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No Increase of Large-scale Mitochondrial DNA Deletions in Peripheral Blood Cells in Residents of Kazakhstan around Semipalatinsk Nuclear Test Site

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The study was set out to elucidate whether there are detectable differences in the molecular features of mitochondrial DNA (mtDNA) from peripheral blood lymphocytes (PBLs) between individuals possibly exposed to radiation due to fallouts from Semipalatinsk Nuclear Test Site and those who lived in non-contaminated area in Kazakhstan. Subjects were residents of three villages in Kazakhstan considered to be affected with radionuclides in the past. Control individuals were from one non-contaminated settlement in Kazakhstan and age-matched Japanese volunteers. Two parameters, the relative mtDNA content and abundance of large-scale deletions (LSDs) in mtDNA were estimated by real-time PCR and a PCR adjusted to sensitively reveal the LSDs, respectively. Relative content of mtDNA as well as the abundance of LSDs were found to differ non-significantly both in Kazakhstan radiation exposed, control Kazakhstan and Japanese individuals. LSD scores displayed no regular or statistically significant association with the mtDNA content in any group tested. Lack of correlation between the number of LSDs and mtDNA level occurred irrespectively the radiation history of a blood donor or individual's age. We conclude that the molecular parameters of PBL mtDNA measured in this work are unlikely to be informative as a bioindicator of radiation exposure.

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Introduction

Most of mammalian cells possess dual genetic system comprised by the nuclear and mitochondrial genomes. Each organelle contains 2-10 copies of double-stranded circular DNA molecules, and there may be from a few to up to several thousand of mitochondria per cell depending on the tissue type and/or cell's energy requirements.[1-3] Given there are many copies of mitochondrial DNA (mtDNA) in a cell, a situation referred to as heteroplasmy may occur when a mutation or various mutations take(s) place in one or different mtDNA molecules leading to the coexistence of wild type and mutant mtDNA species within one cell or perhaps even within one organelle.

mtDNA mutations such as base substitutions and rearrangements have been implicated in degenerative diseases, aging, and cancer.[4-6] Although precise molecular mechanisms of each particular entity may remain unclear, mutated mtDNA-associated distortion of cell function is likely to be a driving force or may contribute to the pathogenesis of a disease, at least in some degenerative disorders. Importantly, low levels of altered mtDNA species, both disease-associated and non-associated ones, have been found even in healthy tissues.[5,7] Their abundance appears to increase with age in different tissues, but total level of mutated mtDNA usually remains very low as compared to that of wild-type molecules.[5,7]

mtDNA possesses 10- to 100-fold higher mutational rate than the nuclear DNA. The enhanced mutagenesis in the mitochondrion are associated with spatial proximity of the mtDNA molecules to the respiratory chain characterized by an aggressive environment, lack of the protective protein sheath such as histones in the nucleus, at

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tenuated mitochondrial DNA repair machinery and infidelity of DNA polymerase gamma responsible for the mtDNA replication.[8-11] Most of mtDNA alterations will likely result from the errors taking place during the mtDNA replication and misrepair of mutations evoked by the endogenous production of reactive radicals from the respiratory chain, while exogenous factors such as chemicals and ionizing radiation can also influence the mutation rate. Both point mutations and large-scale deletions (LSDs) in mtDNA have been shown to arise following DNA-damaging exposure.[12]

Ionizing radiation is one of genotoxic factors that may contribute to the excessive mutagenesis of nucleic acids. We reported that a concordant increase in the relative mtDNA content and the number of mtDNA deletions was detected more frequently in radiation-associated post-Chernobyl papillary thyroid carcinoma than in sporadic cases.[13]

Residents of the territories around Semipalatinsk Nuclear Test Site (SNTS) in Kazakhstan, amounting to several hundred thousand, are considered to have probably been affected by external and internal radiation caused by about 400 nuclear tests conducted at SNTS during 1949-1989.[14] Takada et al.[15] reported external doses up to 100 cGy for residents around SNTS by evaluating with the thermoluminescence technique the external dose from exposed bricks sampled at several settlements around SNTS in 1995-1996.

This study was undertaken to address a question whether specific molecular features of mtDNA from peripheral lymphocytes (PBLs) of the individuals differ in various age group between those proba bly exposed to radiation and those who have lived in non-contaminated areas in Kazakhstan. Blood was chosen as a facile biomaterial accessible by safe and minimally invasive procedure. We note that previous studies detected no mutant mtDNA in the blood of healthy individuals,[16,17] while introduction of highly sensitive and specific methods have enabled the detection of low-abundant mutant mtDNA in the blood cells of healthy individuals.[18,19] We quantitatively examined relative mtDNA levels and number of LSDs in PBL DNA specimens of residents of Kazakhstan and Japan by the method similar to that we used in the study of mtDNA in radiation-associated post-Chernobyl thyroid tumors.[13]

Materials and Methods

Subjects and blood sampling

The study subjects were 61 Kazakh women and 15 Japanese women who were healthy and not suffered from any systemic or hematological diseases. Among 61 Kazaks, 45 were living in 3 villages located within 100 km from the SNTS and 16 were residents of a village located 250 km southeast off the SNTS. We regarded the former 3 villages and the latter one village as highly contaminated with radioactive fallout from the nuclear test and non-radiocontaminated, respectively. Fifteen Japanese women, matched to 16 Kazakh women in the above-mentioned non-radiocontaminated village by age distribution, were recruited from healthy blood donors.

We collected 5 ml of venous blood from respective subjects after we obtained the informed consent individually. We isolated lymphocytes using a Lymphoprep density gradient medium (Nycomed, Denmark) and stored them in the RNAlater reagent (Ambion, USA) at -20°C until use. Total DNA was extracted by standard ProteinaseK/phenol-cholroform procedure.

We classified the subjects into 3 groups by the year of birth according to the status of the nuclear tests at SNTS. The first group consisted of those born before 1949 when nuclear tests were commenced at SNTS. The second group consisted of those born from 1949 to 1962 when the nuclear tests were conducted on the ground and in the atmosphere. The third group consisted of those born from 1962-1989 when all of the nuclear tests were conducted underground. We compared the relative mtDNA content and number of large scale deletions, and their correlation in subjects by residence and the year of birth. Table 1 presents the age distribution (mean ±SD) of subjects by residence and the year of birth.

Table 1 The number of subjects and their age (in years) at the time of examination (mean±SD) by residence and year of birth

Year of birth Type of nuclear tests at SNTS	Contaminated areas Kazakhstan	Control area Kazakhstan	Japan
Before 1949 No tests	13 58.3±5.30	5 54.8±1.30	5 53.2±2.59
1949-1962 Ground/Atmosphere	15 46.3±4.53	5 42.0±3.40	5 42.0±1.79
1962-1989 Underground	17 31.7±5.73	6 38.0±1.63	5 34.4±3.47

Determination of relative mtDNA content by real-time PCR

The level of mtDNA in each sample was assayed by real-time PCR with a pair of primers located to the 12S rRNA region of human mitochondrial genome using the SYBR Green Assay (PE Applied Biosystems, USA). The primer sequences were as follows: 5′-CACT GAAAATGTTTAGACGGGC-3′ and 5′-TGCTTGTCCCTTTTGAT CGT-3′. Twenty-five nanograms of total DNA were used as a template, and PCR reactions were performed in triplicates in total volume of 25μl in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, USA). The nuclear DNA content was measured by real-time PCR with human ß-actin TaqMan reagent kit (PE Applied Biosystems, USA) according to manufacturer's guidelines. The standard curves were generated with DNA Template Reagent (PE Applied Biosystems, USA) which is the DNA extracted from Jurkat cells. The relative mtDNA content was calculated as a ratio of mtDNA/nuclear DNA, each of which was measured in arbitrary but the same unit, thus yielding a dimensionless quantity representing the mtDNA level normalized by the nuclear DNA content in a given DNA specimen.

Detection of large-scale mitochondrial DNA deletions

To obtain a spectrum of deleted mtDNA fragments, 100 ng of DNA was subjected to 40 PCR cycles in the presence of a pair of

primers consisting of a forward primer (5´-AGGATGAATAATA GCAGTTCTACCG-3´, mtDNA bases: 5050-5054) in the ND2 region of mitochondrial genome and a reverse primer labeled at the 5´with Texas Red (5´-TGGGCTATTTTCTGCGG-3´, mtDNA bases: 13130-13149) in the ND5. The primer concentrations were 200 nM of both forward and reverse oligonucleotides. The PCR thermal profile was as follows: 94°C for 10 min, then 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. After PCR, the products were denatured, quickly chilled in an ice waterbath and loaded on a sequencing gel in the Hitachi 5500 DNA Sequencer (Hitachi Instruments, Japan). The gel images were generated with Hitachi DNAsis software. To calculate average number of bands per sample, each DNA specimen was subjected to the PCR analysis three to five times.

We used PCR approach for revealing large-scale deletions in mtDNA. We set the elongation time of reaction as short as 1 minute so that the size of the amplified products did not exceed 1.5 kb. Since the primers reside in the mtDNA regions about 8 kb apart from each other, only templates with interstitial fragment deleted would yield amplicons. Use of fluorescent-labeled primer made it possible to visualize the products with a sequencing device. We could obtain the spectrum of various amplicons representing LSDs for each DNA specimen (Figure 1). To confirm the structure of PCR products, we re-amplified some of them after elution of bands randomly selected from an agarose or native acrylamide gel and then sequenced. The amplicons were determined to be authentic human mtDNA species with 7-7.7 kb fragments deleted between the ND2 and ND5 regions of mitochondrial genome (data not shown).

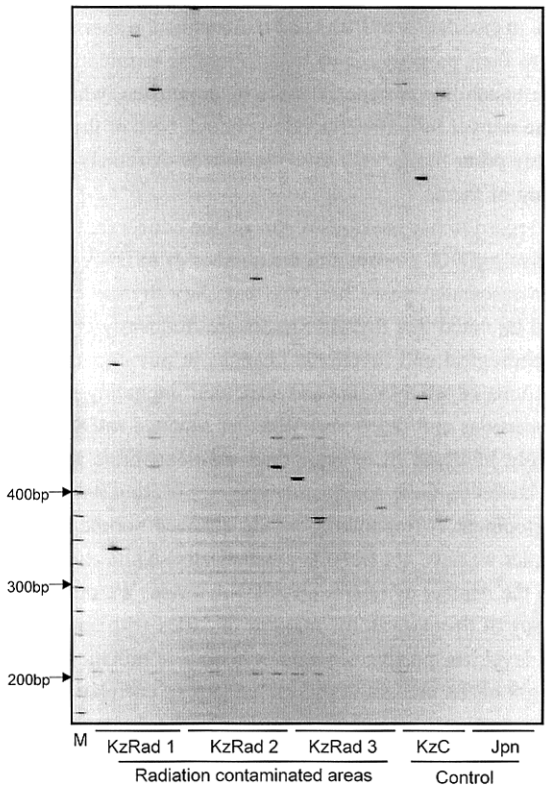

Figure 1. The spectrum of aberrant mtDNA fragments with large-scale deletions. The sequencing gel image was generated by computer program. KzRad 1, 2 and 3 denote three groups of subjects in the three radio-contaminated villages in Kazakhstan, respectively; KzC denotes a group of subjects in the non-contaminated village in Kazakhstan; and Jpn denotes a group of Japanese.

Results

No significant difference was observed in the relative mtDNA content in the PBLs among three age groups for each group of residence (Figure 2).

Similarly, no significant difference was observed in the number of large-scale mtDNA deletions among three age groups for each group of residence (Figure 3).

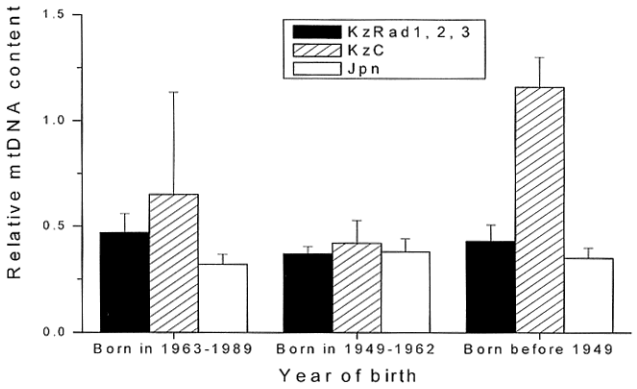

Figure 2. The relative mtDNA content in the PBLs of subjects by age group and residence. KzRad 1, 2, 3 denotes a combination of three groups (KzRad 1, KzRad 2 and KzRad 3) of subjects in the three radio-contaminated villages in Kazakhstan; KzC denotes a group of subjects in the non-contaminated village in Kazakhstan; and Jpn denotes a group of Japanese. The whisker denotes the standard error.

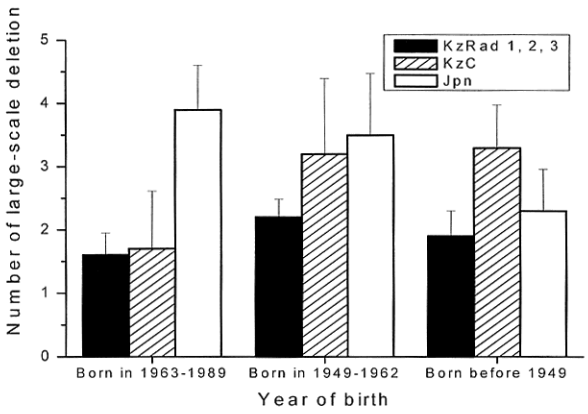

Figure 3. The mean number of large-scale deletions in mtDNA per PBL DNA sample by age group and residence. KzRad 1, 2, 3 denotes a combination of three groups (KzRad 1, KzRad 2 and KzRad 3) of subjects in the three radio-contaminated villages in Kazakhstan; KzC denotes a group of subjects in the non-contaminated village in Kazakhstan; and Jpn denotes a group of Japanese. The whisker denotes the standard error.

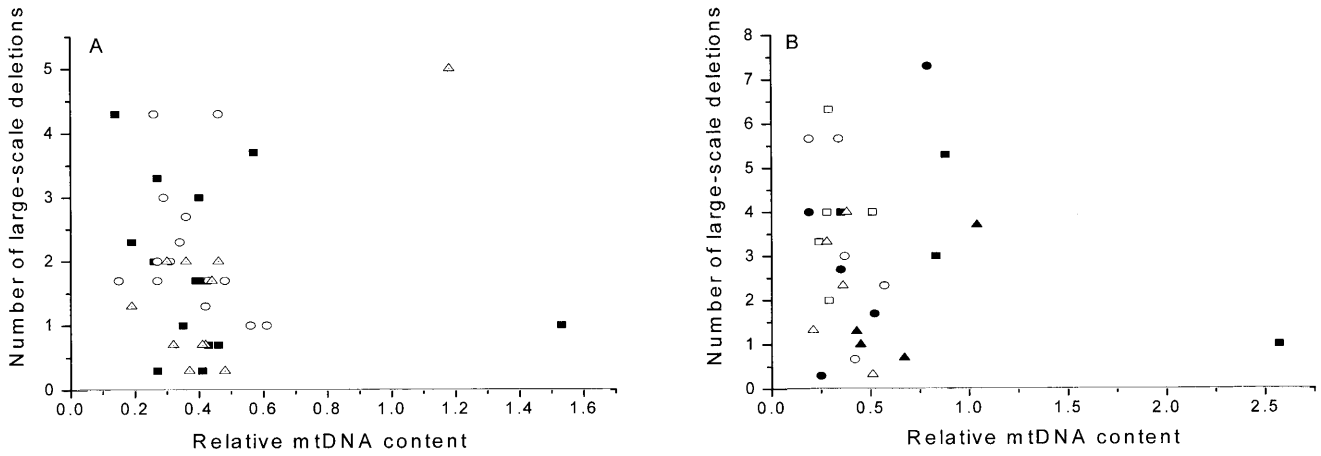

Figure 4. Scatter plots of the relative mtDNA content and the number of large scale deletions by age group in the PBLs of subjects living in the radio-contaminate areas of Kazakhstan (A, left panel) and those living in non-contaminated area of Kazakhstan and Japan (B, right panel). The correlation (p-value) between the relative mtDNA content and the number of large-scale deletions in each group was as follows: in A, r=0.79 (p=0.004) for the group (△) of those born before 1949; r=-0.32 (p=0.258) for the group (○) of those born in 1949-1962; and r=-0.26 (p=0.373) for the group (■) of those born in 1963-1989; and in B, r=0.85 (p=0.153) for the group (▲) of Kazakhs born before 1949; r=0.66 (p=0.221) for the group (●) of Kazakhs born in 1949-1962; r=-0.81 (p=0.192) for the group (■) of Kazakhs born in 1963-1989; r=-0.30 (p=0.624) for the group (△) of Japanese born before 1949; r=-0.69 (p=0.915) for the group (○) of Japaneses born in 1949-1962; and r=0.07 (p=0.916) for the group (□) of Japaneses born in 1963-1989.

Figure 4 depicts the scatter plots of the relative mtDNA content and the number of large-scale deletions by age group in subjects living in the radio-contaminated areas of Kazakhstan (A) and those living in Japan and the control area of Kazakhstan (B). No significant correlation was observed in either group except for the one consisting of Kazakh women in the radio-contaminated areas who were born before 1949.

Discussion

The method used in the present study to estimate the number of LSDs in mtDNA was demonstrated by the study[13] using DNA extracted from thyroid tissue that it reproduces estimates and that it does not result in artificial distortion of data. Furthermore, another experiment we conducted using cultured human cells demonstrated a dose-dependent increase in the number of LSDs in the cells after X-ray irradiation. Additionally, sequencing of the PCR products confirmed the presence of appropriately truncated authentic aberrant mtDNA species, once more attesting to the validity of the technology.

The present study, however, showed a positive or a negative correlation, though not statistically significant except for one, between the relative mtDNA content and the number of large-deletions in Kazakh women in the radio-contaminated areas as well as those in the contaminated area and Japanese women.

One of the reasons that the present study resulted in negative findings may partly be associated with the biology of both PBLs and mtDNA. Results of the flow cytometry enumeration of lymphocytes with mutant T-cell receptor in exposed individuals demonstrated that such variant cells could not be detected in the bloodstream long time (2-4 years) after exposure.[20,21] Thus, in our study, mtDNA aberrations that could probably be revealed in the used material should either i) have had taken place in exposed (?) hematopoetic stem cells that gave rise to circulation of PBLs and transmitted the aberrant mtDNA species to their progeny, or ii) be attributable to radiation-induced genomic instability in exposed cells or organisms, whose mechanisms are not yet fully understood. Although both of these and perhaps some other things may take place, it is extremely difficult to prove any of them.

With regard to our previous report on the concordant increase in the relative mtDNA content and the number of mtDNA deletions in radiation-associated post-Chernobyl papillary thyroid carcinoma,[13] it should be noted that thyroid tumors are frequently characterized by morphological and functional changes in mitochondri[22] as well as alterations of mtDNA size and structure[23] including point mutations, insertions and deletions.[6] Whether mutated mtDNA may be causatively involved in or comprises carcinogenesis remains unknown; however, there are speculations implicating its role in cancer development.[6,24] Importantly, in the matched normal thyroid tissue samples we have not found any statistically significant association between the number of LSDs and mtDNA levels. We observed only a tendency of decrease in the number of LSDs with an increase in mtDNA level irrespective of patient's history of radiation exposure. In the light of the present study, such a lack of correlation between the two parameters would be plausible to ascribe to "normal" or "physiological" situation occurring in a healthy tissue without gross abnormalities or pathology. The results of our following animal experiment support in part this reasoning. We irradiated rats by X-ray of various doses and examined the parameters of mtDNA in either resting or mitogen-stimulated PBLs as well as in healthy solid tissues of many other types. No statistically significant correlation

was observed between the abundance of LSDs and mtDNA content, while the number of LSDs showed a tendency to decrease with an increase in mtDNA content (data not shown); this fact parallels the data obtained in the present study.

Although the number of subjects in the present study was relatively small and the radiation dose received by the subjects was unknown, we may conclude from our results that characteristics of mtDNA in PBLs of healthy adults are unlikely to be informative as a biological indicator of radiation exposure.

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