SPANDIDOS PUBLICATIONS

Loss of mutant mitochondrial DNA harboring the MELAS A3243G mutation in human cybrid cells after cell-cell fusion with normal tissue-derived fibroblast cells

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Abstract. Mutant mitochondrial (mt) DNA variants are related to human disease and have been investigated using cytoplasmic hybrid (cybrid) cells generated from human tumor cells in which mutant mt maintenance depends on the cell line. It is, however, unclear whether human intercellular fusion of non-tumorous cells influences the maintenance of disease-related mutant mt. A preliminary experiment of cell-cell fusion between a human skin fibroblast cell line from a Lesch-Nyhan syndrome patient and an osteosarcoma cybrid cell line harboring the mitochondrial tRNA$^{Leu(UUR)}$A3243G mutation showed a decrease of A3243G mutant mtDNA in fused cells during passages. In order to confirm the decrease of mutant mtDNA, we performed cell-cell fusion experiments using another human lung fibroblastic cell line. When the hygromycin-resistant osteosarcoma cybrid cell line was fused with the fibroblasts without any A3243G mtDNA mutations, the proportion of A3243G mutant mtDNA in the hybrid cells gradually decreased during cell culture and almost completely disappeared in all hybrid clones at the end of 15 passages. These results indicated that A3243G mutant specific mtDNA decreases in the hybrid background when normal fibroblast-derived cell contents, including the nucleus and mt, were introduced. Thus, we are hypothesizing that the non-tumorigenic fibroblast cellular components induce a difference in replication efficacy between the mtDNAs with and without the A3243G mutant sequence, which may be related to the decrease of disease-related mutant mtDNA in the hybrid cells.

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Introduction

Mitochondrial DNA (mtDNA) mutations induce a wide spectrum of human diseases caused by mitochondrial dysfunction. A number of disease-associated mtDNA point mutations have been identified (MITOMAP, human mitochondrial genome database, www.mitomap.org, 2009). Mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), one of the mitochondrial diseases, is a syndrome characterized by lactic acidosis, episodic vomiting, seizures and recurrent cerebral insults resembling strokes that lead to hemiparesis, hemianopsia or cortical blindness (1). The A3243G mutation in mitochondrial leucine transfer RNA (tRNA) (UUR) genes is the most common mtDNA mutation and accounts for the majority (80%) of cases of MELAS (2,3). King *et al* generated a human tumor cell line devoid of endogenous mtDNA (ϱ^0 cells) (4), and the transfer of exogenous mitochondrial genomes with the A3243G mutation to the ϱ^0 cells resulted in globally impaired mito-chondrial protein synthesis and affected all respiratory chain complexes containing mtDNA-encoded subunits, which were related to respiratory deficiency in MELAS (5). The A3243G mutation decreased steady-state levels of mitochondrial tRNA$^{Leu(UUR)}$ (6), caused translational defect by the taurine-modification deficiency at the anticodon wobble position of tRNA$^{Leu(UUR)}$ (7) and induced decreased levels of amino-acylation of tRNA$^{Leu(UUR)}$ (8) in MELAS.

The A3243G mutant mtDNA in MELAS is present with a mixture of wild-type mtDNA in the same cell. Such a condition is called heteroplasmy, which maintains the oxidative phosphorylation (OXPHOS) function by compensating for the mutant mtDNA under a certain threshold level of the mutation (9). The levels of mtDNA heteroplasmy vary in cells and even in tissues (10). So far, the regulating mechanism of the copy number of mutant mtDNA in cells has been poorly understood. The cybrid study showed that levels of mutant mtDNA with

Table I. Genetic characteristics and mutation percentages of parental cells and nuclear hybrids.

Cell lines	Drug resistance	Cross	Selection	% A3243G
Tumor cell line				
143B	HAT^s, $Hygro^s$			0.0
Parent cell lines				
Cybrid cell line				
2SD	HAT^s, $Hygro^s$			94.0
$2SD^{hygro}$	HAT^s $Hygro^r$			86.6
Fibroblast cell line				
MiTen[a]	HAT^s, $Hygro^s$			0.0
TIG-3-20	HAT^r, $Hygro^s$			0.0
Hybrid cell clones	HAT^r, $Hygro^r$	$2SD^{hygro}$ x TIG-3-20	HAT^+ Hygro	
Hy-1				21.3
Hy-2				17.5
Hy-3				15.2
Hy-4				82.6
Hy-5				32.7
Hy-6				15.5

[a]Human skin fibroblast cell line from Lesch-Nyhan syndrome patient; [s]drug sensitive; [r]drug resistance.

the A3243G mutation depend on nuclear backgrounds (11). It is critical that the mtDNA heteroplasmy fluctuation is analyzed in various nuclear backgrounds in order to understand the pathology and prognosis of the disease. Although conventional cybrid cell studies have been performed using immortal tumor cell lines (4), it has not been made clear whether the non-tumorigenic nucleus and cytoplasm influence the levels of heteroplasmy.

In the current study, we performed an investigation of cell fusion manipulation using two human non-tumorigenic fibroblast-derived cell lines and found a decrease of the A3243G mutant mtDNA in human hybrid cells.

Materials and methods

Cell culture. A human skin fibroblast cell line from a Lesch-Nyhan syndrome patient, MiTen (JCRB0072), and a human diploid fibroblast cell line, TIG-3-20 (JCRB0506) were obtained from the Health Science Research Resources Bank (Osaka, Japan). The human 143B osteosarcoma cell line and its derivative, 2SD, a cybrid cell line harboring the mitochondrial A3243G mutation (12), are generous gifts from Dr Masafumi Ito of Gifu International Institute of Biotechnology.

Table I shows the genetic characteristics and mutation percentages of parental cells and their nuclear hybrid clones used in this study. MiTen is hypoxanthine phosphoribosyl transferase deficient ($HPRT^-$) and 143B cells, 2SD and $2SD^{hygro}$ cells are thymidine kinase deficient (TK^-). $HPRT^-$ or TK^- cells are sensitive to HAT. The cybrid and hybrid cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mg/ml sodium pyruvate and 50 mg/ml uridine (complete DMEM).

Fluorescence activated cell sorting (FACS) analysis. The mitochondrial membrane potential of 2SD cybrid cells was determined by FACS analysis with the cationic lipophilic dye MitoTracker Orange CMTMRos (Invitrogen, Tokyo, Japan). Cybrid cells were incubated with the MitoTracker Orange dye at a concentration of 100 nM for 30 min at 37˚C in the dark. After staining with dye, cybrid cells were washed and harvested by trypsinization and then subjected to the FACS-Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Generation of hygromycin-resistant 2SD cybrid cells. A hygromycin-resistant 2SD cybrid cell line ($2SD^{hygro}$), was generated by the transfection of cells with linearized pcDNA3.1/Hygromycin (Invitrogen, Tokyo, Japan) using the Lipofectamine PLUS (Invitrogen) method.

Intercellular fusion. Intercellular fusion of both 2SD and $2SD^{hygro}$ cybrid cells and fibroblast MiTen or TIG-3-20 was achieved using 50% (w/v) polyethylene glycol 1450 (Sigma-Aldrich, Tokyo, Japan). Briefly, a mixture of 10^6 of each parental cell was washed with DMEM without serum before fusion. Polyethylene glycol (0.4 ml) was added to the pellet of the mixed parental cells, and they were suspended. After 90 sec of suspension, polyethylene glycol was removed by washing cells with the medium. Then, fusion mixture was plated out in the complete DMEM. Two days after fusion, cells in the fusion mixture were cultivated in selection medium [complete DMEM containing HAT (GibcoBRL, Tokyo, Japan) or complete DMEM containing HAT and 0.3 mg/ml hygromycin (Invitrogen)]. Fused hybrid cells between MiTen ($HPRT^-/TK^+$) and 2SD ($HPRT^+/TK^-$) were resistant to HAT and cells between TIG-3-20 ($Hygro^s/TK^+$) and $2SD^{hygro}$

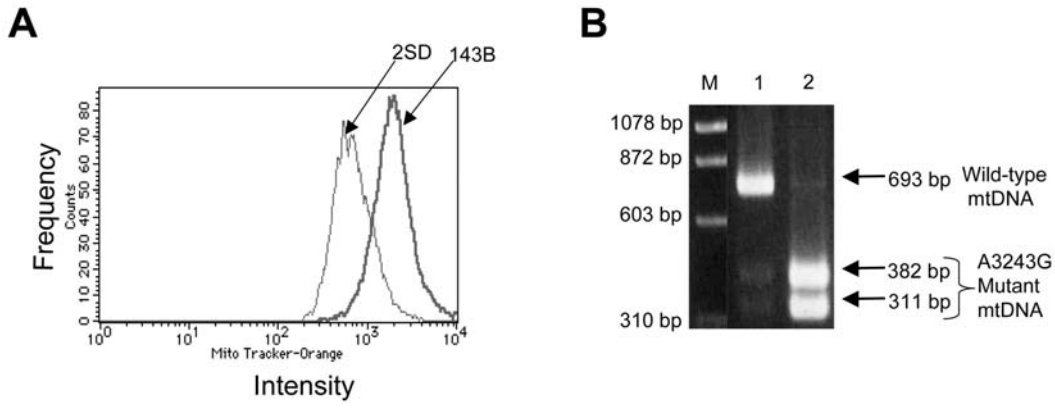

Figure 1. (A) FACS analysis for the intracellular mitochondrial membrane potential of 2SD cybrid cells with MitoTracker Orange. The left and right peaks indicate the mitochondrial membrane potential of 2SD cybrid cells and 143B osteosarcoma cells, respectively. (B) mtDNA genotyping of hybrid cells generated from MiTen (skin-derived fibroblast cell line) and 2SD cybrid cells. Lane M, molecular weight marker; lane 1, 2SD-MiTen hybrid cells and lane 2, 2SD cybrid cells. A decrease in A3243G mutant mtDNA was observed in lane 1 of the 2SD-MiTen hybrid cells.

(Hygro^r/TK$^-$) were resistant to both HAT and hygromycin. On days 14-17 after the fusion, hybrid colonies grown in selection medium were cloned by the cylinder method, and the hybrid clones were then cultivated in complete DMEM.

Examination of the heterozygous state of the hybrid cells by amplifying a polymorphic nuclear marker MCT118 at the variable number of tandem repeat (VNTR) locus. The successful fusion was examined as a heterozygous state of nuclear DNA by analysis of nuclear genomic polymorphisms, MCT118 VNTR nuclear marker. Total DNA was extracted from the cells used in this study by using a DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. Primers used for the amplification were, forward primer 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3' and reverse primer 5'-GTCTTGTTGGAGATGCACGTGCC CCTTGC-3'. The thermal cycling condition was 30 cycles of 94˚C for 60 sec, 66˚C for 15 sec, and 72˚C for 120 sec. PCR was performed in a DNA thermal cycler (PTC-100 Peltier Thermal Cycler; MJ Research Inc., Waltham, MA, USA). The size of amplified fragments was determined by 2% agarose gel electrophoresis.

Mitochondrial genotyping by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Primers used for the amplification of the region encompassing the mitochondrial nucleotide position 3243 were, forward primer 5'-TCACCAGTCAAAGCGAACTA-3' and reverse primer 5'-AGAAGAGCGATGGTGAGAGC-3'. The thermal cycling condition was 24 cycles (normal PCR, 28 cycles) of 94˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 60 sec. Then, the last-hot cycle PCR-RFLP method was performed. PCR products were labeled with [α-^{32}P]-dCTP in the last cycle of PCR, digested with *Apa*I and run on 6% non-denaturing polyacrylamide gels. The gel was exposed to an imaging plate (Fuji Film, Tokyo, Japan), and the radioactivity of the bands was quantified with a bioimaging analyzer, BASS5000 (Fuji Film). While 2SD-MiTen hybrid cells were harvested in bulk, the A3243G mutation was analyzed by 2% agarose gel electrophoresis after the non-radioisotope PCR-RFLP method.

Results

Decrease in mitochondrial membrane potential of 2SD cells. Fig. 1A shows the mitochondrial membrane potential of 2SD cybrids cells analyzed by FACS. The mitochondrial membrane potential was remarkably decreased in osteosarcoma derivative 2SD cybrid cells (left peak), which harbored the A3243G mutation, compared with that of 143B osteosarcoma cells used as a control (right peak), indicating that serious mitochondrial dysfunction in the OXPHOS system was caused by the mitochondrial pathogenic mutation in the cybrid cells.

Decrease of A3243G mutant mtDNA levels in bulk culture of 2SD-MiTen hybrid cells. 2SD-MiTen hybrid cells were selected in complete DMEM containing HAT and harvested by trypsinization in bulk. After total DNA extraction, mtDNA genotyping was performed by PCR-RFLP. We observed a decrease in A3243G mutant mtDNA levels in a bulk culture of hybrid cells (Fig. 1B). This preliminary experiment encouraged us to design and perform a subsequent experiment in which another fibroblast cell line, TIG-3-20, was used for intercellular fusion with 2SD^{hygro} cybrid cells in order to confirm the decrease of mutant mtDNA in hybrid cells between oncogenic and non-tumorigenic cells without the X-linked Lesch-Nyhan mutation.

Establishment of hybrid clones between 2SD^{hygro} cybrid cells and TIG-3-20. The cybrid cells that were fused with TIG-3-20 lung fibroblast cells survived in the selection medium because parental 2SD^{hygro} cells are thymidine kinase-deficient. Any residual 2SD^{hygro} cybrid and TIG-3-20 cells that did not fuse were killed by HAT and hygromycin in the selective medium, respectively. Two weeks after the cell fusion, double-resistant hybrid cells appeared in the selection medium and formed their colonies. Hybrid cells grew rapidly in the complete medium; however, they developed contact inhibition, like many non-tumorigenic cells. Cell-growth was arrested at the point of their confluent state. Fig. 2 shows an analysis of successful cell fusion by amplification of the polymorphic nuclear variable number of tandem repeat (VNTR) marker, MCT118. All the

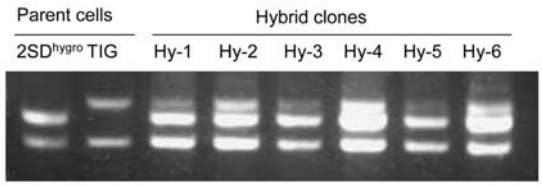

Figure 2. Examination of heterozygous state of cell fusions by amplification using MCT118 nuclear marker. MCT118 is a variable number of tandem repeat (VNTR), which was used for the identification of genetic polymorphism between parental cell lines. The 2SD^{hygro} cybrid cells and TIG-3-20 cells have two polymorphic bands and shared the lower band. The upper bands were polymorphic between the 2SD^{hygro} cybrid cells and TIG-3-20 cells. All six hybrid clones (Hy-1 to -6) have a lower non-polymorphic band and two more upper bands that are from a polymorphic marker between the two parental cells.

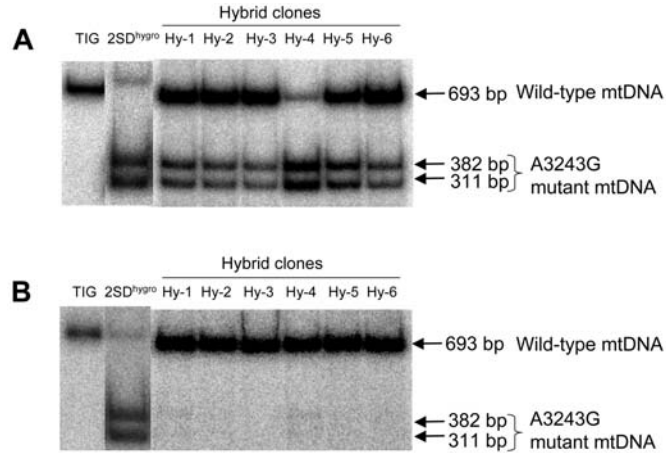

Figure 3. Mitochondrial genotyping by last-hot cycle PCR method in hybrid cells. The levels of mutant mtDNA sequence of hybrid clones (A) in early passage (3 passages) and (B) late passage (15 passages) are shown. Six hybrid cell clones (Hy-1 to -6) were isolated from each single colony. PCR-amplified fragments derived from the A3243G mutant mtDNA have one *Apa*I-cutting site that cleaves a 693-base fragment into 382- and 311-base fragments. The *Apa*I-digestion patterns show the ratio of mutant mtDNA load in each parental cell and hybrid clones at either early passage (three passages) (A) or late passage (15 passages) (B).

hybrid clones showed the two upper polymorphic bands, suggesting the presence of the nuclear component forms in both parental cells (Fig. 2).

mtDNA composition of 2SD^{hygro}-TIG hybrid clones. mtDNA composition was analyzed by a last-hot cycle PCR-RFLP to precisely assess the mutant-specific mtDNA decrease. The PCR amplifies the 693-bp fragment. The A to G transition at mitochondrial nucleotide position 3243 creates an additional *Apa*I restriction site in the mutant mtDNA. After cleavage with *Apa*I, 382- and 311-bp fragments are produced. *Apa*I-digestion patterns of mtDNA from the hybrid clones showed that hybrid clones contained predominantly wild-type mtDNA, except hybrid clone Hy-4. In the case of Hy-4, the proportion of A3243G mutant mtDNA was 82.6% in the early passage (3 passages) (Table I, Figs. 3A and 4). In later passages (15 passages), all hybrid clones investigated showed almost no detectable A3243G mutant mtDNA sequence, whereas no discernible change in the proportion of A3243G was seen in the 15 passages of 2SD^{hygro} used as a control (Fig. 3B).

The rate of decrease in mutant mtDNA. Fig. 4 shows quantitative monitoring of the mutant mtDNA levels of each of the hybrid clones. For 115 days, DNA samples were extracted from hybrid cells and analyzed. The rate of decrease in the levels of A3243G mutant mtDNA in hybrid clones varied but was exponentially decreased in each passage.

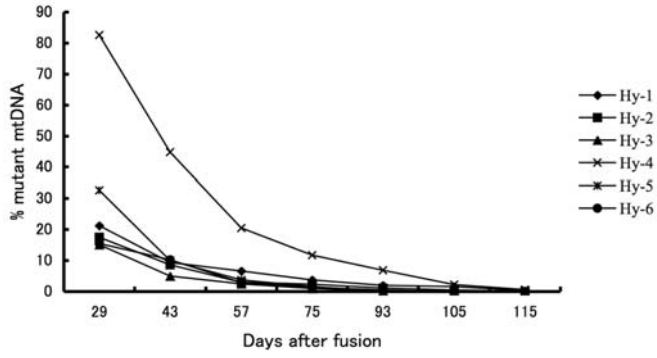

Figure 4. Quantitative monitoring of the level of mutant mtDNA sequence of each hybrid clone. Percentage of mutant mtDNA is measured by the last-hot cycle PCR method at seven time points (29, 43, 57, 75, 93, 105 and 115 days). After, cell fusion is plotted for each hybrid clone (Hy-1 to -6) in a multiple-line graph.

Discussion

In the present study, we demonstrated that the cell-cell fusion between normal lung tissue derived fibroblastic cells and osteosarcoma cybrids with A3243G mutant mtDNA caused the preferential loss of the mutant mtDNA sequence. An almost complete loss of the A3243G mutant mtDNA occurred in all six hybrid clones during the process of 15 passages. It has been reported that long-term cell culture of human osteosarcoma cybrid cells with the A3243G mtDNA mutation resulted in an increase in levels of A3243G mutant mtDNA or stochastic segregation (13,14). However, in long-term cell cultures of >100 days, we observed a different phenomenon. The directional progressive shift of the mitochondrial genotype toward wild-type mtDNA in all hybrid clones, indicating that A3243G mutant mtDNA cannot be maintained in the osteosarcoma hybrid cells whose nuclear genetic background is altered by the introduction of human non-tumorigenic fibroblast cell contents, including the nucleus.

It was reported that the selective decrease of paternal mtDNA in the fertilization of mammals was due to the destruction of the ubiquitinated sperm mitochondria (15). However, such a selective decrease of mtDNA was seen in a relatively short time, whereas the decrease of mutant mtDNA observed in this study gradually progressed, suggesting that a different mechanism in the decrease of mutant mtDNA operates in the hybrid cells generated by the fusion of the cybrid cell with fibroblastic cells. Furthermore, it was reported that mitochondria were fused with each other in the hybrid cells in two weeks (16). Our hybrid cells carried both wild-type and A3243G mutant mtDNA sequences, perhaps the covalent bond of mitochondrial DNA also existed in the early passage. The selective decrease in levels of A3243G mutant

sequence continued for more than two weeks after 1 in our present study. Therefore, it is not explained sufficiently whether introduced fibroblastic cell contents destroyed the fused mitochondrion that contained A3243G mutant sequence and wild-type mtDNA.

It may be hypothesized that the cause of the decrease of A3243G mutant mtDNA lies in the difference in the replication or maintenance mechanism between the wild-type and mutant mtDNA in the hybrid cells. It was reported that human mtDNA is preferentially maintained over ape mtDNA in human cybrid and hybrid cells (17). The report indicated that the human nucleus had a strong preference for human mtDNA rather than ape normal mtDNA, even if pathogenic mutations exist in the human mtDNA. Therefore, the selection of mtDNA is not always dependent on functional mtDNA haplotypes. Human nuclear mtDNA replication factor(s) could preferentially bind to human mtDNA rather than ape mtDNA.

It is possible that there are some nuclear factor(s) involved as determinants whose affinity for mtDNA is affected by the presence of an A3243G mutation in mtDNA selection. Mitochondrial transcription termination factor (mTERF) has been widely known to bind to a tridecamer sequence, the location of the A3243G mutation, which is essential for transcription termination (18). The A3243G mutation provoked a severe affinity defect in mTERF protein for the target sequence template and was thought to impair two proper mitochondrial rRNA syntheses and eventually produce the disease phenotype (19). In studies using cybrid cells and tissues from MELAS patients, mitochondrial transcription termination is not as severely impaired by the affinity defect of mTERF-binding as was previously expected, suggesting that the affinity defect is compensated for *in vivo* by a certain mechanism (12,20). However, an unprocessed RNA transcript (16S rRNA + $tRNA^{Leu(UUR)}$ + ND1 genes) called RNA 19, which was thought to be accompanied by the incompetence of mitochondrial transcription termination by the affinity defect, was identified in cybrid cells with the A3243G mutation (5). There may be a somatic mutation maintaining the A3243G mutation in osteosarcoma cells, such as 2SD and cells from MELAS patients.

The main function of mTERF is believed to be a mitochondrial transcription terminator in the modulation of mitochondrial transcriptions (21). However, it was reported that mTERF was a modulator of mtDNA replication as well as mitochondrial transcription, and that it also played a role in replication-pausing in human mtDNA (22). Although mTERF protein bound to the mitochondrial target sequence where nucleotide position 3243 is located, the affinity of mTERF protein-binding with the wild-type 3243 site was different in those with the mutant-type DNA sequences. In this study, we found loss of the mutant mtDNA sequence after the introduction of non-tumorigenic normal tissue-derived fibroblastic cellular contents. An adaptation of the replication modulator binding, such as an mTERF binding inhibition, may be distinct and regulated differently from wild-type mtDNA after cell fusion.

Clarifying the mechanism of the selective decrease of mutant mtDNA in cybrid cells could lead to the application of gene therapy for mitochondrial disease caused by an A3243G mitochondrial mutation such as MELAS. In this study, we performed an introduction of normal tissue-derived fibroblast cell components into an osteosarcoma background using a cell-fusion technique. Further investigation is needed to scrutinize which cell component includes the mitochondrial replication modulation factors for the decrease of A3243G mutant mtDNA.

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