Mitochondrial DNA Mutations Induced by Carbon Ions Radiation: A Preliminary Study

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Abstract
Heavy-ion irradiation-induced nuclear DNA damage and mutations have been studied comprehensively. However, there is no information about the deleterious effect of heavy-ion irradiation on mitochondrial DNA (mtDNA). In this study, 2 typical mtDNA mutations were examined, including 4977 deletions and D310 point mutations. The 4977 deletions were quantified by real-time polymerase chain reaction, and D310 point mutations were analyzed by direct sequencing and a specific enzyme digestion genotyping method. Results showed that carbon ions radiation can induce temporal fluctuation of mtDNA 4977 deletions in 72 hours after irradiation, while survived clones were free from this deletion. Carbon ions induced more D310 mutations than X-rays, and the single-cell heteroplasmy was eliminated. This is the first study investigating mtDNA mutations induced by carbon ions irradiation in vitro. These findings would provide fundamental information for further investigation of radiation-induced mitochondrial biogenesis.

Keywords
carbon ions, mtDNA 4977 deletions, D310, colony formation

Introduction
Among all the biomolecules that makeup a living organism, DNA is the most crucial target of ionizing radiation (IR). When a single track of IR hits the DNA directly or passes close by the DNA strand, multiple lesions can be produced, referred as clustered DNA damage.¹ This type of damage is considered to result in double-strand breaks in a DNA molecule, which is lethal for cells if not repaired. Radiation-induced DNA damage can cause cell death or even worse for multicellular organisms, mutations that might lead to genomic instability and eventually to cancer. However, current knowledge about radiation-induced DNA damage is mostly limited to nuclear DNA damage, the radiation-induced damage on the extranuclear genome in human cells—mitochondrial DNA (mtDNA) has not been well studied.

Due to its close proximity to the electron transport chain and lack of protecting histones, mtDNA is susceptible to oxidative damage. Hence, mtDNA is one of the main targets to radiation-induced oxidative damage.² More importantly, mutations in mtDNA are associated with a number of hereditary human diseases and are maternally inherited, including Kearns-Sayre syndrome, Leber hereditary optic neuropathy, Pearson syndrome, and chronic progressive external ophthalmoplegia.³ It is necessary to evaluate mtDNA mutations after exposure to IR. Currently, there is no information available for heavy-ion irradiation-induced mtDNA mutations.

The increased lethal effectiveness (cell inactivation) of high-linear energy transfer (LET) radiation contributes to new methods for using radiation therapy, but it is also necessary to study the enhanced mutagenic effect of high-LET radiation, because higher frequencies of mutation can be expected to provide higher rates of carcinogenicity with human exposure. The aims of this study were to investigate whether carbon ions could induce specific mtDNA mutations and whether the mutations can be inherited by the progenies. There are 2 major types of mutations in mtDNA: large deletions and single-nucleotide

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polymorphisms (SNPs). Mitochondrial DNA 4977 deletions, as the most common deletions in tumor and aging cells,4,5 and D310 repeat, as the mutation hotspot in mtDNA,6,7 were chosen. The profile of mutations in HeLa cells after carbon ion irradiation was genotyped and quantified. To date, this is the first evidence of specific mtDNA mutation induced by carbon ion irradiation.

Materials and Methods

Cell Culture and Irradiation

The human cervical cancer cell line HeLa was purchased from the American Type Culture Collection and maintained in Dulbecco modified Eagle medium (Gibco, USA) with 10% fetal bovine serum. Cells were maintained at 37°C using humidified air supplemented with 5% CO2. Cells in exponential growth were irradiated with 200 keV X-rays or 12C6 ions beam at energy 50 MeV/u and LET 45.2 keV/m generated by the irradiation equipment at the Heavy-ion Research Facility in Lanzhou (Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China), with a dose rate of 1 Gy/min. The collimation of the beams to the place irradiated was controlled by a microcomputer. The acquisition of data (preset numbers converted by doses of irradiation) was automatically accomplished using a microcomputer during irradiation. Particle fluence was determined from air ionization chamber signal according to the calibration of the detector (type: PTW-UNIDOS, PTW-FREIBURG Co, Germany).

Survival Assay

The sensitivity of cells to carbon ions and X-rays radiation was measured using a colony-formation assay. Confluent cultures were replated within 1 hour postirradiation into 100-mm dishes in quadruplicate at numbers estimated to yield 50 to 100 clonogenic colonies per dish. After incubation for 2 weeks, the cultures were fixed in methanol and stained with Crystal violet. These stained colonies were observed with an Olympus (Olympus BX51, Tokyo, Japan) light microscope. Colonies with 50 or more cells were scored as clonogenic colonies. D10, the dose required to reduce the surviving fraction to 10%, was selected in the subsequent experiment involved in mutagenic effect assay.

D310 Sequence Genotyping

For detection of D310 mutation in 72 hours after irradiation, a restriction enzyme digestion-based genotyping assay was used.8 The primer sequences were as follows: forward 5′-ACAAATTTAGTCTTTGACAGCCACTT-3′ and reverse 5′-GGCCAGAGATGTTTAAATGCTG-3′, Polymerase chain reaction (PCR) amplifications were performed in a 50 μL volume containing 200 μmol/L of each deoxynucleotide (dNTP), 12.5 pmol of each of the forward and reverse primers, 50 mmol/L KCl, 10 mmol/L Tris–HCl (pH 9.0), 1.5 mmol/L MgCl2, and 1 U of Taq DNA polymerase (Fermentas). The PCR cycling conditions were as follows: 96°C for 90 seconds, followed by 40 cycles at 96°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, then extension at 72°C for 5 minutes (Eppendorf matrycycle PCR system, Germany); 109-bp PCR products were checked by 1.8% agarose gel electrophoresis. The PCR products were subjected to restriction enzyme digestion. The PCR product of 5 μL was mixed with 3 U of BsaXI (New England Biolabs, Frankfurt, Germany) in 1× buffer containing 50 mmol/L potassium acetate, 20 mmol/L Tris–acetate, 10 mmol/L magnesium acetate, 1 mmol/L Dithiothreitol (DTT), and pH 7.9. The samples were incubated at 37°C for 24 hours to ensure complete digestion. Restriction products were separated on 4% agarose gel.

For D310 amplification of the survived clones, each clone was picked and lysed in a 50 μL PCR buffer with a heat denaturation procedure. After that, 1 U Taq was added to the mixture and then subjected to the PCR protocol as described earlier. The PCR products were then sent to Sangon Biotech Corporation (Sangon Biotech Corporation, Shanghai, China) and sequenced. All the results were further validated by at least a new independent PCR amplification and genotyping.

Quantification of mtDNA 4977 Deletions

Real-time PCR quantifications were performed using the light cycle 2.0 real-time PCR system (Roche Applied Science, Basel, Switzerland). The PCR reaction was carried out for 45 cycles in a 20-μL reaction mixture containing 1 U Taq polymerase (MBI Fermentas, USA), 1X SYBR Green I supermix, 1× PCR buffer (MBI, 2 mmol/L Tris–HCl, pH 8.4, 5 mmol/L KCl), 200 mmol/L dNTP, 4 mmol/L MgCl2, 200 mmol/L each primer, and 20 ng DNA. After an initial denaturation at 95°C for 1 minute, 45 cycles of 3-step PCR followed: 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, followed by melting curves analysis to certify product specificity. Relative quantification approach (ΔΔCt) was used to validate the fluctuation in mtDNA deletions in HeLa cells, according to method previously described.9 Each test was carried out in triplicate.

Results

Clonogenic Survival

Radiation-induced loss of clonogenicity was examined with the colony-formation assay. The respective survival curves after exposure to carbon ions and X-rays are shown in Figure 1. Cells exposed to carbon ions showed higher radiation sensitivity when compared to X-rays. The relative biological effect value for the surviving fractions at D10 was 3.1 (1.5 Gy for carbon ions and 4.65 Gy for X-rays). Our data confirmed that carbon ions are more effective than X-rays in cell killing.

Temporal Fluctuation of mtDNA 4977 Deletions

In order to investigate the occurrence of mtDNA 4977 deletions after irradiation, real-time PCR was used to quantify mtDNA 4977 deletions. As shown in Figure 2, mtDNA 4977 deletions...
reached a maximum at 24 hours after exposure to both type of radiations and then decreased sharply. There was no mtDNA 4977 deletions detectable in survived clones 14 days after radiation. These results also indicated that the survived cells after radiation return to a homoplasmy state rather than heteroplasmy.

**Accumulation of D310 Mutations After Radiation**

No significant D310 mutations were detectable by the restriction enzyme digestion-based genotyping assay or by direct sequencing of PCR product. This may due to the dominance of C7 tract in the population of tumor cells. By sequencing the single clone after 14 days’ incubation, the accumulation of D310 mutations induced by radiation was detectable. As shown in Table 1, both types of radiation resulted in increased D310 C-tract deletion (C6) and insertion (C8), but the mutagenic effect was higher in carbon ions radiation than in X-ray radiation. Interestingly, the heteroplastic cells (e.g., C6/C7, C7/C8) were not detected after radiation.

**Discussion**

To further understand the mutagenic effect of high-LET radiation, we analyzed 2 typical mutations in mtDNA after exposure to carbon ions radiation. Our results showed that both high- and low-LET radiations can induce temporal mtDNA 4977 deletions in a short time after exposure but not in survived clones. The results indicate that cells with mtDNA 4977 deletions are eventually eliminated in proliferative cells. This phenomenon was also observed by Wang et al using a cell sorting method. In our results, we found a transit induction of mtDNA 4977 deletions after both irradiations, but these deletions cannot be inherited in the survival clones. Dani et al suggested that the absence of mtDNA 4977 deletions is required for neoplastic growth. In agreement with them, we propose that mtDNA 4977 deletions are intolerable in cells under stress such as in cancer cells, in which high-energy metabolism is required. However, the mechanism involved in the elimination of mtDNA 4977 deletions remains elusive. Programmed cell death and/or autophagy might be involved. Further studies are needed to investigate the potential mechanism in the IR-induced mtDNA mutations.

Different from the temporal pattern of large deletions induced by radiation, D310 mutations increased in progeny cells after X-rays and carbon ion radiation. This result proved that a clonal selection mechanism may take place after radiation. Certain mutants that confer disadvantages to the cell would be eliminated, such as 4977 deletions, whereas those that possess survival advantage or neutrality will be inherited, such as D310 mutants. Ling et al found that certain level of reactive oxygen species (ROS) can promote mt-allele segregation toward wild-type and mutant mtDNA homoplasmy. Similar to their results, we also found that D310 heteroplasmy was not detectable after IR, which might be due to ROS-induced mtDNA segregation.

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**Table 1. D310 Polymorphism Distribution in the 3 Groups of Survived Clones.**

| D310 C-Tract | Unirradiated (%) | X-Ray (%) | Carbon Ions (%) |
|-------------|------------------|-----------|----------------|
| C6          | 4.8              | 8.16      | 12.24          |
| C6/C7       | 2.4              | 0         | 0              |
| C7          | 37.74            | 35.70     | 29.58          |
| C8          | 6.12             | 7.14      | 9.18           |
| C7/C8       | 1.2              | 0         | 0              |
In summary, both high- and low-LET radiations will lead to rapid shift from heteroplasmy to homoplasmy in single cell. The mtDNA 4977 deletions were induced after exposure to radiation irrespective of the quality of radiation. The induced large deletions cannot be retained in survived cells. On the other hand, mtDNA D310 SNP increased after both irradiations, but mutagenic effect was higher in carbon ions radiation than X-rays radiation. In conclusion, carbon ions radiation-induced mtDNA mutation is essentially the same as X-rays. However, the mutagenic effect in carbon ions radiation is higher than in X-rays even at the same cell killing level.

The Novelty and Impact of the Study
Carbon ions radiation-induced mtDNA mutations have not been studied before. This study provides the first report about the mutagenic effect of carbon ions radiation on mtDNA, including 2 typical mutations: mtDNA 4977 deletions as the large deletion and D310 polymorphism as the SNP.

Authors’ Note
Yong Chen and Haining Gao contributed equally to this work and are considered cofirst authors.

Declaration of Conflicting Interests
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