reveal that MnSOD and CuZnSOD are less sensitive to oxidative damage than other antioxidant enzymes such as CAT, GPx and GST. This was also confirmed by tBHP treatment, a short treatment of cells with tBHP resulted in drastic decline in activity of GPx, CAT and GST, but not a significant decline in SOD was observed by the same treatment.

Our results suggested that complex I deficiency causes an elevated ROS in childhood encephalomyopathies. There is an increase in the majority of the antioxidant enzymes at transcription or translational level, suggesting compensatory mechanisms to overcome the deleterious consequences of oxidative damage. However, the activities of crucial peroxisomal and cytosolic antioxidant enzymes such as CAT, GPx and GST are impaired, being ROS sensitive. The activity of SOD probably MnSOD remained to be elevated in patient-derived OXPHOS deficient cell lines, most active in cells having severe complex I deficiency compared to those having a partial defect. As MnSOD is the mitochondrial enzyme, the overexpression of which also reflects that the ROS accumulated in patient cells is of mitochondrial origin and possibly generated by defective respiratory chain. The survival and growth of these defective cell lines *in vitro* is probably because of proper maintenance of glutathione levels and GR activity. In our experiments, we did not directly measure c-glutamylcysteine synthetase (CGS) expression or activity. However, the possibility is that the patient cells may respond to oxidative stress by increasing their GSH content through increased CGS activity [41] and increased transcription of regulatory sub-unit of CGS [42].

We could not find a very good correlation of ROS production with complex I defect and antioxidant enzyme activity in all the studied patients. It can be clearly seen from Table III that the SOD activity was increased in G4812C, T3866C, C4640 and T3394C, whereas the activities of CAT and GPx were lowered in the same cell lines as compared to controls. A higher ROS (all three species H_2O_2, O_2^- $OH^{\bullet}$) production by cell line T3394C was correlating well with the severe complex I assembly defect in this cell line. However, the same was not true with the T11916A cell line which was also displaying a complex I assembly defect. A possible reason for this could be that the T11916A mtDNA mutation in this cell line was heteroplasmic in nature, which maintained some of the complex I in assembled form (Figure 1) and thereby a low ROS level compared to T3394C cells (Figure 2B). From these results, it seems that ROS production by complex I defective cell lines depends upon the status of mtDNA mutation, e.g. its degree of heteroplasmy or pathogenecity. Also a high O_2^- production was well correlating with low SOD activity in T3866C cell line. Overall, a perfect correlation of ROS production with mitochondrial respiratory chain defect and antioxidant enzyme activity is difficult to establish *in vitro* due to instability of ROS and limitations of measurement techniques.

Further studies are needed to support the above results by analysing ROS and antioxidant parameters in more patients with mitochondrial disease involving complex I deficiency. However, we are not sure whether there is any role played by the variants found in the mitochondrial DNA of these complex I deficient cell lines. The role of mtDNA variants cannot be ignored and the clear-cut analysis whether the OXPHOS deficiency is due to mtDNA or nuclear DNA remains to be determined. However, database search and bioinformatics analysis showed that the variants are unique and may have some pathogenic significance [13].

In earlier studies [43], it was found that lipid peroxides and fluorescent adducts of aldehyde with plasma proteins are elevated in blood cells of patients with CPEO syndrome. Hence, our results also suggest that peripheral lymphocytes can be a suitable system to study the status of ROS and antioxidant defenses in patients with ROS specific clinical symptoms, particularly involving complex I deficiency.

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References

[1] DiMauro S, Hirano M. Mitochondrial encephalomyopathies: an update. Neuromuscul Disord 2005;15:276–286.

[2] Suzuki H, Kumagai T, Goto A, Sugiura T. Increase in intracellular hydrogen peroxide and upregulation of nuclear respiratory gene evoked by impairment of mitochondrial electron transfer in human cells. Biochem Biophys Res Commun 1998;249:542–545.

[3] Esposito LA, Melov S, Panvo A, Cottrell BA, Wallace DC. Mitochondrial disease in mouse results in increased oxidative stress. Proc Natl Acad Sci USA 1999;96:4820–4825.

[4] Wallace DC. Mitochondrial disease in man and mouse. Science 1999;283:1482–1488.

[5] Luo X, Pitkanen S, Kassovska-Bratinova S, Robinson BH, Lehotay D. Excessive formation of hydroxyl radicals and aldehydic lipid peroxidation products in cultured skin fibroblasts from patients with Complex I deficiency. J Clin Invest 1997;99:2877–2882.

[6] Jazin EE, Cavelier L, Eriksson I, Oreland L, Gyllensten U. Human brain contains high levels of heteroplasmy in the noncoding regions of mitochondrial DNA. Proc Natl Acad Sci USA 1996;93:12382–12387.

[7] de la Asuncion JG, Millán A, Pla R, Bruseghini L, Esteras A, Pallardo FV, Sastre J, Viña J. Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA. FASEB J 1996;10:333–338.

[8] Hayakawa M, Hattori K, Sugiyama S, Ozawa T. Age-associated oxygen damage and mutations in mitochondrial DNA in human heart. Biochem Biophys Res Commun 1992;189:979–985.

[9] Turrens JF. Superoxide production by the mitochondrial respiratory chain. Biosci Rep 1997;17:3–8.

[10] Du G, Mouithys-Mickalad A, Sluse FE. Generation of superoxide anion by mitochondria and impairment of their functions during anoxia and reoxygenation in vitro. Free Radic Biol Med 1998;25:1066–1074.

[11] Bolter CJ, Chefurka W. Extramitochondrial release of hydrogen peroxide from insect and mouse liver mitochondria using the respiratory inhibitors phosphine, myxothiazol, and antimycin and spectral analysis of inhibited cytochromes. Arch Biochem Biophys 1990;278:65–72.

[12] Pitkanen S, Robinson BH. Mitochondrial complex I deficiency leads to increases production of superoxide radicals and induction of superoxide dismutase. J Clin Invest 1996;98:345–351.

[13] Wani AA, Ahanger SH, Bapat SA, Rangrez AY, Hingankar N, Suresh CG, Barnabas S, Patole MS, Shouche YS. Analysis of mitochondrial DNA sequences in childhood encephalomyopathies reveals new disease-associated variants. PLoS ONE 2007;29:e942.

[14] Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P, Hewitt WR. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. Toxicol Appl Pharmacol 1985;78:473–483.

[15] Nijtamans LG, Henderson NS, Holt IJ. Blue native electrophoresis to study mitochondrial and other protein complexes. Methods 2002;26:327–334.

[16] Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 2004; 142:231–255.

[17] El Mouatassim S, Guerin P, Menezo Y. Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. Mol Hum Reprod 1999; 5:720–725.

[18] Del Maestro RF, McDonald W. Oxidative enzymes in tissue homogenates. In: RA Greenwald, editor. Handbook of methods for oxygen radical research. Boca Raton, FL: CRC Press; 1985. p 291–296.

[19] Flohe L, Gunzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114–121.

[20] Oberley LW, Spitz DZ. Assay of superoxide dismutase activity in tumor tissue. Methods Enzymol 1984;105:457–464.

[21] Habig WH, Pabst MJ, Jakoby WB. Gluthatione-Stransferase:the first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–7139.

[22] Halliwell B, Gutteridge JM. The definition and measurement of antioxidants in biological systems. Free Radic Biol Med 1995;18:125–126.

[23] Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem J 1980;191:421–427.

[24] Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys 1985;237: 408–414.

[25] Chan TS, Teng S, Wilson JX, Galati G, Khan S, O'Brien PJ. Coenzyme Q cytoprotective mechanisms for mitochondrial complex I cytopathies involves NAD(P)H: quinone oxidoreductase 1(NQO1). Free Radic Res 2002;36:421–427.

[26] Acin-Perez R, Bayona-Bafaluy MP, Fernandez-Silva P, Moreno-Loshuertos R, Perez-Martos A, Bruno C, Moraes CT, Enriquez JA. Respiratory complex III is required to maintain complex I in mammalian mitochondria. Mol Cell 2004; 13:805–815.

[27] Pang CY, Lee HC, Wei YH. Enhanced oxidative damage in human cells harboring A3243G mutation of mitochondrial DNA: implication of oxidative stress in the pathogenesis of mitochondrial diabetes. Diabet Res Clin Pract 2001;54(Suppl 2):S45–S56.

[28] Wei YH, Lu CY, Wei CY, Ma YS, Lee HC. Oxidative stress in human aging and mitochondrial disease-consequences of defective mitochondrial respiration and impaired antioxidant enzyme system. Chin J Physiol 2001;44:1–11.

[29] Mattiazzi M, Vijayvergiya C, Gajewski CD, DeVivo DC, Lenaz G, Wiedmann M, Manfredi G. The mtDNA T8993G (NARP) mutation results in an impairment of oxidative phosphorylation that can be improved by antioxidants. Hum Mol Genet 2004;13:869–879.

[30] Beretta S, Mattavelli L, Sala G, Tremolizzo L, Schapira AHV, Martinuzzi A, Carelli V, Ferrarese C. Leber hereditary optic neuropathy mtDNA mutations disrupt glutamate transport in cybrid cell lines. Brain 2004;127:2183–2189.

[31] Floreani M, Napoli E, Martinuzzi A, Pantano G, De Riva V, Trevisan R, Bisetto E, Valente L, Carelli V, Dabbeni-Sala F. Antioxidant defences in cybrids harboring mtDNA mutations associated with Leber's hereditary optic neuropathy. FEBS J 2005;272:1124–1135.

[32] Wong A, Cavelier L, Collins-Schramm HE, Seldin MF, McGrogan M, Savontaus ML, Cortopassi GA. Differentiation-specific effects of LHON mutations introduced into neuronal NT2 cells. Hum Mol Genet 2002;11:431–438.
[33] Chowdhury SK, Raha S, Tarnopolsky MA, Singh G. Increased expression of mitochondrial glycerophosphate dehydrogenase and antioxidant enzymes in prostate cancer cell lines/cancer. Free Radic Res 2007;41:1116–1124.
[34] Vives-Bauza C, Gonzalo R, Manfredi G, Garcia-Arumi E, Andreu AL. Enhanced ROS production and antioxidant defenses in cybrids harbouring mutations in mtDNA. Neurosci Lett 2006;391:136–141.
[35] Vergani L, Florean M, Russell A, Ceccon M, Napoli E, Cabrelle A, Valente L, Bragantini F, Leger B, Dabbeni-Sala F. Antioxidant defences and homeostasis of reactive oxygen species in different human mitochondrial DNA-depleted cell lines. Eur J Biochem 2004;271:3646–3656.
[36] de Haan JB, Cristiano F, Iannello R, Bladier C, Kelner MJ, Kola I. Elevation in the ratio of Cu,Zn-superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydrogen peroxide. Hum Mol Genet 1996;5:283–292.
[37] Li N, Oberley TD, Oberley LW, Zhong W. Inhibition of cell growth in NIH/3T3 fibroblasts by overexpression of manganese superoxide dismutase: Mechanistic studies. J Cell Physiol 1998;175:359–369.
[38] Pigeolet E, Remacle J. Susceptibility of glutathione peroxidase to proteolysis after oxidative alterations by peroxides and hydroxyl radicals. Free Radic Biol Med 1991;11:191–195.
[39] Pigeolet E, Corbisier P, Houbion A, Lambert D, Michileis C, Raes M, Zachary MD, Remacle J. Glutathione peroxidase, superoxide dismutase and Catalase inactivation by peroxides and oxygen derived free radicals. Mech Ageing Dev 1990; 15:283–297.
[40] Cyrne L, Martins L, Fernandes L, Marinho HS. Regulation of antioxidant enzymes gene expression in the yeast Saccharomyces cerevisiae during stationary phase. Free Radic Biol Med 2003;34:385–393.
[41] Shi MM, Kugelman A, Iwamoto T, Tian L, Forman HJ. Quinone-induced oxidative stress elevates glutathione and induces c-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. J Biol Chem 1994;269:26512–26517.
[42] Tian L, Shi MM, Forman HJ. Increased transcription of the regulatory subunit of gamma-glutamylcysteine synthetase in rat lung epithelial L2 cells exposed to oxidative stress or glutathione depletion. Arch Biochem Biophys 1997;342:126–133.
[43] Piccolo G, Banfi P, Azan G, Rizzuto R, Sandona D, Bellomo G. Biological markers of oxidative stress in mitochondrial myopathies with progressive external ophthalmoplegia. J Neurol Sci 1991;105:57–60.

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