Mutation at position 5628 in the mitochondrial tRNAAla gene in a Chinese pedigree with maternally diabetes mellitus

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Abstract

Background: To investigate the clinical, genetic and molecular characteristics of mitochondrial diabetes mellitus (MDM).

Methods: Resultant variants were evaluated for evolutionary conservation, allelic frequencies, and structural and functional consequences. The mitochondrial function including mitochondrial tRNAAla levels, protein synthesis, membrane potential, adenosine triphosphate (ATP) production, and reactive oxygen species (ROS) generation were measured using lymphoblastoid cell lines carrying the m.5628T>C mutation and 2 controls .

Results: We observed differences in the severity and age of onset in diabetes in affected maternally-related individuals, and through amolecular of the complete mitochondrial genome in this family, we identified a homoplasmic m.5628T>C mutation, located at conventional position 31 of tRNAAla, and we further detected distinct sets of mtDNA polymorphisms belonging to haplogroup L1. The identified mutation was further found be important for tRNA identity and stability. Using cellular models, we were able to determine that the respiratory deficiency caused arising as a consequence of the m.5628T>C mutation led to decreased efficiency of mitochondrial tRNAAla levels, protein synthesis, mitochondrial ATP synthesis and a reduced mitochondrial membrane potential.These mitochondrial dysfunctions caused an increase in the production of reactive oxygen species in the mutant cell lines.

Conclusions: These data provide a direct evidence that novel m.5628T>C mutation may be associated with MDM, thus, offering novel insights into the understanding of pathophysiology of MDM.

1. Background

Diabetes mellitus (DM) is a serious disease affecting upwards of 10% of the adult population in China [1,2]. This disease is multifactorial in nature, arising in a heterogeneous fashion owing to interactions between both genetic and environmental factors[3]. As mitochondrial diabetes mellitus (MDM) is a serious metabolic disease, and mitochondria serve asessential metabolic regulators within cells, these organelles are thought to be central to the pathogenesis of MDM [4]. As multiple studies have found that MDM risk can be transmitted in a matrilineal fashion, this further supports the potential for a mitochondrial role in the development of this disease [2,5]. Indeed, some studies have proposed that mutations within mitochondrial tRNA sequences are key factors influencing the development of MDM and other metabolic diseases [6]. The 3243A>G, and m.3264T>C mutations in the $tRNA^{Leu(UUR)}$, the m.4291T>C mutation in the $tRNA^{Ile}$, the m.14709T>C and m.14692A>G mutations in the $tRNA^{Glu}$, and the m.10003T>C mutation in the $tRNA^{Gly}$ have all been identified as potential mutations affecting MDM disease risk [7–12]. Exactly how these mutations ultimately contribute to disease onset, however, remains poorly understood, and as such there is a clear need for additional research into how mitochondrial dysfunction can act to mediate the onset or progression of MDM.

During a MDM genetic screening effort among 100 Chinese subjects, we were able to detect a T-to-C transition at position 5628 (conventional position 31) in the mitochondrial light(L)-strand in individuals with a familial history of matrilineal MDM transmission, and we further detected a set of variants belonging to the Asian haplogroup L1. The identified mutation is likely to disrupt normal base-pairing (31A–39U) for the $tRNA^{Ala}$ anticodon in the mitochondrial heavy(H)-strand, thereby resulting in its abnormal functionality and structure, thereby likely resulting in mitochondrial dysfunction. We therefore sought to explore the significance of this m.5628T>C mutation using lymphoblastoid cell lines in order to observe the effects of this mutation on mitochondrial functionality, with a particular focus on processes including mitochondrial $tRNA^{Ala}$ levels, protein synthesis, membrane potential, adenosine triphosphate (ATP) production, and reactive oxygen species (ROS) generation.

2. Methods

We initially recruited a Chinese MDM proband at the First Affiliated Hospital of Soochow University, China. After obtaining informed consent, we then conducted clinical evaluations and isolated blood samples from all family members who agreed to participate in this study. Any family or personal history of metabolic disease was identified through thorough historical and physical exams, and the proband and family members also underwent blood glucose testing at our hospital. In total, we recruited 100 control Chinese volunteers in order to screen for any mitochondrial tRNA mutations identical to those identified in the family the MDM patient. The Ethics Committees of Soochow University approved all study protocols. The principles of the Declaration of Helsinki were observed in this study [13].

2.2. Mitochondrial genome mutation analysis

We isolated total genomic and mitochondrial DNA from patient whole blood samples using a DNA Extraction Kit (QIAGEN: 51104). We then conducted PCR amplification of the whole mitochondrial genome of the proband subject with MDM (SZDM001-III–1), generating 24 overlapping PCR product fragments through the use of primers specific to the light and heavy strands, as in previous studies [12]. We then isolated these fragments and used an ABI 3700 automated DNA sequencer and a BigDye Terminator Cycle sequencing reaction kit to determine their sequences, which were compared to the updated consensus Cambridge sequence (GenBank accession number: NC_012920) [14].

In order to detect the m.5628T>C mutation of the mitochondrial $tRNA^{Ala}$ gene, we used the following primers in order to specifically amplify the 5238–6050 region of the mitochondrial DNA via PCR: F:5’-CTA ACC GGC TTT TTG CCC–3’ and R:5’-ACC TAG AAG GTT GCC TGG CT–3’, as in previous reports [11]. We then isolated and assessed these fragments as in previous reports [12].

2.3. Phylogenetic analyses

In order to perform interspecific analyses, as in previous studies, we compared human mtDNA nucleotide variation frequencies to sequences in 16 other species of vertebrates in order to determine the appropriate conservation index (CI) [15,16]. Based on this CI value, it was then possible to determine what percentage of these 16 species exhibit a conserved wild-type base at the position of interest.

We further used an online application (www.mitotool.org/genomeRSRS.html) in order to determine the mitochondrial haplogroup of the proband and related individuals, consistent with current haplogroup nomenclature [17,18].

2.4. Cell culture

The Epstein-Barr virus was used in order to generate immortalized lymphoblastoid cell lines, as reported previously [12]. Immortalized cells from the proband (III–1) and another subject (III–10) bearing the m.5628T>C mutation who had MDM, as well as from two controls (SZC1-control and SZC2-control) without the mutation but from the same haplogroup, were cultured using RPMI 1640 (Invitrogen) containing 10% FBS as well as 1% penicillin and streptomycin.

2.5 mtRNA Northern blotting

We extracted mitochondria from our control and mutant immortalized lines, and then isolated total mitochondrial RNA (mtRNA) using TRIzol, as in previous reports [19, 20]. We then electrophoretically separated 2 mg of this mtRNA onto a nylon membrane (Roche), prior to conducting a hybridization analysis using appropriate oligodeoxynucleotides as probes. We used digoxigenin (DIG)-labeled probes specific to 5S RNA, $tRNA^{Ala}$, $tRNA^{Thr}$, $tRNA^{Glu}$, or $tRNA^{His}$, and then used these for hybridization reactions and subsequent densitometric quantification, as in previous reports[19, 20].

2.6 Western blotting

Western blotting was conducted as in previous studies[19, 20]. Primary antibodies used herein include those targeting ND6(abcam ab81212), ND4(abcam ab219822), ATP6(proteintech 55313–1-AP), ND1(proteintech 19703–1-AP), and VDAC(proteintech 10866–1-AP). We used the peroxidase Affinipure goat anti-mouse IgG and goat anti-rabbit IgG (Jackson) as secondary antibodies, and an enhanced chemiluminescence (ECL) system (Millipore) was used for protein detection, after which densitometric calculations were performed.

2.7. Assessment of ATP levels

We utilized a CellTiter-Glo Luminescent cell viability assay (Promega, G7572) in order to assess levels of mitochondrial ATP based on a modified protocol provided by the manufacturer, with additional previously detailed modifications[20].

2.8. Mitochondrial membrane potential measurement

A JC–10 Mitochondrial Membrane Potential Microplate Assay Kit (Abcam, ab112134) was utilized based on provided protocols, as in previous studies, as a means of assessing the mitochondrial membrane potential [20]. Excitation/emission ratios of Ex/Em = 490/590 and 490/525nm were determined as a means of measuring ΔΨm in samples, with the relative ratio of these two values in mutants and controls being calculated to determine the ΔΨm value.

2.9. ROS measurements

The MitoSOX Red Mitochondrial Superoxide Indicator (Invitrogen, M36008) was used to assess ROS levels within live cells based on provided protocols, as in previous studies [21,22]. ROS production rates were determined based on geometric mean intensities in samples, and ratios of these intensity values between unstimulated and H_2O_2-stimulated cells were used to determine whether ROS generation was altered under oxidative stress conditions.

2.10. Statistical analysis

Microsoft Excel 2016 was used for all statistical testing, comparing values via unpaired, two-tailed t-tests. $P < 0.05$ was the threshold of significance unless otherwise indicated.

3. Results

3.1. Familial MDM presentation in a Han Chinese family

We identified a Chinese individual (SZDM001) that had visited the Diabetes Clinic at the First Affiliated Hospital of Soochow University (as shown in Figure 1). This patient underwent full physical examinations in an effort to identify any clinical or genetic factors associated with this instance of familial MDM. The patient was had been diagnosed with DM based upon standard diagnostic criteria: (1) fasting plasma glucose ≥ 7.0 mmol/L, (2) oral glucose tolerance ≥ 11.1 mmol/L, or (3) ≥6.5% glycated hemoglobin (HbA1c). Patients were diagnosed as having an elevated diabetes risk if they did not have a history of diabetes and met the following criteria: (1) fasting plasma glucose from 5.5–7.0 mmol/L, (2) 2 hour plasma glucose levels from 7.8–11.1 mmol/L, or (3) a HbA1c concentration of 5.7–6.4%. When a total of 13 matrilineal relatives were surveyed, a total of 5 were determined to have MDM (2 males and 3 females; as shown in Table 1). MDM symptoms were first diagnosed in these patients at the average age of 56 (range: 42–65), and other matrilineal relatives exhibited an elevated risk of diabetes based on the diagnostic criteria described above (data not shown). We did not observe any other significantly abnormal clinical conditions in these related individuals, such as cardiac, muscular, or neurological

diseases. No other surveyed members of this family exhibited MDM, leading to our determination that this family exhibited matrilineally-inherited MDM.

3.2. Identification of MDM-associated mitochondrial mutations

We next sequenced the mitochondrial genomes of the proband patient SZDM001-III–1 patient in an effort to determine whether there were specific mitochondrial mutations underlying the observed matrilineal MDM pattern of inheritance. After comparing our sequences from this patient to the Cambridge consensus sequence, we detected a total of 67 single nucleotide polymorphisms in the mitochondrial genome, and additionally determined them to be of the Eastern Asian mitochondrial haplogroup L1. Of these 67 mutations, 9 were previously described D-loop variants, 2 were known 12S rRNA gene variants, 3 were known 16SrRNA variants, one was the newly characterized m.5628T>C $tRNA^{Ala}$ mutation (as shown in Figure 2), 10 were known to be silent variants, and 8 were known missense mutations affecting protein-coding genes which were as follows: m.3316T>C(Ala>Thr) in the *ND1* gene, m.8860A>G (Thr>Ala) in the *ATP6* gene, m.10609T>C (Met>Thr) in the ND4L gene, m.12406G>A(Val>Ile) and m.13928G>C(Ser>Thr) in *ND5* gene, and m.14766C>T(Thr>Ile), m.15326A>G (Thr>Ala), and 15402C>T(Thr>Ile) in the *CYTB* gene. We next performed phylogenetic comparisons of these variants to known sequences of these same genes in 16 additional primate species in order to establish to what extent they were conserved. This comparison revealed the $tRNA^{Ala}$ m.5628T>C mutation to be 100% conserved across species, whereas there was no clear functional significance of the other surveyed variants. We also did not detect this m.5628T>C mutation in 100 Chinese control subjects, and we thus found no evidence that that any detected mutations other than m.5628T>C were evolutionarily conserved.

3.3. Decreased $tRNA^{Ala}$ levels

We found that mutant cell lines exhibited significantly decreased $tRNA^{Ala}$ levels as compared with controls, with the 5S RNA used for normalization within cells (as shown in Figure 3). Indeed, mutant cells exhibited $tRNA^{Ala}$ levels which were 80.41% ($P<0.05$) of those in control cells following normalization. In comparison, these mutant cells had steady-state $tRNA^{Thr,}$ $tRNA^{Glu}$, and $tRNA^{His}$ levels of 99.74%, 91.30% and 90.32%, respectively, relative to controls (as shown in Figure 3).

3.4. Reduced mitochondrial protein levels

We next assessed the levels of respiratory chain components encoded by the mitochondrial genome via Western blotting (as shown in Figure 4), using VDAC to control for equal protein loading. As shown in Figure 4, we found that levels of four proteins associated with mitochondrial translation in mutant cells were present at levels 8.05% - 26.04%, (average: 17.31%) relative to control cells ($P<0.05$). Cells bearing the m.5628T>C mutation also exhibited significant 18.60%, 26.04%, and 16.55% reductions in levels of the proteins ND6, ATP6, and ND1 proteins, respectively. These proteins contain between 4.6 and 8.8%

alanine codons. In contrast, reductions of the ND4 protein which contains 5.7% alanine codons was relatively limited, at 8.05% relative to control.

3.5. ATP generation is reduced in mutant cells

We next assessed how the m.5628T>C mutation influences ATP generation in the mitochondria of affected cells using a luciferin/luciferase assay system. Cells were combined with media containing glucose, with or without oligomycin (to inhibit ATP synthase and promote glycolysis) or with a combination of pyruvate and 2-deoxy-d-glucose (to inhibit glycolysis and promote oxidative phosphorylation). As shown in Figure 5, we found that glycolysis activity was comparable between mutant and control cells, whereas ATP production via oxidative phosphorylation was significantly reduced in mutant cells relative to controls, with ATP production being 68.28% and 65.24% (average 66.76%) that observed in control cells (P = 0.009).

3.6. Mitochondrial membrane potential changes

We next assessed how m.5628T>C mutations altered mitochondrial membrane potential (ΔΨm) using the JC–10 fluorescent probe. As shown in Figure 6, we observed decreased ΔΨm values in mutant cells bearing the m.5628T>C mutation, with decreases averaging 69.16% of control (68.50% and 69.82%) (P = 0.002). We found that ΔΨm in mutant cells treated with FCCP were similar to values in control cells.

3.7. Elevated ROS production

We used flow cytometry to assess ROS production by mutant and control cells both upon H_2O_2 stimulation and at baseline. As shown in Figure 7, we found that mutant cells bearing the m.5628T>C mutation exhibited increased ROS production, with an average increase of 120.46% relative to controls (P = 0.005).

4. Discussion

Herein, we determined that the $tRNA^{Ala}$ m.5628T>C mutation is associated with the matrilineal transmission of MDM risk in a Chinese family. This m.5628T>C mutation affects coding at position 31 of this tRNA, and the presence of an adenine at this position is highly evolutionarily conserved among species, suggesting it is essential for normal tRNA function or pre-tRNA processing [23,24]. We were only able to detect this mutation in members of a Chinese family exhibiting MDM, and not in 100 control subjects. As shown in Table 2, we observed impaired glucose tolerance in 5 (2 males/3 females) out of a total of 13 matrilineal relatives, compared with the average penetrance of MDM in other Chinese families carrying the m.10003T>C and m.14692A>G mutations [11, 12]. Average age of diabetes onset in those with the m.5628T>C mutation was 56 (range: 42—65), while the average ages in those families with

m.10003T>C and m.14692A>G mutations were 45 and 60 years, respectively [11,12]. As families with identical m.5628T>C mutations exhibit significant variability, this suggests that nuclear genes also play a role in modifying phenotypic manifestations of MDM in these individuals.

The observed mutation at the position 5628 (conventional position 31) is expected to lead to functional defects by interfering with normal base-pairing (31A–39U) in the mitochondrial H-strand of the $tRNA^{Ala}$ anticodon stem. These structural and functional shifts in this tRNA in turn mediate impaired mitochondrial functionality. We were able to utilize cell lines bearing this m.5628T>C mutation to provide clear evidence of multiple forms of mitochondrial dysfunction in these cells. Indeed, we found $tRNA^{Ala}$ levels to be clearly decreased in these mutant cells relative to controls (80.41%). It is possible that this mutated form of $tRNA^{Ala}$ has less metabolic stability, resulting in its more rapid degradation and thus reducing effective levels of the tRNA, as in the case of the of $tRNA^{Leu(UUR)}$ m.3243A > G mutation in the [25,26]. This mutation was also linked to significant reductions in 7 mitochondrially-encoded proteins, and all of these reductions were associated with a marked reduction in ATP synthesis, as well as increased oxidative stress that may be linked to defects in normal energetic processes. The observed 33.24% decrease in mitochondrial ATP in cells bearing the m.5628T>C mutation was similar to that in cells with the m.14692A>G mutation [11]. Impaired respiratory chain activity can also result in impaired mitochondrial membrane potential, which is closely linked to cell viability, and we found clear reductions in this potential by roughly 30.84% in mutant cells, indicating increased that mutant mitochondria were suffering from increased electron leakage during oxidative phosphorylation. These dysregulated mitochondrial activities were associated with excessive ROS generation in mutant cells, with a roughly 20.46% increase relative to controls. ROS production can in turn result in substantial damage to proximal proteins and other macromolecules, thereby impairing cell functionality.

- Conclusion

This work is the first to detect a link between the m.5628T>C mutation and MDM. Disrupted normal metabolism of this tRNA as a result of this mutation appears to significantly impair normal mitochondrial functionality, with impaired mitochondrial protein synthesis, membrane potential, ATP production, and ROS generation being evident in mutated cells. As such, this m.5628T>C mutation is clearly a relevant candidate biomarker for heritable MDM risk, making it useful for molecular diagnostics. Our results therefore have the potential to improve current understanding of MDM, offering new insights into disease development and suggesting potential avenues for treatment or prevention.

Declarations

Abbreviations

MDM: mitochondrial diabetes mellitus; DM: diabetes mellitus; ATP:adenosine triphosphate; ROS: reactive oxygen species; CI: conservation index.

Ethics approval and consent to participate

All subjects were willing to participate in the study and the written informed consent for clinical evaluations and genetic analysis were obtained from each participant. In addition, the protocol of the study was approved by the medical ethics committee of the Institutional Review Board of the First Affiliated Hospital of Soochow University.

Consent for publication

Written informed consent for publication of clinical details and/or clinical images was obtained from all participants.

Availability of data and material

We used an online application (www.mitotool.org/genomeRSRS.html) in order to determine the mitochondrial haplogroup.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KL and LW contributed to conception or design of the work, data collection, data analysis and interpretation, and drafting of the article. WL,TZ and QQ contributed to data collection, drafting of the article, and critical revision of the article. JL, WH, YS, XL and WC contributed to the data analysis and interpretation. KL contributed to conception or design of the work, data analysis and interpretation, and critical revision of the article. All authors approved the final version to be published.

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Tables

Table 1 Summary of clinical and biochemical data of members in one Chinese pedigree with T2DM.

Subjects	Gender	Age at onset (yrs)	Fasting glucose (mmol/L)	Oral glucose tolerance (mmol/L)	HbA1c (%)
III-1	M	50	16.1	17.9	7.7
III-10	F	36	9.1	17.7	6.2
II-3	M	58	10.1	16.5	7.1
II-6	F	56	11.3	15.9	7.3
I-2	F	85	6.8	10.1	5.9
II-2	F	61	6.5	10.8	5.3
II-7	M	55	6.5	9.8	4.1
III-4	F	46	6.7	8.4	3.8
III-5	M	38	4.8	8.9	4.6
III-7	M	35	5.1	7.7	5.1
III-11	M	27	5.6	7.9	4.3
IV-2	M	20	5.5	8.3	4.2
IV-6	F	19	4.2	8.1	4.8

Table 2. mtDNA variants in a Chinese pedigree with T2DM

Gene	Position	CRS	Replacement	AA change	Conservation (H/B/M/X)	Previously reported[a]
D-loop	73	A	G	-		Yes
	146	T	C	-		Yes
	152	T	C	-		Yes
	249	A	delA	-		Yes
	263	A	G	-		Yes
	310	T	CCTC	-		Yes
	514-515	CA	delCA	-		Yes
	16304	T	C	-		Yes
	16519	T	C	-		Yes
12S rRNA	750	A	G	-	A/A/A/-	Yes
	1438	A	G	-	A/A/A/G	Yes
16S rRNA	1734	C	T	-	C/C/C/T	Yes
	2706	A	G	-	A/G/A/A	Yes
	3316	G	A	Ala-Thr	A/I/I/I	Yes
ND1	3970	C	T	-		Yes
ND2	4769	A	G	-		Yes
$tRNA^{Ala}$	5628	T	C	-	T/T/T/T	No
CO1	6392	T	C	-		Yes
	6962	G	A	-		Yes
	7028	C	T	-		Yes
	7214	C	T	-		Yes
	7738	T	C	-		Yes
ATP6	8860	A	G	Thr-Ala	T/A/A/T	Yes
CO3	10310	G	A	-		Yes
ND3	10609	T	C	Met-Thr	M/T/T/T	Yes
ND4	11719	A	G	-		Yes
ND5	12406	G	A	Val-Ile		Yes
	12882	C	T	-		Yes
	13928	G	C	Ser-Thr	S/T/S/T	Yes
	14766	C	T	Thr-Ile	T/S/T/S	Yes
ND6	15326	A	G	Thr-Ala	T/M/I/I	Yes
Cytb	15402	C	T	Thr-Ile	T/P/P/P	Yes

a Conservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and Xenopus laevis (X). b CRS, Cambridge reference sequence [20]. c See online mitochondrial genome databases http://www.mitomap.org and http://www.genpat.uu.se/mtDB/.

Figures

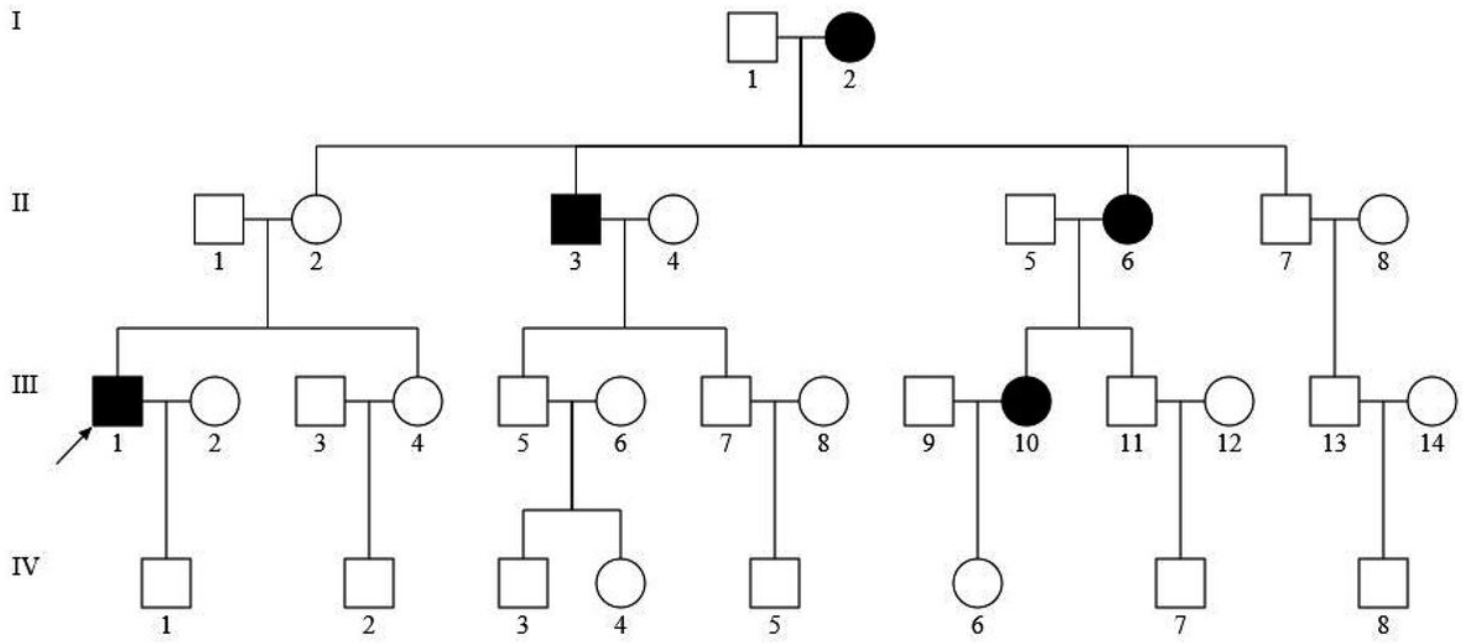

Figure 1

The Chinese pedigree with MDM. Vision-impaired individuals are indicated by blackened symbols.

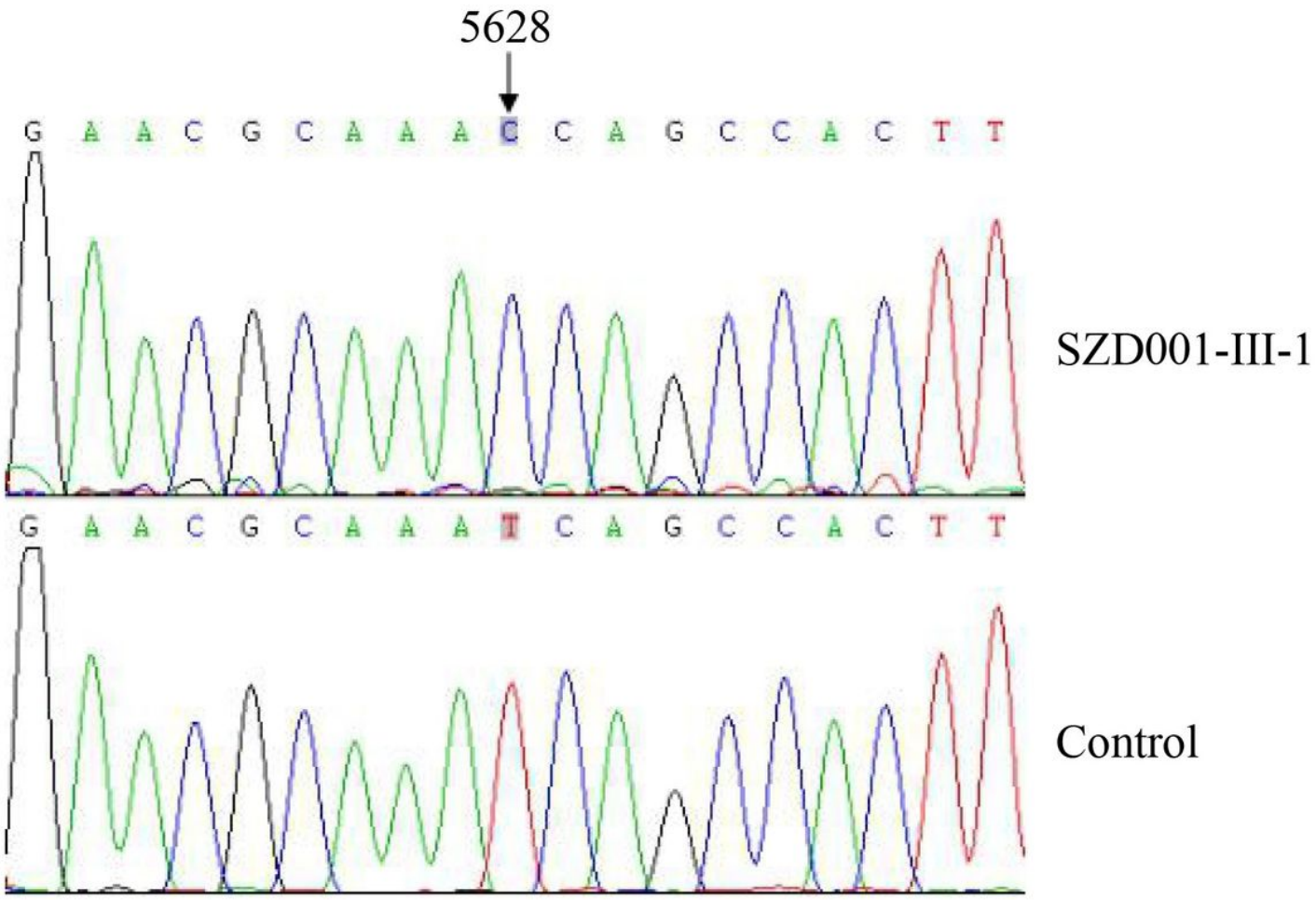

Figure 2

Identification of the m.5628T>C mutation in the tRNA gene. Partial sequences chromatograms of tRNA gene from the proband and one Chinese control. An arrow indicates the location of the base changes at position 5628.

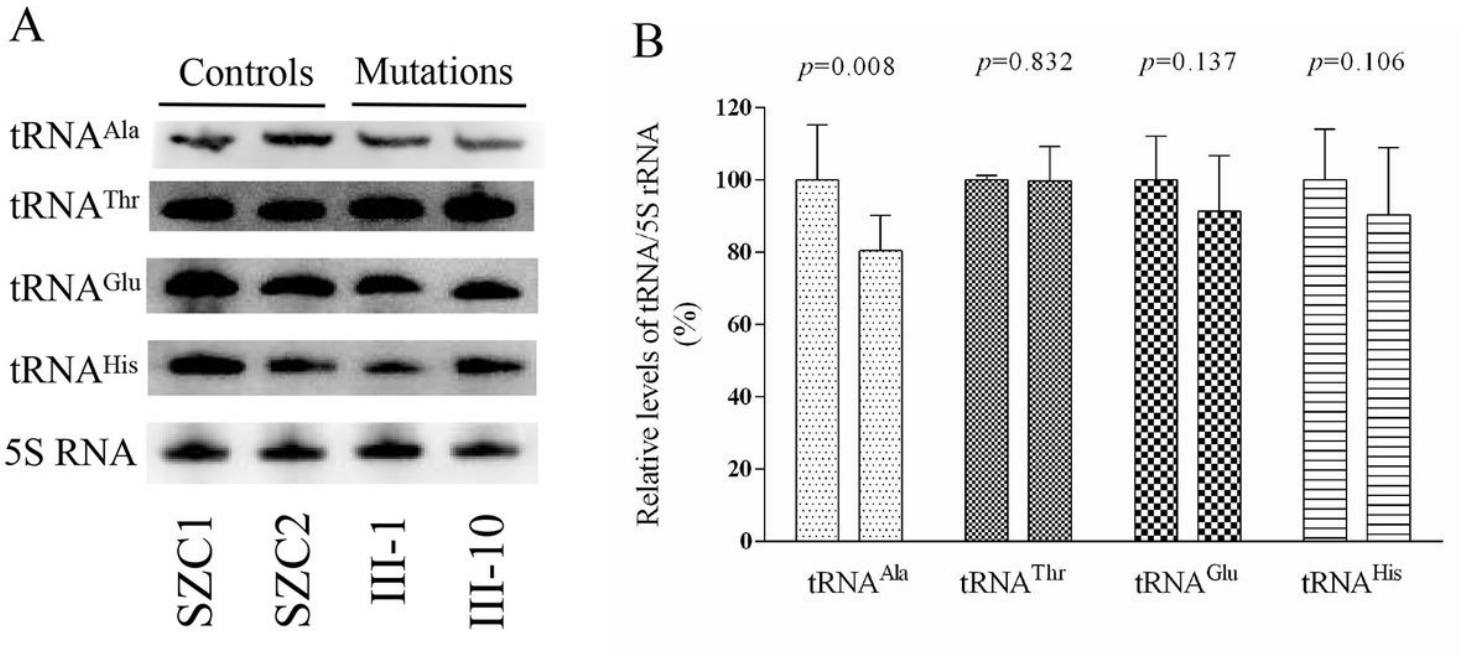

Figure 3

Northern blot analysis of mitochondrial tRNA. (A) Equal amounts of total mitochondrial RNA from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with DIG-labeled oligonucleotide probes specific for the tRNAAla, tRNAThr, tRNAGlu, and tRNAHis, respectively. (B) Quantification of tRNA levels. Average relative tRNA content per cell, was normalized to the average content per cell of 5S rRNA in two mutant cell lines carrying the m.5628T>C variant and two control cell lines (SZC1, SZC2). The values for the latter are expressed as percentages of the average values for the control cell lines.

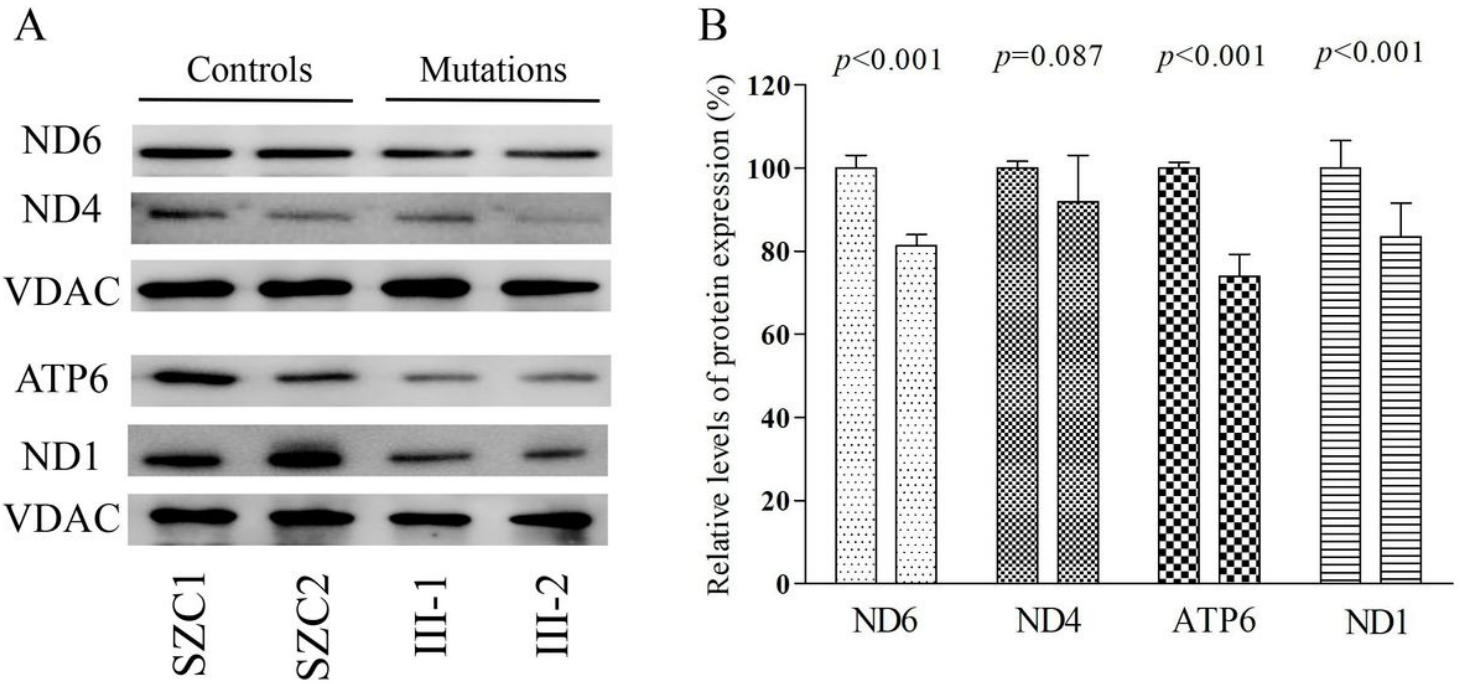

Figure 4

Western blot analysis of mitochondrial proteins. (A) Twenty micrograms of total cellular proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with respiratory complex subunits in mutant and control cells with VDAC as a loading control. (B) Quantification of 6 respiratory complex subunits. The levels of ND6, ND4, ATP6, and ND1 in two mutant cell lines and two control cell lines were determined. The error bars indicate two standard errors of the means. p indicates the significance, according to the t-test, of the differences between mutant and control cell lines.

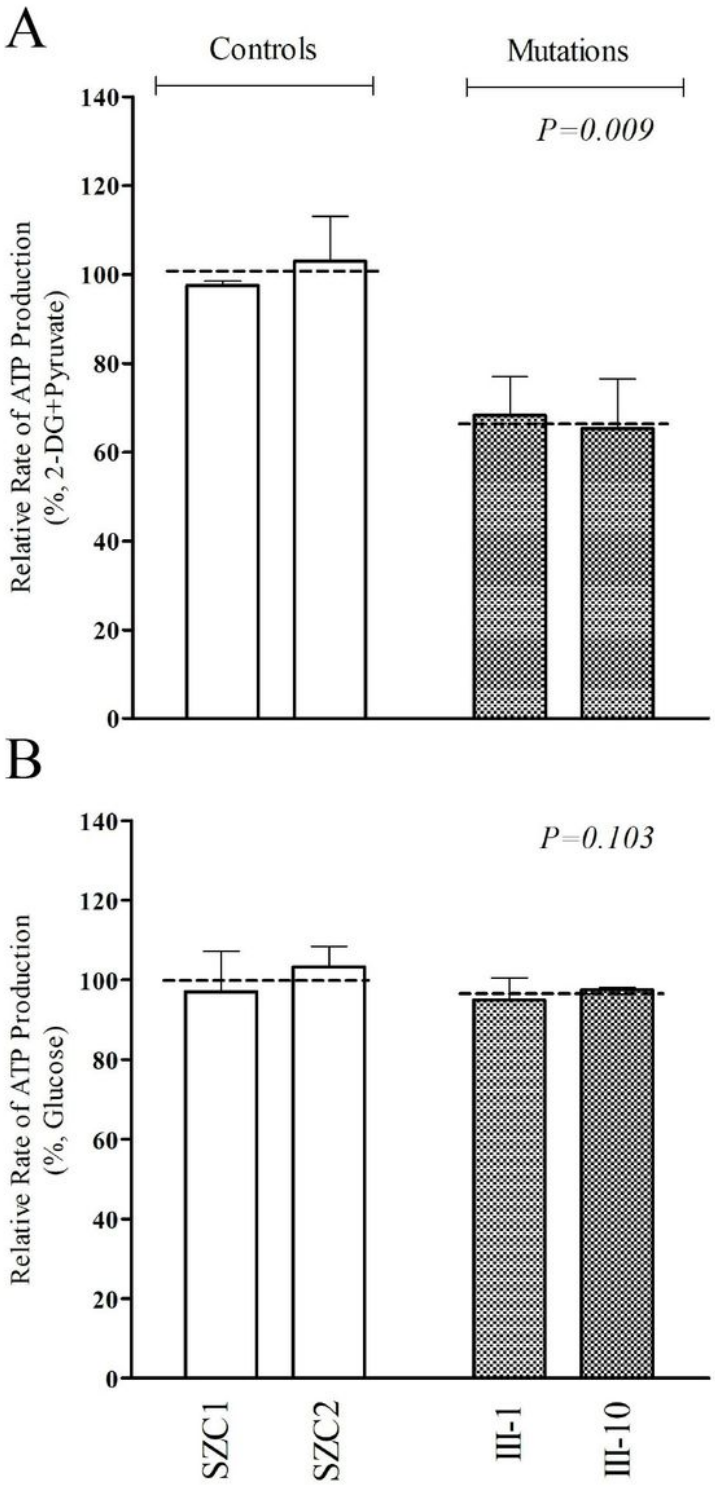

Figure 5

mitochondrial ATP levels. Mutant and control cell lines were incubated with 10 mM glucose or 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 in mitochondria are show. The determinations were made for each cell line.The calculations were based on the independent determinations in each cell line.

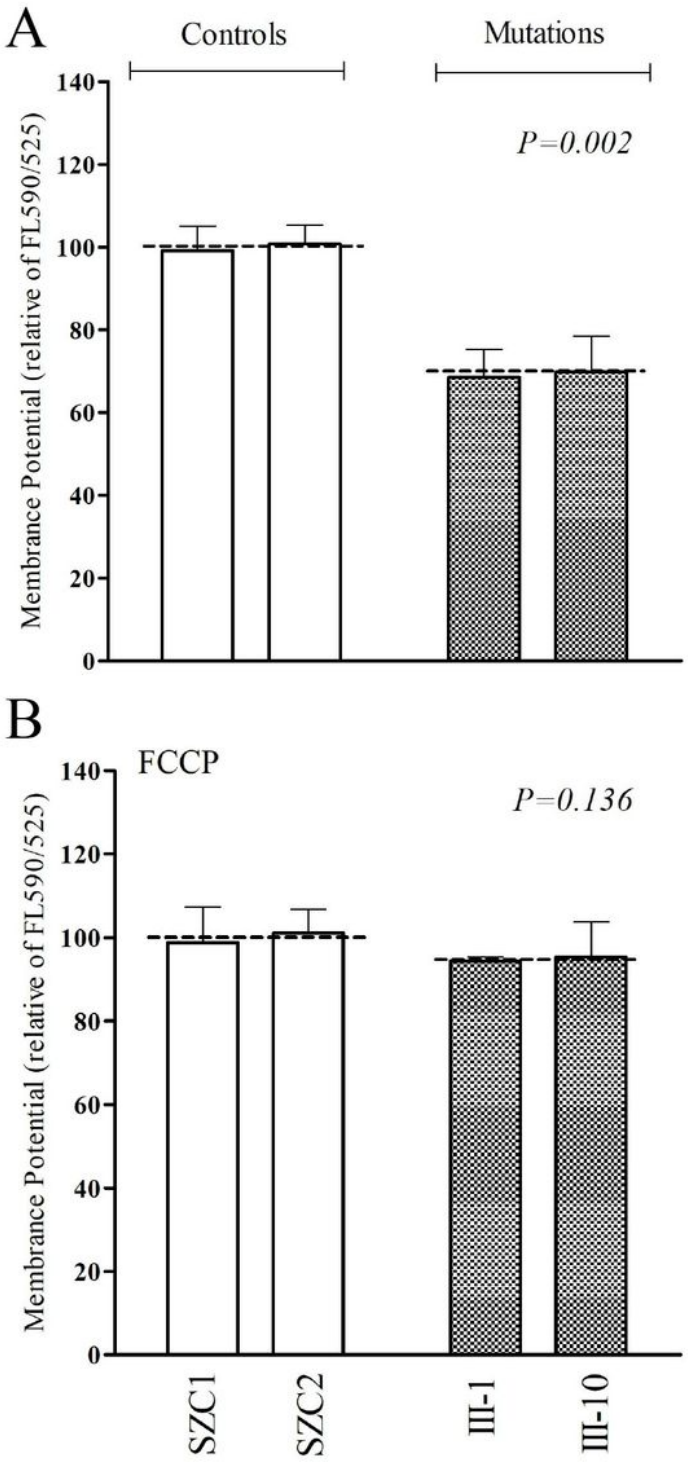

Figure 6

Mitochondrial membrane potential analysis. The mitochondrial membrane potential (ΔΨm) was measured in mutant and control cell lines using a fluorescence probe JC-10 assay system. The ratio of fluorescence intensities Ex/Em=490/590 nm and 490/530 nm (FL590/FL530) were recorded to delineate the ΔΨm level of each sample. The relative ratios of FL590/FL530 geometric mean between mutant and control cell lines were calculated to reflect the level of ΔΨm. Relative ratio of JC-10 fluorescence

intensities at Ex/Em = 490/530 nm and 490/590 nm in absence and presence of 10μM of carbonyl cyanide 3-chlorophenylhydrazone (FCCP). The average of 3 determinations for each cell line is shown.

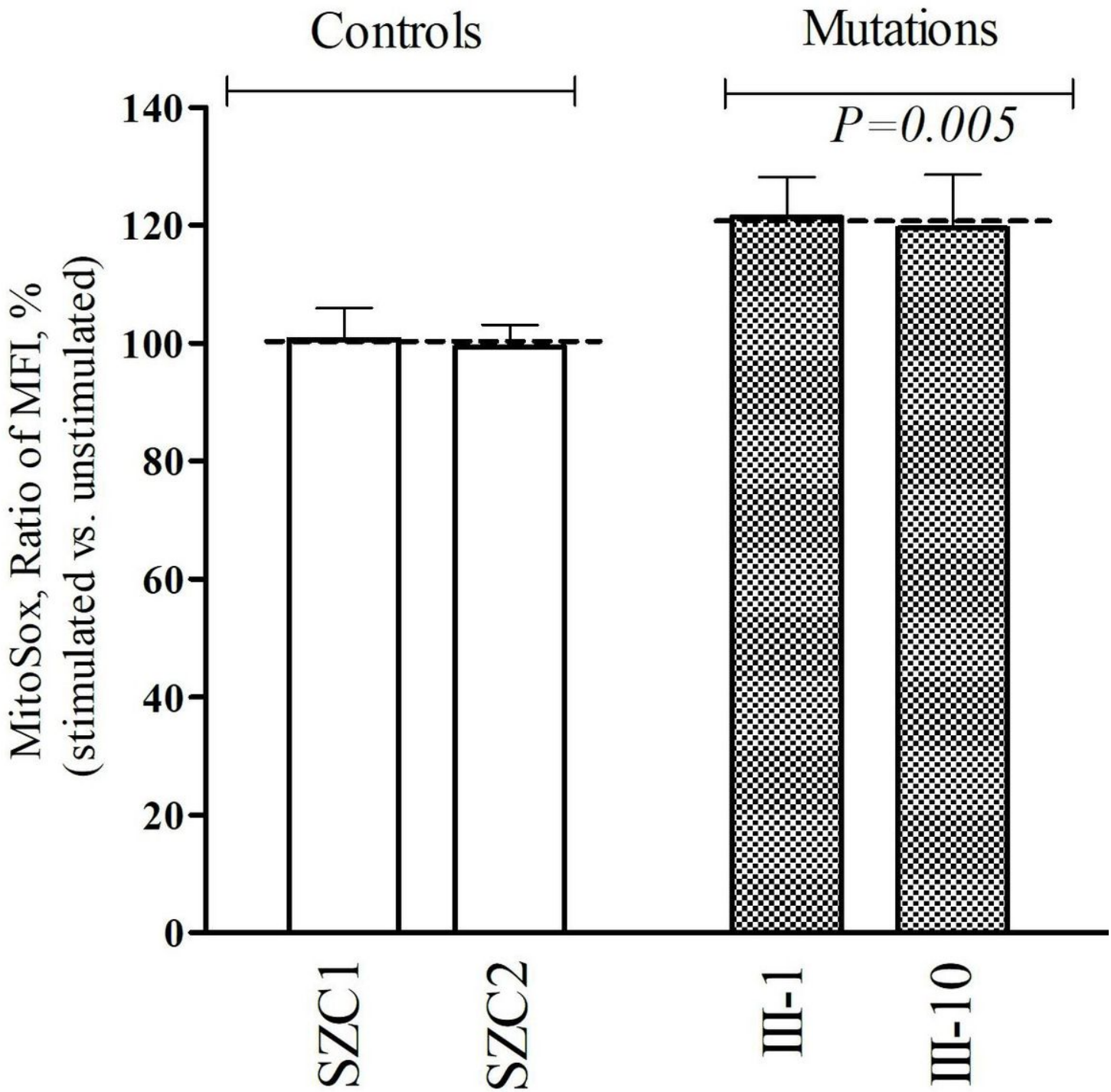

Figure 7

Ratio of geometric mean intensity. Measurement of mitoROS. The levels of ROS generation by mitochondria in living cells from mutant and control cell lines were determined using the mitochondrial superoxide indicator MitoSOX-Red. The average of the determinations for each cell line is shown. The

error bars indicate two standard errors of the means. p indicates the significance, according to the t-test, of the differences between mutant and control cell lines.