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The Acquisition of an Inheritable 50-bp Deletion in the Human mtDNA Control Region Does Not Affect the mtDNA Copy Number in Peripheral Blood Cells

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ABSTRACT: The mitochondrial DNA (mtDNA) control region is believed to play an important biological role in mtDNA replication. Large deletions in this region are rarely found, but when they do occur they might be expected to interfere with the replication of the molecule, thus leading to a reduction of mtDNA copy number. During a survey for mtDNA sequence variations in 5,559 individuals from the general Chinese population and 2,538 individuals with medical disorders, we identified a 50-bp deletion (m.298_347del50) in the mtDNA control region in a member of a healthy Han Chinese family belonging to haplogroup B4c1b2, as suggested by complete mtDNA genome sequencing. This deletion removes the conserved sequence block II (CSBII; region 299–315) and the replication primer location (region 317–321). However, quantification of the mtDNA copy number in this subject showed a value within a range that was observed in 20 healthy subjects without the deletion. The deletion was detected in the hair samples of the maternal relatives of the subject and exhibited variable heteroplasmy. Our current observation, together with a recent report for a benign 154-bp deletion in the mtDNA control region, suggests that the control of mtDNA replication may be more complex than we had thought. Hum Mutat 31:538–543, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: mtDNA; 50-bp deletion; replication; Chinese

Introduction

Mitochondria play an important role in cellular metabolism, energy production, reactive oxygen species generation, apoptosis regulation, and also act as a center for antiviral response [Moore and Ting, 2008; Wallace, 2005, 2007]. Each mitochondrion contains DNA and the mitochondrial DNA (mtDNA) copy number varies in different kinds of cells. The mtDNA control region, or the D-loop region, is a noncoding region that is located in region 16024-576 relative to the Cambridge reference sequence, and contains three hypervariable segments [Anderson et al., 1981]. Many functional sequence elements, such as the conserved sequence blocks (CSB) I-III, the Heavy (H) strand origin (O_H), the termination-associated sequence (TAS), and the mitochondrial transcription factor 1 (mtTF1) binding site, are located in this region (cf. reviews [Clayton, 1991; Falkenberg et al., 2007]). Nevertheless, there is a debate regarding the strand-displacement model versus the strand-coupled model for mtDNA replication [Bogenhagen and Clayton, 2003; Brown et al., 2005; Fish et al., 2004; Holt and Jacobs, 2003; Holt et al., 2000; Yasukawa et al., 2005]. However, the essential role of the control region during mtDNA replication has never been questioned. Large deletions in the mtDNA control region, which deprive the mtDNA of these functional sequence elements, may affect mtDNA replication. Considering the available mtDNA deletion pattern (cf. MITOMAP, http://www.mitomap.org/cgi-bin/tbl10gen.pl), large deletions have not been reported in this region, and this gives indirect support for the necessary presence of the control region during mtDNA maintenance [Bogenhagen and Clayton, 2003].

Recently, a 154-bp deletion (m.16154_16307del154) that spanned positions 16154 to 16307 in mtDNA control region was identified in a healthy family. This deletion removes a TAS and another important region for mtDNA replication [Behar et al., 2008]. The presence of the 154-bp deletion in a healthy family apparently challenges the notion for a critical role of the control region during the regulation of mtDNA replication [Behar et al., 2008]. In this study, we have identified a 50-bp deletion (m.298_347del50) in mtDNA control region from a healthy woman during a survey for mtDNA sequence variations in Chinese. Although the deleted region removes the conserved sequence block II (CSBII) and the replication primer location, the

Additional Supporting Information may be found in the online version of this article.

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deletion does not appear to affect the mtDNA copy number in the peripheral blood cells of the subject.

Materials and Methods

Sample Collection, DNA Extraction, and Amplification

We collected blood samples from 5,559 Chinese individuals across China as part of our project on the characterization of mtDNA sequence variations in general Chinese populations. In addition, 2,538 individuals with medical disorders, such as suspected or confirmed Leber hereditary optic neuropathy, schizophrenia, leprosy, and hematological disorders, were also studied. Each subject was given informed consent and the study was approved by the institutional review board of the Kunming Institute of Zoology. Total genomic DNA was isolated from blood using a standard phenol/chloroform method. The second hypervariable segment of the mtDNA control region was amplified in a volume of 20-μl reaction mixture containing 1 × PCR buffer, 125 μM of each dNTP, 0.15 μM of each primer (L29: 5′-ggTCTATCACCCTATTAACCAC-3′ and H408: 5′-CTgTTAAA-AgTgCATACCgCCA-3′), 0.5 unit of Taq DNA polymerase, and 20 ng DNA during the initial survey.

The 50-bp deletion was identified in a 33-year-old woman (II:3 in family ZJ170) from Zhejiang, China (Fig. 1). This woman is healthy and without any mitochondrial disease. Family members of the subject, including her mother (I:1), sister (II:1), brother-in-law (II:2), and nephew (III:1), are all healthy and were invited to donate hair and/or other samples. Hair roots were either pooled together or were analyzed individually and genomic DNA was extracted using a described method [Yao et al., 2007]. To confirm the deletion, we amplified part of the control region using another pair of primers L16209 (5′-CCCCATgCTTACAAgCAAgT-3′)/H575 (5′-TgAggAggTAAgCTACATAAACTg-3′), which covered a longer region. The PCR product was purified and directly sequenced.

Detecting Heteroplasmy of the 50-bp Deletion

We employed two approaches to detect the minimal level of heteroplasmy of the 50-bp deletion. In the first round, PCR products amplified by primer pair L29/H408 were purified and cloned into pGM-T vector according the manufacturer's manual (Tiangen Bio Inc., Beijing, China). *Escherichia coli* colonies were screened by using colony PCR with the following thermal condition (one cycle at 94°C for 7 min; 30 cycles of 94°C for 30 sec, 49°C for 30 sec, and 72°C for 30 sec; one cycle at 72°C for 7 min). The universal primer pair T7/SP6 was used in a volume of 20-μl reaction mixture to detect the positive clones, which were further confirmed by direct sequencing. In the second round, we used primer pair L332 (5′-CCCgCTTCTggCCACAgCAC-3′)/H575 (5′-TgAggAggTAAgCTACATAAACTg-3′) to selectively amplify the nondeleted mtDNA molecules in these DNA samples with or without apparent homogeneity of the deletion.

Complete mtDNA Genome Sequencing

The entire mtDNA genome of subject II:3 was amplified by using nine pair of primers (for primer list, cf. ref [Wang et al., 2008]) and was sequenced. Sequences were handled with DNAstar software package (DNAStar Inc., Madison, WI, USA) and nucleotide variations were scored relative to the revised Cambridge Reference Sequence (rCRS) [Andrews et al., 1999]. The sequence variations in sample II:3 are also presented in a tree profile, together with two reported mtDNAs (EF429141

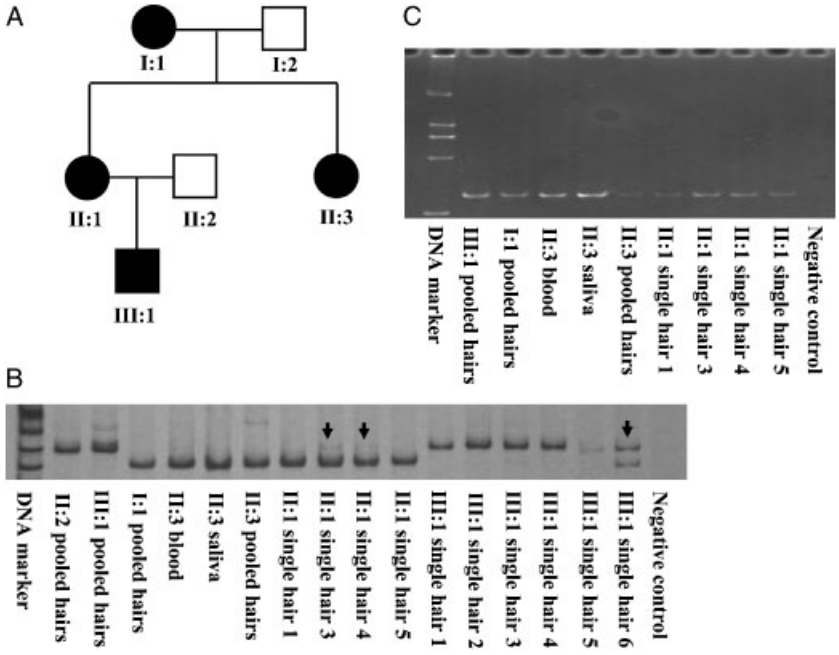

Figure 1. Characterization of the 50-bp deletion in mtDNA control region in a healthy family. **A:** Pedigree showing the presence of the 50-bp deletion (marked in black). **B:** Distribution of the 50-bp deletion in different tissue samples from the family. PCR products amplified by using primers L29 and H408 were separated on 8% polyacrylamide gel electrophoresis. Arrows refer to the heteroplasmic status of the nondeleted mtDNAs in two single hairs from subject II-1 and one single hair from subject III-1. **C:** PCR amplification using primer pair L332/H575 to detect the presence of the nondeleted mtDNAs.

[Wang et al., 2007] and AP010718 [Bilal et al., 2008]) that closely match our sample, following the same approach as described in our recent studies [Ji et al., 2008; Kong et al., 2006; Wang et al., 2008].

Quantification of the mtDNA Copy Number

The mitochondrial DNA copy number was measured using the real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method [Schmittgen et al., 2000; Winer et al., 1999]. mtDNA content was normalized to a single copy nuclear β-globin gene. The following primers were used for real-time PCR analysis: for β-globin gene, HBB502f (5′-CTATgggACgCTTgATgT-3′)/HBB614r (5′-gCAATCATTCgTCTgTTT-3′); for mtDNA, L394 (5′-CACCAgCCTAACCAgATTTC-3′)/H475 (5′-gggTTgTATTgATgAgATTAgT-3′). We first generated standard curves for both fragments and calculated their respective amplification efficiencies to test if using the $2^{-\Delta\Delta CT}$ method was appropriate. The quantitative real-time PCR was then conducted for the calibrator DNA, sample II:3, and samples from 20 healthy individuals without any deletion in the mtDNA control region.

Results and Discussion

Identification, Tissue Distribution, and Transmission of the 50-bp Deletion

We recently launched a comprehensive study to analyze mtDNA sequence variations in Chinese populations and 8,097 Chinese individuals have been analyzed. Among them, a sample from Zhejiang Province, China (II:3 in family ZJ170) presented a faster mobility compared with the other samples during electrophoresis, and the difference remained when using different primer pairs to amplify the genomic DNA isolated from peripheral blood (Fig. 1 and Supp. Fig. S1). Analysis of the DNA extracted from saliva and hair roots of subject II:3 also showed the presence of the deletion, suggesting a wide tissue distribution (Fig. 1). Sequencing analysis of the PCR product identified a 50-bp deletion (m.298_347del50) from position 298 to 347 in the second hypervariable segment (HVS-II) (Fig. 2). The deletion was not observed in the available mtDNA dataset, which contains more than 6,700 complete or near complete mtDNA genomes [van Oven and Kayser 2009] and more than 20,000 HVS-II sequences. It was also absent in >8,000 Chinese mtDNAs that we had analyzed for the control region variation.

To characterize further whether the deletion was inherited or not, we collected hair samples from her mother (I:1 in family ZJ170), her sister (II:1), and a nephew (III:1) of the subject II:3 (Fig. 1). The hair samples were either pooled or analyzed individually. Initially, the deletion was found in the hair samples from the mother and the sister, but not in the pooled hair DNA of the nephew. This unexpected pattern suggested that the deletion might exist in a heteroplasmic instead of a homoplasmic status as discerned in subject II:3. Indeed, analysis of the single hair roots from the sister (four hair roots) and nephew (six hair roots) showed that the deletion was heteroplasmic in several hair samples (Fig. 1).

We used the TA-cloning method to analyze the presence of the deleted mtDNA molecules in the pooled hair DNA (I:1 and III:1), the blood DNA (II:3), and individual hairs (II:1 and III:1), and confirmed the presence of deletion in these hair samples, albeit at different level of heteroplasmy (Supp. Table S1). The pattern of the results confirmed the maternal inheritance of the deletion and we speculate that there may be a minimum level of nondeleted molecules in the subject II:3 and her mother, which was beyond the detection sensitivity of the TA-cloning method. Otherwise, it would be very hard to explain why the nephew had an apparently homogenous level of nondeleted mtDNA. We used primers L332 and H575, which were located in the middle of the deleted region and downstream of the deletion, respectively, to optionally amplify the nondeleted mtDNAs in the genomic DNA. All the analyzed samples yielded the expected bands (Fig. 1), suggesting that there was indeed a low level of heteroplasmy of the non-deleted mtDNA in the maternally related individuals.

The 50-bp Deletion Does Not Affect mtDNA Copy Number

Mitochondrial DNA deletions often occur in a region flanked by sequence repeats [Samuels et al., 2004] and in this study two short homologous direct repeats of CCAAACCCC flanked the 50-bp deletion (Fig. 2). The deleted region is located in the region for H-strand origin and includes the conserved sequence block II and the replication primer location (region 317–321) [Chang and Clayton, 1985, 1987; Kang et al., 1997; Pham et al., 2006].

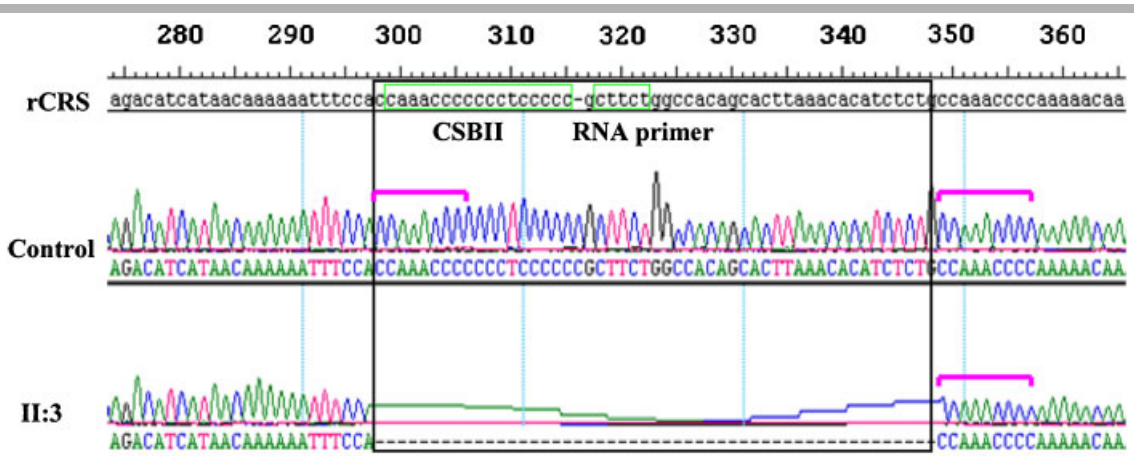

Figure 2. Sequencing results of the subject with the 50-bp deletion and a control mtDNA without the deletion. The sequences were aligned together with the revised Cambridge reference sequence (rCRS). Note that the rCRS has a rare deletion of a cytosine compared to the other mtDNAs [Andrews et al., 1999]. The flanked repeats (CCAAACCCC) are marked by pink line. Functional sequence elements in this region are marked by green boxes.

The guanine-rich CSBII directs transcription termination and primer formation in mtDNA replication [Pham et al., 2006]. The transcription-coupled R-loop formation and the assembly of stable R-loops, which are crucial for initiating mtDNA replication, are completely abolished by mutations in this sequence-dependent transcription termination element [Lee and Clayton, 1998]. Considering the absence of the functional elements for mtDNA replication in subject II:3, we hypothesized that this individual might encounter a problem with mtDNA replication and would have a reduced mtDNA copy number within cells. However, we found that the relative value of the mtDNA copy number in this individual was within the range of those of healthy individuals without the deletion and had no difference from the majority of the samples (Fig. 3). It should be mentioned that three control samples presented a higher value of mtDNA copy number. This might be caused by the DNA extraction method employed in this study, as recent reports have shown that the phenol/chloroform method can give randomly false high values during the quantification of mtDNA content [Andreu et al., 2009; Guo et al., 2009]. Taken together, our data suggested that mtDNA copy number in sample II:3 was not affected by the 50-bp deletion.

Absence of Other Sequence Anomaly in the mtDNA with the 50-bp Deletion

To see whether subject II:3 harbored other mtDNA deletions or sequence anomalies, we amplified the entire genome by using nine overlapping fragments and separated the PCR products on 1.5% agarose gel, together with a control mtDNA which had been fully sequenced in our previous study [Ji et al., 2008] and shown to be free of deletions. We observed no obvious size difference of the respective bands between the II:3 and control sample on the gel except for the first fragment that covers the 50-bp deletion (Supp. Fig. S2). To exclude further the possibility of any small deletion in the mtDNA of subject II:3, which would be unable to be resolved in the gel, we sequenced the entire mtDNA genome and the sequence has been deposited in GenBank under accession number GQ161177. We did find a 9-bp deletion (CCCCCTCTA) in the region 8281–8289; and this deletion, together with a string of specific variants such as m.709G>A, m.1119T>C, m.3497C>T, m.3571C>T, m.8772T>C, m.15346G>A, m.16189T>C, and m.16217T>C, unambiguously indicated a haplogroup status of B4c1b2 [Kong et al., 2006]. The sequence variations of II:3 and two near-matching sequences from published sources [Bilal et al.,

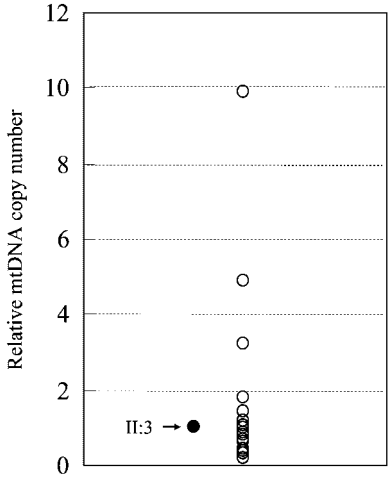

Figure 3. Quantification of mtDNA copy number in subject II:3 with the 50-bp deletion (marked by an arrow) and 20 age-matched healthy controls without the deletion.

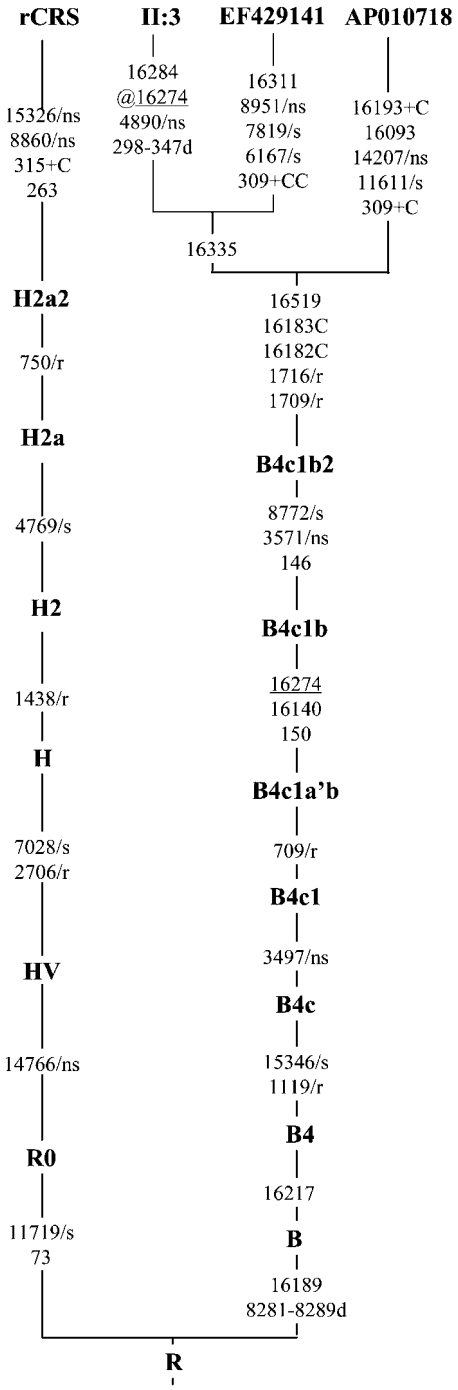

Figure 4. Classification tree of the complete mtDNA sequences of subject II:3 and two mtDNAs from published resources (EF429141 [Wang et al., 2007] and AP010718 [Bilal et al., 2008]), plus the revised Cambridge reference sequence (rCRS) [Andrews et al., 1999]. The order of mutations on each uninterrupted branch section is arbitrary. Recurrent mutations are underlined, reversions to the rCRS are marked by "@," whereas suffix "d" indicates a deletion. The synonymous and nonsynonymous coding-region variants in the four mtDNAs are further denoted by "/s" and "/ns," respectively. Variations in the ribosomal RNA genes are denoted by "/r."

2008; Wang et al., 2007] are presented in a tree profile (Fig. 4). Apparently, the three mtDNAs belong to an unnamed subclade of haplogroup B4c1b2, which is characterized by two variants (m.1709G>A and m.1716T>C) in the *MT-RNR2* gene.

Implications for mtDNA Replication and Transmission

Compared to the mtDNA coding region, the rate of nucleotide substitutions in the control region is much higher, and most of the variations are located within three hypervariable segments. Deletions that remove three base pairs or more are seldom observed. The absence of large deletions within the control region has been interpreted as showing the functional necessity of this region during the origin and termination of mtDNA replication [Bogenhagen and Clayton 2003; Samuels et al., 2004; Yasukawa et al., 2005]. Indeed, in our previous study that focused on the occurrence of indels (insertions/deletions) in the mtDNA control region, we only observed the sporadic occurrence insertions, such as m.43_44insC, m.16259_16262insC, m.16259insA, and m.16470_16474insG in Chinese [Yao et al., 2003]. Deletion of an adenine at position 249 is characteristic of haplogroups F and CZ in East Asia [Kong et al., 2006], whereas deletion or insertion of a tandem repeat AC in region 515–524 and the length mutations of the C-stretch in regions 303–309, 568–573, and 16184–16193 constitute well-known mutational hot spots. In Native American populations, some haplogroup A2 lineages bear a 6-bp deletion (GGAGCA; m.106_111del6) in region 106–111 and this deletion has been regarded as a valuable marker for studying Amerindian history [Santos and Barrantes 1994a, 1994b]. Intriguingly, another 6-bp deletion (CGGAGC; m.105_110del6), with one base pair shift of the deletion m.106_111del6, was noticed in four haplogroup C1 lineages [Álvarez-Iglesias et al., 2007]. Because all of the above mini-indels do not remove functional elements for mtDNA replication, the overall structure of the control region may not be greatly altered, and we do not expect that these mini-indels affect mtDNA replication. However, if the deleted region were large enough to remove the functional elements, a reduced mtDNA replication efficiency would be naturally expected. The 50-bp deletion found in our current study removes the CSBII and the replication primer location, but we did not observe a reduction of mtDNA copy number in this subject as expected. One possible explanation is that this deletion is a rare polymorphism and does not affect mtDNA copy number, or there is an alternative mechanism for mtDNA replication in this individual. Indeed, new data generated using two-dimensional agarose gel electrophoresis of mtDNA replication intermediates have showed that replication may be initiated from multiple origins across a broad zone [Bowmaker et al., 2003]. The newly reported 154-bp deletion, which removed a region containing essential elements for the regulation of mtDNA replication according to both the asynchronous displacement model and the strand-coupled replication model [Behar et al., 2008], did not pose a deleterious effect. Despite the fact that the mtDNA copy number was not measured in the sample with the 154-bp deletion, we would expect that this individual would have a similar mtDNA copy number compared to the control samples. Similarly, the deletions m.106_111del6 and m.105_110del6 that may affect the initiation of mtDNA replication in the region around nucleotide 110 [Kang et al., 1997], are also best categorized as polymorphisms.

Despite of the absence of the 50-bp deletion in available mtDNA data sets of world populations, an extensive Web-based search [Bandelt et al., 2009] using "50-bp deletion and mtDNA" yielded several hits for a somatic 50-bp deletion in cancerous tissues, including gastric adenocarcinoma [Burgart et al., 1995], hepatocellular carcinoma [Lee et al., 2004; Okochi et al., 2002], lung cancer [Lee et al., 2005], and primary endometrial carcinoma [Liu et al., 2003]. Except for the pioneer report for the somatic 50-bp deletion [Burgart et al., 1995], the patients in the subsequent reports were most likely of East Asian origin. The exact reason for the seemingly high occurrence of somatic 50-bp deletion in East Asian patients with cancer is unknown. We failed to detect this deletion in tumorous tissues/cells from 299 Chinese patients with cancer, including breast cancer, colorectal cancer, gastric cancer, esophagus cancer, lymphoma, or leukemia. Moreover, there is no occurrence of cancer in the members of family ZJ170, suggesting no direct correlation between tumorigenesis and the 50-bp deletion.

One intriguing question arising from the current study is the transmission of the 50-bp deletion in the pedigree. The analysis of DNA from blood, saliva, and hair samples of subject II:3 and hair samples from her mother and sister showed a prevalence of the deleted mtDNAs, whereas the nephew showed a nearly homoplasmic status of the nondeleted mtDNAs. This pattern suggested that the 50-bp deletion was not generated de novo in subject II:3 and could be transmitted to the offspring. The almost total absence of the 50-bp deletion in the nephew could be interpreted by the genetic drift effect occurring in early oogenesis [Brown et al., 2001; Cree et al., 2008; Jenuth et al., 1996; Wai et al., 2008].

Conclusion

In summary, we characterized a 50-bp deletion in the second hypervariable segment of the mtDNA control region in samples taken from members of a healthy family. The deletion was inheritable and did not affect mtDNA copy number within cells, and therefore should be categorized as a rare polymorphism. Our current finding, together with the recently reported 154-bp deletion in the control region [Behar et al., 2008], suggests that the role of the control region during mtDNA replication may be more complex than we had thought. Further in vitro functional assays using cell lines harboring the deleted and nondeleted mtDNAs or the templates derived from this family are essential to confirm our result that no problems occur with mtDNA replication and transcription in the presence of the 50-bp deletion.

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