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Pathogenesis of the deafness-associated A1555G mitochondrial DNA mutation☆

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Abstract

The pathogenic mechanisms of the A1555G mitochondrial DNA mutation in the 12S rRNA gene, associated with maternally inherited sensorineural deafness, are largely unknown. Previous studies have suggested an involvement of nuclear factor(s). To address this issue cybrids were generated by fusing osteosarcoma cells devoid of mtDNA with enucleated fibroblasts from two genetically unrelated patients. Furthermore, to determine the contribution, if any, of the mitochondrial and nuclear genomes, separately or in combination, in the expression of the disease phenotype, transmitochondrial fibroblasts were constructed using control and patient's fibroblasts as nuclear donors and homoplasmic mutant or wild-type cybrids as mitochondrial donors. Detailed analysis of mutant and wild-type cybrids from both patients and transmitochondrial fibroblast clones did not reveal any respiratory chain dysfunction suggesting that, if nuclear factors do indeed act as modifier agents, they may be tissue-specific. However, in the presence of high concentrations of neomycin or paromomycin, but not of streptomycin, mutant cells exhibit a decrease in the growth rate, when compared to wild-type cells. The decrease did not correlate with the rate of synthesis or stability of mitochondrial DNA-encoded subunits or respiratory chain activity. Further studies are required to determine the underlying biochemical defect.

Keywords: Transmitochondrial; Nuclear; Cybrids; Aminoglycosides; rRNA; Paromomycin; Neomycin; Mutant; Wild type; Tissue-specific

Mitochondrial DNA (mtDNA) mutations have been associated with sensorineural deafness, either in isolation or in conjunction with neuromuscular disease or diabetes (for reviews see [1–3]). At least six different mutations have been identified in families with deafness as the main clinical feature. Five of these (Table 1) are located in the $tRNA^{Ser(UCN)}$ gene. In some cases, hearing loss was accompanied by other features, such as palmoplantar keratoderma [4], ataxia, and myoclonus [5].

The most common mutation associated with maternally inherited deafness is A1555G in the 12S rRNA gene [6], which has been reported in more than 120 affected families of different ethnic origins [1]. Some of these had familial hypersensitivity to aminoglycosides [7–9], while others had no history of aminoglycoside exposure [10–13]. Different clinical phenotypes have been associated with the mutation. In one family, sen-

☆ *Abbreviations:* mtDNA, mitochondrial DNA; COX I, COX II, COX III are subunits I, II, and III of cytochrome oxidase; CS, citrate synthase; ND1, ND2, ND3, ND4, ND4L, and ND5 are subunits of NADH dehydrogenase; A6 and A8 are subunits of ATP synthase; cyt *b*, apocytochrome *b*; SDH, succinate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BrdU, 5-bromodeoxyuridine; TK, thymidine kinase; R6G, rhodamine 6G; PEG, polyethylene glycol; HAT, hypoxanthine–aminopterin–thymidine; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; bp, base pair; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide.

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Table 1
Mutations in the tRNA $^{Ser(UCN)}$ of mtDNA associated with hearing loss

Nucleotide change	Position	References
A to G transition	7445	[4]
Insertion of C	7472	[5]
T to C transition	7511	[50]
T to C transition	7510	[51]
T to C at transition	7512	[52]

sorineural hearing loss was accompanied by Parkinson's disease [14]; in another, with a constellation of digital, spinal, and pigmentary disturbances [15]; and in a third family, Santorelli et al. [16] found the mutation in a 35-year-old woman with restrictive cardiomyopathy without hearing loss.

In contrast to syndromic mutations that usually affect only a fraction of all mtDNA molecules (heteroplasmy), mutations associated with nonsyndromic hearing loss are often homoplasmic and the clinical phenotype varies considerably among family members, ranging from profound congenital deafness to completely normal hearing. This variability is also found at the biochemical and molecular genetic levels. Cells harboring the tRNA$^{Ser(UCN)}$ mutations at or near homoplasmic levels showed only a slight inhibition of growth in glucose-free medium and had normal or near-normal levels of respiratory chain enzymes [17,18]. At the molecular genetic level, even though homoplasmic levels of the tRNA$^{Ser(UCN)}$ mutations seemed to cause a 30–40% decrease in the steady-state level of the tRNA, mitochondrial protein synthesis was impaired only in one family, harboring the A7445G mutation [19]. It is not clear why certain homoplasmic mutations cause deafness although they are present in all tissues. It is also not clear why these mutations express such variable clinical, biochemical, and molecular genetic features.

Several factors, such as nuclear genes, mtDNA haplotype, environmental agents, or tissue-specific effects, acting independently or in combination, may influence the clinical expression. Epidemiological data [20] and biochemical studies [21,22] in symptomatic and asymptomatic members of a family with hearing loss and the A1555G mutation suggested nuclear factors as the cause of these phenotypic differences. Linkage analysis of several pedigrees with the A1555G mutation further suggested that the nuclear factor may be a multigene [23,24] and a locus on chromosome 8 seemed to segregate with the phenotype, at least in some families [25]. However, to date, no nuclear modifier gene has been identified. In addition, an extensive study of 50 Spanish and four Cuban families found no correlation between the mutation and mitochondrial haplotypes [26].

The only environmental factor known to affect the A1555G mutation is the group of aminoglycoside antibiotics. The mutation lies in a highly conserved region of the 12S rRNA, the decoding site, that is also involved in the binding of aminoglycosides in bacteria [27–29]. Mutations in this region alter the ribosome's susceptibility to aminoglycosides and confer resistance to these drugs in bacteria and yeast [30–33]. Hamasaki and Rando [32] have demonstrated specific binding of aminoglycosides to the A1555G transition in a 12S rRNA construct and Guan et al. [34] have shown a decrease in the growth rates of homoplasmic mutant lymphoblastoid cell lines in the presence of these antibiotics. To investigate the potential role of nuclear DNA modifiers, we used a cell culture model to study two families with A1555G mutation, one with hearing loss, the other with cardiomyopathy as the only clinical feature.

Materials and methods

Patient 1 is a 35-year-old woman who had idiopathic restrictive cardiomyopathy without hearing loss or other clinical features of mitochondrial dysfunction [16]. Both the patient's mother and her maternal grandmother were affected, suggesting maternal inheritance. Direct sequencing of the mtDNA-encoded genes for 22 tRNAs, as well as the 12s and the 16s rRNAs, and the cytochrome *c* oxidase (COX) subunits did not reveal any mutations except the A → G change at nt-1555. PCR–RFLP analysis showed that the mutation was heteroplasmic in all the tissues studied, ranging from 50% to 95%.

Patient 2 is a 54-year-old woman with bilateral sensorineural deafness since age 18 unrelated to aminoglycoside administration [10]. Family history revealed matrilineal transmission of the hearing loss. A combination of PCR–SSCP was used to screen the 22 tRNA genes; direct sequencing of the two mtDNA-encoded rRNA genes revealed the A1555G transition. The mutation was homoplasmic in all the family members studied.

Cell culture – generation of cybrids. Skin fibroblasts from both patients 1 and 2 were grown in DMEM medium containing 4.5 mg glucose per ml and 110 μg pyruvate per ml supplemented with 15% FBS. Transmitochondrial cybrids were produced by fusion of enucleated fibroblasts from patient 1 (containing 50% mutation) and from patient 2 (harboring homoplasmic level of the mutation) with the mtDNA-less (ρ°) osteosarcoma (143B) cell line [35]. The cells were replated 24 h after fusion in DMEM containing 100 μg BrdU per ml and supplemented with 10% dialyzed FBS (selection medium). Individual cell clones were isolated 10–20 days later using glass cylinders.

Generation of transmitochondrial fibroblasts. Transmitochondrial fibroblast clones were constructed using fibroblasts from a normal control and from patient 1 (nuclear donors) and homoplasmic mutant (M) or wild-type (WT) cybrids with the TK$^-$ osteosarcoma nuclear background harboring the A1555G mutation (mitochondrial donors) as shown schematically in Fig. 1A. To eliminate the endogenous mtDNA, subconfluent cultures of control and patient fibroblasts were treated with 2.5 μg/ml R6G in DMEM supplemented with 50 μg/ml uridine, [36,37]. They were then fused with cytoplasts derived from either the 100% WT (YA116) or the 100% M (YA117) cybrids enucleated with cytochalasin B, using 50% PEG [35]. The cells were selected in medium supplemented with HAT [36,37] and individual clones were isolated. This protocol yielded four different fibroblast clones harboring WT or M mitochondria in combination with control or patient nuclei. PCR–RFLP analysis of total genomic DNA from the four trans-nuclear fibroblast cell lines confirmed the level (0% or 100%) of the mtDNA mutation present in the original cybrids that were used for fusions (Fig. 1B).

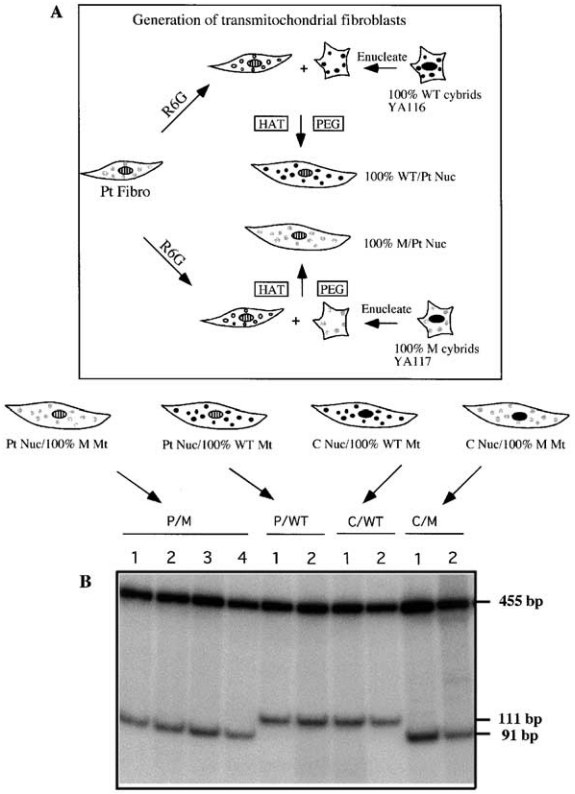


Fig. 1. (A) Generation of transmitochondrial fibroblasts. To eliminate the endogenous mtDNA, control and patient fibroblasts were treated for 5 days with R6G, 2.5 μg/ml in DMEM supplemented with 50 μg/ml uridine. They were then fused with cytoplasts derived from either the 100% WT (YA116) or the 100% M (YA117) cybrids enucleated with cytochalasin B using 50% PEG. Patient nucleus: hatched; Wild-type nucleus: black; Wild-type mitochondria: black; Mutant mitochondria: gray. (B) RFLP of transmitochondrial fibroblasts. Autoradiogram of gel electrophoresis of PCR products digested with *Hae*III. Mutated mtDNA is cleaved in three fragments of 455, 91, and 20 bp (20 bp not shown). Wild type is cleaved in two fragments of 455 and 111 bp. P/M: Patient nucleus/100% mutant mitochondria; P/WT: Patient nucleus/100% wild-type mitochondria; C/WT: Control nucleus/100% wild-type mitochondria; C/M; Control nucleus/100% mutant mitochondria.

DNA analysis. Total genomic DNA was extracted from exponentially growing cells according to standard techniques. To detect the A1555G mutation we used a PCR–RFLP method [10]. To quantitate the percentage of mutant mtDNA, 1 μl of [α-P^{32}]dATP was added at the last PCR cycle. Ten percent of the PCR product was digested with *Hae*III and electrophoresed through a 12% nondenaturing polyacrylamide gel. The intensities of the fragments were measured with a GS-363 Molecular Imager System (Bio Rad, Laboratories). For Southern blot hybridization, 5 μl total genomic DNA was digested with the restriction enzyme *Bam*HI. Gel electrophoresis and capillary transfer to Zeta Probe membranes (Bio Rad Laboratories, California, USA) were performed as recommended by the manufacturer. The blot was co-hybridized with two PCR fragments corresponding to mtDNA (nt 3087–4553) and the nDNA encoded 18S rRNA gene, labeled by Random Primed DNA Labeling Kit (Roche Molecular Biochemicals, New Jersey, USA).

Growth properties. To evaluate the growth characteristics of cybrids, 2.5×10^4 cells were plated in multiple 35 mm^2 dishes and grown in: (a) DMEM supplemented with 10% dialyzed FBS (dFBS), with 2 mmol L-glutamine/liter, 4.5 mg glucose/ml, and 110 μg sodium pyruvate per ml; or (b) the same medium without glucose and supplemented with 5 mM galactose. At various time intervals, cells from individual plates were trypsinized and counted. To test the various cell lines for sensitivity to aminoglycosides, cells were grown in DMEM medium supplemented with 10% dFBS in the presence or absence of different concentrations of streptomycin, neomycin, or paromomycin, with or without uridine (50 μg/ml). The cells were trypsinized and counted at specific time intervals.

Analysis of mitochondrial translation products. Exponentially growing transmitochondrial cybrids and fibroblasts were grown for 4 days in DMEM supplemented with 10% FBS, 2 mmol L-glutamine/liter, 4.5 mg glucose/ml, and 110 μg sodium pyruvate per ml; or the same medium without glucose, supplemented with 5 mM galactose in presence or absence of different concentrations of aminoglycosides. The cells were labeled with 150 μCi of [^{35}S]methionine (1175 Ci/mmol; Expre ^{35}S, NEN Life Science Products) for short pulses of 10 and 30 min (to analyze the rate of labeling) and for long term (90 min) in 2 ml methionine-free DMEM supplemented with 10% dialyzed FBS in the presence of 100 μg emetine per ml [38]. Lysates of the labeled cells were analyzed by loading equal amounts of total protein (Protein Assay Kit, Sigma, St. Louis, MO) onto a 10% Tricine–SDS–polyacrylamide gel [39]. Quantitation of mtDNA-encoded polypeptides was performed with a GS-363 Molecular Imager System (Bio Rad). To examine the stability of mitochondrial subunits, pulse-chase labeling was performed. Cell lines were exposed for 2 h to 200 μCi/ml of [^{35}S]methionine in methionine-free DMEM, supplemented with 10% dialyzed FBS and 100 μg/ml cycloheximide. The labeling medium was then replaced with complete non-radioactive medium and the cells were grown for 16 h at 37 °C before harvesting. For reference, samples were labeled for 2 h and immediately harvested. Analysis and quantitation of mtDNA-encoded polypeptides were performed as described above.

Biochemistry and cytochemistry. The activities of individual respiratory chain complexes in fibroblasts obtained from five normal controls from patients 1 and 2, and in cybrid cell lines were determined using whole cell lysates [40]. To elucidate the biochemical basis of the aminoglycoside sensitivity, cytochrome *c* oxidase (COX) and citrate synthase (CS) activities were determined in cybrids grown for 4 days in medium with or without 2 mg/ml neomycin. All biochemical assays were done in triplicate with concurrent controls.

For COX and succinate dehydrogenase (SDH) cytochemistry, cells grown on glass coverslips were air-dried for 1 h at room temperature, incubated with the respective substrates, mounted as described [41], and viewed with a Zeiss microscope with brightfield optics.

Oxygen consumption. Oxygen consumption of intact cells was determined as described previously [35] in cell lines grown for 4 days in DMEM in the presence or absence of increasing concentrations (0.5–2.5 mg/ml) of neomycin or paromomycin.

ATP synthesis. ATP synthesis in cybrid cell lines from patient 1 was measured in living cells after digitonin permeabilization using the luciferin–luciferase system to determine the ATP content, as described [42].

RNA analysis. Total RNA was isolated from exponentially growing cells in the absence or presence of different concentrations (0.5–2.5 mg/ml) of neomycin using an RNA Isolation Kit, "Totally RNA" (Ambion, Texas, USA). Equal amounts of total RNA from mutant and wild-type cybrids were electrophoresed through a 1.5% agarose–formaldehyde gel, transferred to Zeta-Probe GT membranes (Bio Rad Labs, Hercules, CA, USA), and hybridized in accordance with manufacturer's protocols. PCR fragments corresponding to selected mtDNA genes (12S rRNA, 16S rRNA, ND1, COX I, and COX III) labeled by random primed DNA Labeling Kit (Roche Molecular Biochemicals, USA) were used as probes for mRNAs. The cDNA of β-actin was used as an internal control for quantitation. All experiments were performed in triplicate on at least three different WT and 100% M cybrid clones from patient 1 and three different 100% M clones from patient 2.

Results

Fibroblast biochemistry and cytochemistry

COX activity in fibroblasts from both the patients was not significantly different from that of controls (patient 1: 1.0, patient 2: 0.94, controls: 0.67 ± 0.13 nmol/min/unit of CS activity) (control values are means $\pm$ SD). The cytochemical stain for COX was normal in these cells, even when grown in the presence of neomycin (up to 0.75 mg/ml) (data not shown).

Analysis of cybrids

From patient 1, sixty-eight cybrid clones were isolated from the fusion and screened by PCR–RFLP analysis. Clones harboring a range of mutation levels between 0% and 100% were obtained. For further studies, 3 clones (YA116, YA101, YA124) containing 100% WT mtDNA and 4 clones (YA117, YA120, YA107, YA129) harboring 100% M mtDNA from patient 1 were selected. From patient 2, 12 clones were isolated from the fusion, all of which were homoplasmic for the mutation. Four of these (BM101, BM102, BM103, and BM104) were selected for further analysis (Fig. 2). The mtDNA content of cybrids was estimated by Southern blot analysis using mtDNA-specific and nDNA-specific (18S rRNA gene) probes. All clones were repopulated with mtDNA to the same extent.

Growth characteristics

Analysis of growth rates revealed essentially no difference between WT and M cybrids in medium containing glucose as well as in glucose-free medium supplemented with galactose (data not shown). However, in the presence of neomycin or paromomycin the growth rates were markedly lower in M than in WT cybrids (Fig. 3A). After a three-day treatment with 2 mg/ml neomycin or 1.5 mg/ml paromomycin, the M cells stopped growing and eventually died. However, in the presence of streptomycin, no differences were observed between M and WT and high concentrations of the drug were required to inhibit the growth of both cell lines (Fig. 3B).

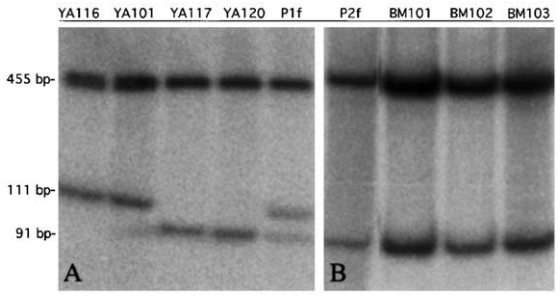


Fig. 2. RFLP analysis of cybrid clones. (A) Two wild-type clones (YA116 and YA101), two mutant clones (YA117 and YA120), and fibroblasts from patient 1 (P1 f) harboring 60% mutation. (B) Three mutant clones (BM101, BM102, BM103) and fibroblasts with 100% mutation (P2 f) from patient 2.

Mitochondrial protein synthesis

There was no difference in mitochondrial protein synthesis between M and WT cybrids. The mtDNA subunits from M cybrids grown in $\pm$ glucose were qualitatively identical to those of the WT cybrids and 143B cells, both in terms of electrophoretic mobility and relative labeling of the various polypeptides. Increasing concentrations of aminoglycosides also did not influence the labeling pattern (Fig. 4). Interestingly, quantitation of the overall rate of labeling of mitochondrial translation products did not reveal differences between M and WT clones (data not shown). The pulse-chase labeling analysis revealed that mtDNA subunits of M cybrids were as stable as those of the WT and that high concentrations (up to 5 mg/ml) of neomycin did not affect their stability.

Respiratory function

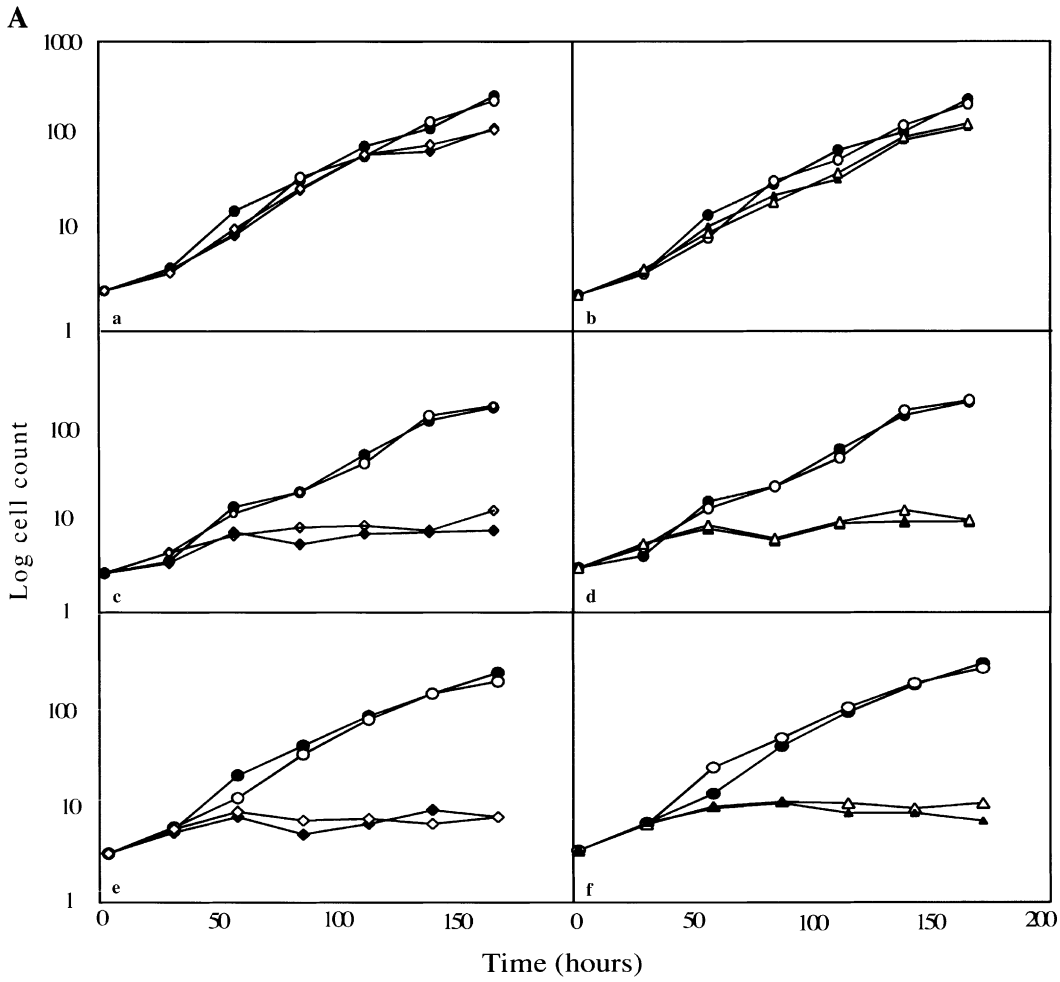
There were no differences in COX activity, oxygen consumption or ATP synthesis (Table 2) between M and WT cells when grown in neomycin. Activities of NADH dehydrogenase, NADH cytochrome *c* reductase, and succinate-cytochrome *c* reductase were also similar in M and WT cybrids derived from patient 1 (data not shown).

RNA analysis

Northern analysis revealed no difference in the size or intensity of mitochondrial transcripts when total RNA extracted from M and WT cybrids grown in the presence of aminoglycosides was probed for 12S rRNA, 16S rRNA, ND1, COX I, and COX III and normalized to β-actin (data not shown).

Transmitochondrial fibroblasts

To study nuclear–mitochondrial interaction in the pathogenesis of the A1555G mutation, we constructed transmitochondrial fibroblasts using fibroblasts from a normal individual and from patient 1 (nuclear donors), harboring homoplasmic mutant (M) or wild-type (WT) mtDNA by fusion with enucleated M (YA117) and WT (YA116) cybrids (mitochondrial donors) as shown schematically in Fig. 1A. By this method, we were able to obtain fibroblast clones with the patient nucleus containing WT and M mtDNA and clones with control nucleus harboring WT and M mtDNA.



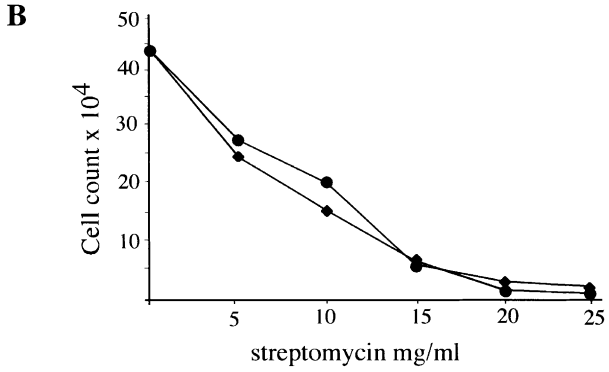


Fig. 3. Growth characteristics of cybrid cell lines. (A) Representative growth curves of wild-type cybrids (top panels) in the presence/absence of neomycin (a), paromomycin (b), and mutant cybrids from patient 1 (middle panels) and patient 2 (bottom panels) in the presence/absence of neomycin (c, e) and paromomycin (d, f). Growth curves were determined by seeding multiple 35-mm petri dishes with a constant number of cells in DMEM supplemented with 10% dFBS and 50 μg of uridine per milliliter (solid symbols) or without uridine (open symbols) in the presence of 2 mg of neomycin per milliliter (diamond), 1.5 mg of paromomycin per milliliter (triangles), or without antibiotics (circles). At various time intervals cells from individual plates were trypsinized and counted. All experiments were performed in triplicate on at least three different WT and four different 100% M cybrids clones from patient 1 and four different 100% M cybrids clones from patient 2. (B) Growth properties of mutant (YA117) and wild-type (YA116) cybrid clones from patient 1 in presence of streptomycin. Cells (2.5×10^4) were plated and grown for 6 days in DMEM supplemented with 10% dialyzed FBS with increasing concentrations of streptomycin. On the sixth day cells were trypsinized and counted. Wild type (circles), mutant (diamonds).

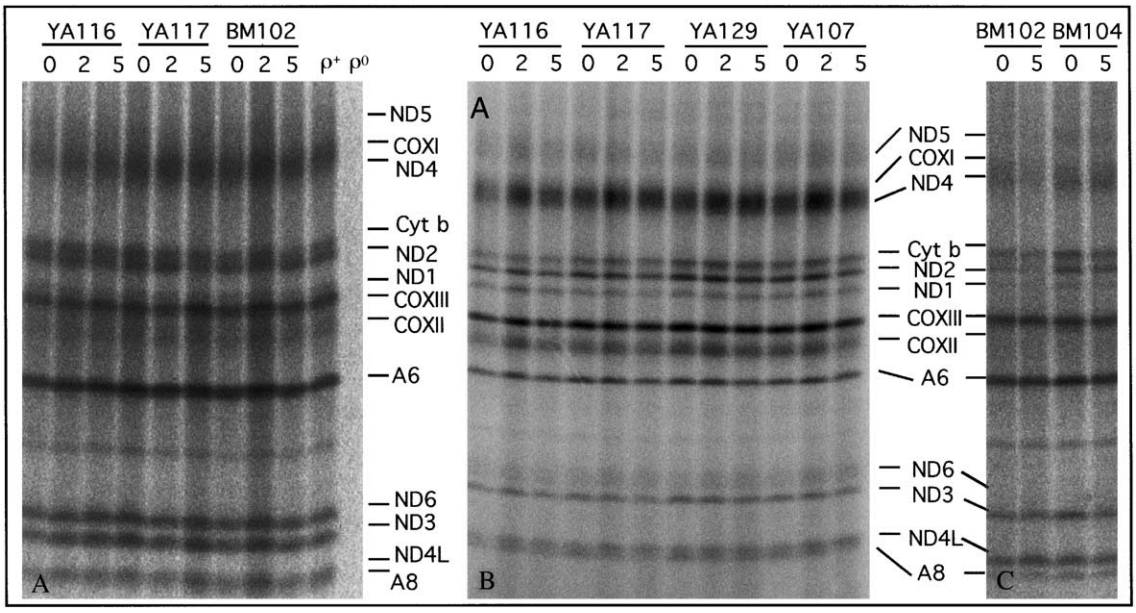


Fig. 4. Analysis of mitochondrial translation products in cybrid cell lines. Representative fluorograms of mitochondrial translation products labeled with [^{35}S]methionine after electrophoresis through a 10% Tricine–SDS–polyacrylamide gel. Wild-type (YA116) and mutant clones (YA117, YA129, YA107) from patient 1 and mutant clones from patient 2 (BM102, BM104) were grown for 4 days in DMEM supplemented with 10% dFBS, in the absence or presence of 2 and 5 mg/ml neomycin (A) and paromomycin (B and C). Cells were labeled for 30 min in the presence of 150 μCi of [^{35}S]methionine and 100 μg emetine per ml. 143B cells containing wild-type mtDNA (ρ^+) and mtDNA-less cells (ρ^0) were also labeled. Equal amount (30 μg) of cellular protein was loaded in each lane. All experiments were performed in triplicate.

Table 2
Respiratory chain function in cybrids harboring the A1555G mtDNA mutation

	COX nmol/min/unit citrate synthase		Oxygen consumption fmol/min/cell		ATP synthesis nmol/mgprotein/mi	
	−neo	+neo	−neo	+neo	−neo	+neo
YA 116	0.78 ± 0.09	0.67 ± 0.05	4.41 ± 0.08	4.7 ± 0.11	46.195 ± 16.5	42.15 ± 11.92
YA 101	0.99 ± 0.14	0.85 ± 0.07	4.38 ± 0.13	4.17 ± 0.03	48.85 ± 17.33	52.94 ± 21.32
YA 117	0.93 ± 0.15	0.74 ± 0.08	4.14 ± 0.60	5.14 ± 0.03	35.61 ± 17.65	49.05 ± 26.55
YA 120	1.08 ± 0.07	0.89 ± 0.04	4.8 ± 0.05	4.9 ± 0.05	49.33 ± 25.91	47.03 ± 22.57
BM 101	0.98 ± 0.05	1.13 ± 0.08	4.1 ± 0.10	4.63 ± 0.09	n.d.	n.d.
BM 102	0.87 ± 0.06	0.97 ± 0.06	3.38 ± 0.06	5.01 ± 0.12	n.d.	n.d.

YA116 and YA101 are WT cybrid clones; YA117 and YA120 are M cybrid clones from patient 1; BM101 and BM102 are M cybrid clones from patient 2. All assays were performed in the presence (+neo) and absence (−neo) of 2 mg/ml neomycin.

PCR–RFLP analysis of DNA from the transmitochondrial fibroblast cell lines corresponded to the percentages of the mtDNA mutation that were transferred from the cybrids. Clone YA116 had 100% WT mtDNA and the clone YA117 had 100% M mtDNA (Fig. 1B). The clones were viable after repeated freezing and thawing and their growth characteristics were similar to those of primary fibroblasts from normal controls and from patients. No respiratory chain dysfunction was observed in transmitochondrial cell lines, as assessed by cytochemical stain for COX and SDH and normal pattern of labeling of mitochondrial translation products (data not shown). Further analyses involving large call numbers were not feasible because the cells were not immortalized.

Discussion

In the present work, we studied three different cell lines derived from two unrelated patients of different ethnic backgrounds and with different clinical phenotypes. Detailed biochemical and molecular analyses were carried out on: (a) patients' primary fibroblasts, (b) transmitochondrial cybrids with a uniform nuclear background, and (c) transmitochondrial fibroblasts with the patient or control nuclear background. This was done to determine the contribution, if any, of the mitochondrial genome (in cybrids) and of the mitochondrial and nuclear genomes in combination (in primary and transmitochondrial fibroblasts) to the phenotype.

Absence of respiratory chain dysfunction

We found no significant respiratory chain dysfunction in primary fibroblasts from two patients harboring 60% (patient 1) and 100% (patient 2) mutations. These results are in contrast with the previous report by Santorelli et al. [16] which showed a decrease in COX activity and mitochondrial protein synthesis in fibroblasts from patient 1. This discrepancy is perhaps due to the very mild phenotype associated with the A1555G mutation which could be further obscured by intercellular variability in uncloned cells, where heteroplasmy may compensate for the partial defect by complementation. Therefore, for the first time, we compared biochemical properties of cybrids with the 143B nuclear background, repopulated with mitochondria harboring 0% and 100% mutations derived from the same patient. The absence of biochemical defect in the cybrids supports the hypothesis that the nuclear background may be responsible for the different degrees of penetrance observed both in vivo and in vitro [20–22]. Additionally, the lack of a biochemical phenotype in cybrids derived from two unrelated patients of different ethnic origins renders the involvement of a second mtDNA mutation highly improbable. Besides, our data support the hypothesis that the mitochondrial DNA haplotype may not be implicated in the pathogenesis.

The analysis of transmitochondrial fibroblasts harboring WT or M mtDNA in combination with nucleus from the patient or from control fibroblasts permitted us to study the contribution of the two genomes in combination as well as separately. Furthermore, primary cloned cells afforded a system without biological alterations such as aneuploidy or spontaneous hypermutability, both of which are typical of immortal cell lines like the 143B osteosarcoma cell line. Surprisingly, these studies did not reveal any abnormalities in COX cytochemistry or mitochondrial protein synthesis, suggesting that, if nuclear factors do act synergistically with the mtDNA mutation to result in the observed clinical phenotype, they may be tissue-specific and not apparent in the nuclear background of fibroblasts. Nevertheless, the cochlear cells may perhaps be selectively more susceptible to the effects of the mutation.

Aminoglycoside sensitivity in cybrids

Aminoglycoside antibiotics bind directly to prokaryotic 16S rRNA at the decoding site 'A' of the ribosome, which results in misreading and inhibition of translocation [27–29]. The A1555G mutation, which is expected to create a new G–C base pair, makes the secondary structure of the 12S rRNA similar to the corresponding region of the *Escherichia coli* 16S rRNA [6]. Therefore, we examined whether the mutation was associated with increased sensitivity to the drugs in cybrids. In the presence of high concentrations of paromomycin or neomycin, the growth rate was markedly lower in homoplasmic mutant cybrids than in wild-type cells, confirming that the mutation did increase the sensitivity of the cells to these drugs. Since the majority of patients with hypersensitivity to aminoglycosides had become deaf after exposure to streptomycin [1], we analyzed the growth characteristics of cybrid cells in the presence of this drug and, surprisingly, we found no difference between mutant and wild-type clones. This result is contrary to the report of Inoue et al. [43] who observed a moderate reduction in mitochondrial translation products in cybrids with the HeLa cell nuclear background. This discrepancy in susceptibility to streptomycin may be perhaps due to the difference in the nuclear backgrounds of the two cell types. Furthermore, binding studies of different aminoglycosides to a 12S rRNA construct harboring the A1555G mutation showed that paromomycin and neomycin bind more tightly than gentamicin or kanamycin and that streptomycin does not bind at all [32]. Absence of streptomycin binding may be explained by the fact that its molecular structure and primary site of action are different from other aminoglycosides [27,44,45]. Moreover, streptomycin resistance in *E. coli* is governed by polymorphisms in ribosomal proteins, such as S4, S5, or S12, rather than by rRNA mutations [46]. In conclusion our data confirm those from previous studies [19,21] that showed the paromomycin and neomycin sensitivities of homoplasmic mutant lymphoblastoid cell lines and provide evidence that the A1555G mutation per se does not increase sensitivity to streptomycin, at least in osteosarcoma cybrid cells.

Biochemical basis of aminoglycoside sensitivity

Although growth rate was decreased in mutant cybrids exposed to high concentrations of paromomycin or neomycin, there was no dysfunction in the respiratory chain activity. Since aminoglycosides are known to exert their antibacterial effect by miscoding and premature termination of protein synthesis, we studied protein synthesis in cybrids in the presence of neomycin, paromomycin or streptomycin. We performed the experiments both in ±glucose medium to amplify differences if any between M and WT cells and found no abnormalities, lending further support to the tissue specificity of the defect. Previous studies of lymphoblastoid cell lines from a single large kindred showed a decrease in the rate of mitochondrial protein synthesis in mutant cells exposed to paromomycin [22,34]. However, segregation of this mutation with the observed phenotype has not been unequivocally established and no correlation was made between the reduction of protein synthesis and respiratory chain dysfunction [22].

In addition to inhibiting the ribosome, aminoglycosides also target other RNAs, such as, the self-splicing group I [47], RNAse P [48], and hammerhead ribozymes [49]. We performed Northern blot analysis to evaluate proper processing of the mtRNA polycistronic precursor in the presence of neomycin and found no difference in the size or intensity of the transcripts. Besides, there was no difference in the ratio of 12S/16S RNA indicating that binding of neomycin to the small 12S subunit of the rRNA, if any, did not reduce its stability nor inhibit incorporation into actively translating ribosomes (data not shown).

In conclusion, our results have shown that the A1555G mutation per se is not sufficient to cause a respiratory chain dysfunction in the neutral nuclear background of cybrids or with the patients' fibroblast nuclear background. These data suggest that tissue-specific factors involving the hair cells of the cochlea or cardiomyocytes may render these cells selectively vulnerable to the effects of the mutation, leading to impairment in nuclear–mitochondrial interactions. In addition, our findings demonstrate that the A1555G mutation increases cell sensitivity to aminoglycosides of the neomycin family and is associated with a decrease in growth rate in cybrids. Further studies are required to determine the underlying biochemical defect which does not appear to be related to respiratory chain dysfunction. Finally, our data indicate that the A1555G mutation does not increase sensitivity of cybrids to streptomycin. Therefore, families with streptomycin-induced hearing loss should also be considered for linkage studies and candidate gene screening for nuclear modifiers which may predispose them to hearing loss.

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