Alterations of mtDNA copy number and 4977 bp deletion induced by ionizing radiation in human peripheral blood

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Abstract Alterations of mitochondria DNA (mtDNA) 4977 bp common deletion (CD) and mtDNA copy number induced by ionizing radiation were observed in human different cell lines and total body irradiation patients. However, only few experiments have evaluated the levels of the CD and mtDNA copy number in human peripheral blood exposed to ionizing radiation till now. The aim of this study is to analyze the mtDNA alterations in irradiated human peripheral blood from healthy donors as well as to explore their feasibility as biomarkers for constructing new biodosimeter. Peripheral blood samples were collected from six healthy donors, and exposed to ^{60}Co gamma ray with the doses of 0 Gy, 1 Gy, 2 Gy, 3 Gy, 4 Gy and 5 Gy. Levels of the CD and mtDNA copy number in irradiated samples after 2h or 24h incubation were detected using TaqMan real-time PCR, and the CD ratio was calculated. The results showed that the mean of the CD ratio and the CD copy number exhibited a dose-dependent increase 2 h in the dose range from 0–5 Gy, and of the mtDNA copy number significantly increased 24 h in irradiated groups compared with 0 Gy group after irradiation. It indicates that the parameters in human peripheral blood may be considered as molecular biomarkers to applying construction of new biodosimeter.

Key words Ionizing radiation, Human peripheral blood, Mitochondria DNA, 4977 bp common deletion, TaqMan real-time PCR

1 Introduction

The mitochondria DNA (mtDNA) with 4977 deletion mutation from base-pairs 8 470 to 13 446 in human mtDNA map of Anderson is a common type of mtDNA deletion mutation, referred to as the common deletion (CD)[1]. The CD removes the genes or parts of the genes encoding for ATPase 8 and 6, COXIII, ND3, ND4 and ND4L and ND5 of the mtDNA[2], resulting in an impairment of the mitochondrial oxidative phosphorylation[3], which has been shown in various tissues from aging humans, mitochondrial myopathy and cancer[4–9].

Chemical reagents and radiation produce more lesions to mtDNA than to a comparable nDNA fragment in cells. Shieh *et al*. has reported that N, *N*-dimethylformamide damaged mtDNA and resulted in increasing of the proportion of CD in human blood[10]. UV can increase the accumulation of CD in human skin[11,12]. In a nested PCR approach, Gattermann and his coworkers were first detected successfully the deletion in human blood[13]. Improvement of PCR conditions allowed detection of the deleted mtDNA in blood of younger and older individuals without employing two different primer pairs[14]. Using in situ PCR technology, a dose-

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dependent increase in the frequency of human peripheral blood lymphocytes (HPBL) with CD when exposed to gamma radiation between 0.5 and 2.0 Gy had been observed in cultured HPBL in interphase[15]. In our previous study, the higher CD levels were observed in the mtDNA samples from 6 health donor's blood exposed to 1–5 Gy ^{60}Co gamma ray when compared with the samples exposed to 0 Gy at 2h after exposure by real-time PCR, but both the CD levels and the exposure doses were not obviously correlated[16]. Recently, Duan *et al*.[17] detected the CD in human peripheral blood exposed to ^{60}Co gamma ray using relatively quantitative real-time PCR, and found that the relative amount of the CD showed certain dose-effect relation in the dose range from 0–8 Gy at 2 h after exposure. But it is still unknown if the deletion would have dose-effect in a wider range of time after irradiation in human peripheral whole blood by real-time PCR.

Furthermore, it was reported that accumulation of mtDNA copy number was observed in peripheral blood cells from different types of cancer patients after radiotherapy or chemoradiotherapy[18,19]. The increase of mtDNA copy number was also found in the mtDNA samples of human peripheral blood nucleated cells exposed to 1–5 Gy ^{60}Co gamma ray 2 h after irradiation in the previous study[16]. But changes of mtDNA copy number in genomic DNAs of human peripheral blood exposed to ionizing radiation or in a wider range of time after irradiation still remains unclear *in vitro*.

Therefore the purpose of the present study was to detect the levels of the CD and mtDNA copy number in genomic DNAs of human peripheral blood samples exposed to ^{60}Co gamma ray with a dose range of 0–5 Gy in different incubation time after irradiation using TaqMan real-time PCR assays. Furthermore, we investigated the correlation between levels of the parameter change and exposure dose to explore feasibility of the parameters as new biodosimeter.

2 Materials and Methods

2.1 Blood sample collection

Peripheral blood samples from six donors (3 males and 3 females) aged 25–30 years were collected to validate the dose-effect relationship of radiation-induced mtDNA CD. This work was conducted at the Henan Institute of Occupational Medicine (HIOM). The scope of the study was explained for each subject and written informed consents were obtained. The Ethics Committee of HIOM approved all experiments. All subjects were healthy, and did not have any history of chronic disease, substance abuse or toxic chemical exposure. No radiation exposure or viral infection during the months preceding the study was documented. 24 mL peripheral blood samples were collected from each donor by venipuncture into vacutainers containing EDTA, and divided into six equal aliquots for the dose-effect relationship analysis.

2.2 *In vitro* irradiation

Single fraction irradiation was carried out on a gamma -installation containing ^{60}Co source at a dose rate of 0.620 Gy/min in Cancer Hospital of Henan Province, China. The source radioactivity was 9.3×10^{13} Bq, and the uniform exposure field was 20 cm×20 cm. Blood samples from every donor were exposed to different doses of ^{60}Co gamma ray (0 Gy, 1 Gy, 2 Gy, 3 Gy, 4 Gy and 5 Gy) at 25ºC. After irradiation, the blood samples were diluted 1:2 with RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (SunBao Biotech. Co. Ltd, Shanghai, China) and were incubated for 2h or 24h at 37ºC in an incubator prior to collection for DNA extraction.

2.3 Extraction of total DNA

After incubation, the red blood cells in the samples were discarded first as previously described[20]. The total DNAs in the white blood cells remained (including mitochondrial and nuclear DNA) was extracted using the AxyPrep Whole Blood Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, USA) according to the manufacturer's instructions, and diluted in 100 μL TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Total DNA's quality and quantity were tested by separating aliquots of the DNA on 1.0% agarose gels stained with ethidium bromide

and the signal intensity to known amounts of DL 2000 DNA Marker (Takara, Dalian, China) was compared using a gel imaging system (Syngene, Cambridge, UK). Remaining DNA sample was stored at −20ºC.

2.4 TaqMan Real-Time PCR

PCR primers for the quantitative PCR (Q-PCR) were designed according to MITOMAP Human Cambridge Sequence data (www.mitomap.org). A 151-bp region of the 12S rRNA gene in the heavy strand was used to represent the total amount of mtDNA since this region is relatively conserved. Another 151-bp region spanned the deletion junction was also used to represent CD. The forward primers (12S rRNA: 5'-AA ATCCACCTTCGACCCTTAAGT-3'; CD: 5'-ACCC CCATACTCCTTACACTATTCCT-3'), reverse primers (12SrRNA: 5'-AACCCTGATG AAGGCTA CAAAGTAA-3'; CD: 5'-CGGTTTCGATGATGTG GTCTTT-3') and TaqMan hybridization probes (12S rRNA: 5'- FCCATTTCTTGCCACCTCATGGGCTA CP-3'; CD: 5'-FCCACCTACCTCCCTCACCATTG GCAP-3') were synthesized by Gene Core Bio Technologies Co. Ltd., Shanghai, China. Both plasmids containing the breakpoint and the 12S rRNA region were previously constructed by our laboratory [20]. Dose-dependent plasmid-constructed 12S rRNA and CD standards were used in each run of real-time PCR. All TaqMan reactions were carried out in 96-well plates on an ABI 7500 Real-Time PCR instrument (Applied Biosystems, CA, USA) using the real-time PCR Master Mix kit from Takara Co.(Dalian, China). Each PCR reaction was carried out in total volume of 20 μL containing 100 ng total DNA template, 200 nM primer, and 200 nM TaqMan probe. After an initial denaturation step at 95ºC for 20 second, 40 PCR cycles of 5 s at 95ºC, 34 s at 60ºC were performed. Real-time PCR of all samples and standards were performed in triplicate. The data from a PCR run were rejected if the correlation coefficient of the standard curve was less than 0.98.

2.5 Data analysis

The CD rates were calculated as the copy numbers of CD molecules per total mtDNA molecules ($CD/mtDNA^{total}$). All statistical analyses were conducted using SPSS, version 15.0 (SPSS, Chicago, USA). Data were expressed as the mean (±*SD*) value. Differences in mtDNA and CD levels after irradiation were analyzed by Univariate analysis of variance and Student-Newman-Keuls post hoc test or chi-square test. The Pearson's correlation test was used to explore correlation between mtDNA/CD levels and irradiated dose. $P<0.05$ are considered statistically significant. All reported *P* values are two sided.

3 Results

3.1 Reliability and reproducibility of the TaqMan PCR assay

The levels of mtDNA and CD copy number from human peripheral blood cells were determined in a set of independent experiments. The first TaqMan assay was designed to the 12S rRNA region in the heavy strand to measure the total amount of mtDNA copy number. The second TaqMan assay targeted the CD breakpoint to quantitate the abundance of the CD copy number in the samples. The standard curve equation for the total mtDNA copy number was $y=40.8-3.34\log x$ ($r^2=0.998$), and the CD copy number was $y=42.9-3.34\log x$($r^2=0.995$) (Fig.1). It demonstrated that employed TaqMan assay was sensitive enough to detect single molecule of CD and high linearity was found in the range of standard samples. CD levels in most samples were detected between Ct 31 and 40 (Fig.1). PCR products were amplified within the linear range of assays ($r^2>0.98$) in all examined samples. These results suggested that the TaqMan PCR approach may generate high sensitivity, and could give reliable data in this study.

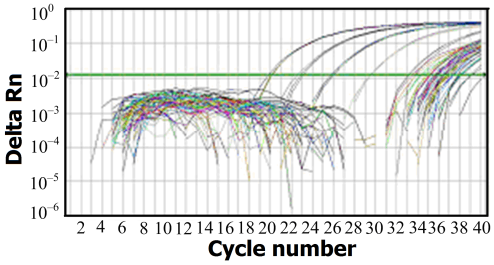

Fig.1 TaqMan PCR assay for measuring the common mitochondrial deletion in genomic DNA extracted from human peripheral blood. This figure showed the amplification plot for the standard curve and the amplification plot for blood samples.

3.2 Copy number of CD in the irradiated samples

The CD copy number in each sample was identified from 78.99 to 723.65 according to calibration curves (Table 1). The mean CD copy number was significantly increased in irradiation group compared with 0 Gy group (χ^2=15.146, P=0.010), and the relationship between the copy number and the exposure dose from 0 to 5Gy was significant at 2 h after irradiation (y=59.343x+149.338, R^2=0.445, F=20.059，P<0.001; Fig.2A). The CD copy number had also increasing trend with the elevated dose at 24h after exposure, but both of the correlation was not obvious (F=0.375, P=0.844 as shown in Fig.2B).

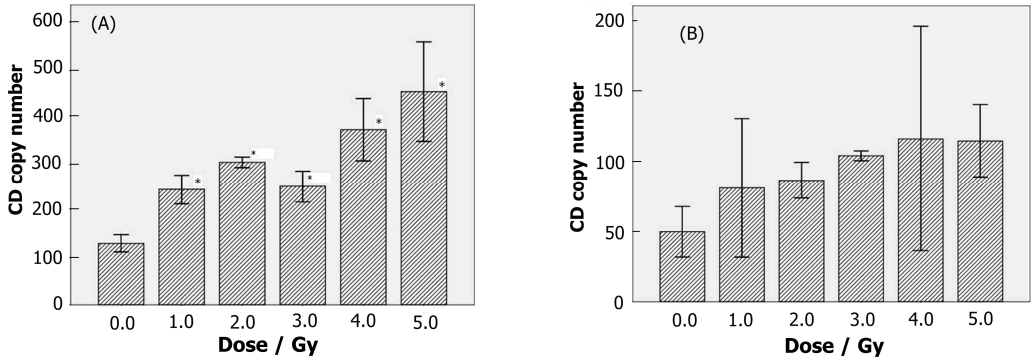

Fig.2 Changes of CD copy number in human peripheral blood exposed to different irradiation dose. A. CD copy number at 2h after exposure; B. CD copy number at 24 h after exposure. *Compared with 0 Gy group, P<0.05.

Table 1 mtDNA alterations in peripheral blood from healthy donors at 2 h after irradiation (n=6)

Dose (Gy)	CD copy number	mtDNA copy number	CD ratio / %
0	128.76±36.29	(1.93±1.00)×10^6	0.0091±0.0077
1	242.60±60.91	(1.38±0.52)×10^6	0.0186±0.0037
2	300.75±25.42	(1.86±1.11)×10^6	0.0204±0.0097
3	249.08±73.89	(1.19±0.50)×10^6	0.0261±0.0151
4	418.91±158.48	(1.65±1.38)×10^6	0.0327±0.0162
5	449.42±209.32	(1.22±0.34)×10^6	0.0386±0.0193

3.3 Copy number of mtDNA in the irradiated samples

The total mtDNA copy number in each sample was from 5.61×10^5 to 4.08×10^6 copy whose Ct values ranged from 18.14 to 22.13, and the mean of mtDNA copy number among each group of exposure dose did not show statistical difference (F=0.710, P=0.622; Table 1; Fig.3A) at 2 h after irradiation. However, the mean of mtDNA copy number obviously increased in irradiation groups compared with 0 Gy group at 24h after exposure (F=16.130, P=0.011; Fig.3B)

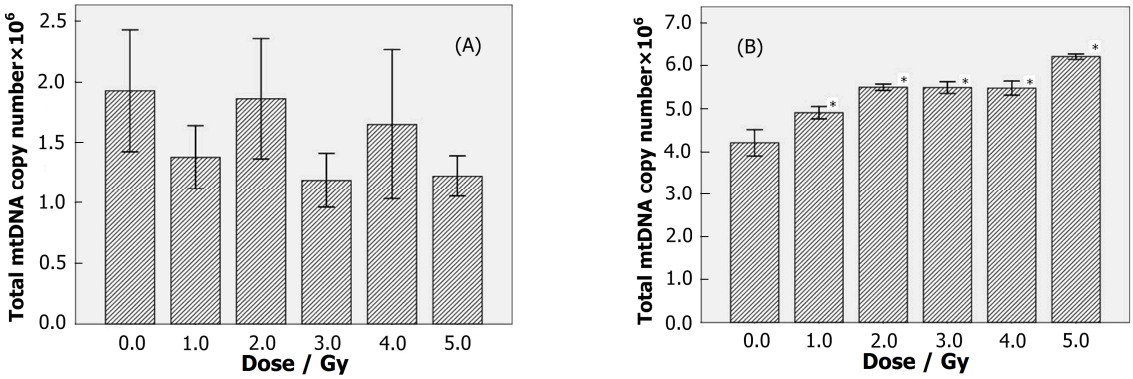

Fig.3 Changes of total mtDNA copy number in human peripheral blood exposed to different irradiation dose. (A) total mtDNA copy number at 2 h after exposure; (B) total mtDNA copy number at 24 h after exposure. *Compared with 0 Gy group, P<0.05.

3.4 Rate of CD in the irradiated samples

The rate of CD in the genomic DNA was calculated and analysed above-mentioned in Materials and Methods. The mean of CD ratio in each sample was from 0.00429% to 0.0569% (Table 1), and showed a dose-dependent increase in the dose range from 0 to 5 Gy (y=0.055991x+0.0101878, R^2=0.380, F=15.309, P=0.001; Fig.4A) at 2h after irradiation, but had not obvious difference among each group with elevated dose at 24 h after irradiation (F=1.373, P=0.404 as shown in Fig.4 B).

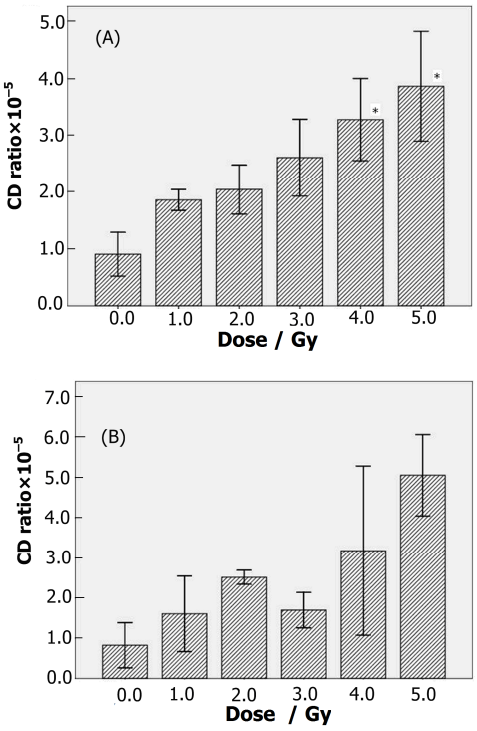

Fig.4 Changes of CD ratio in human peripheral blood exposed to different irradiation dose. A. CD ratio at 2 h after exposure; B. CD ratio at 24 h after exposure. *Compared with 0 Gy group, P<0.05.

4 Discussions

It has been reported that ionizing radiation induces the CD in different human cell lines or human peripheral blood. Using polymerase chain reaction (PCR), the CD could be detected in genomic DNAs of differently radiosensitive cells (the radiosensitive AT cells, the radiosensitive squamous cell carcinoma cells or the radioresistant squamous cell carcinoma cells) after X-ray radiation dose of 1, 2 or 10 Gy, namely ionizing radiation induces the CD in human cells and the radiation doses required to induce the deletion reflect the sensitivity of cells to radiation[21]. And by PCR, levels of the CD significantly accumulated 72 h after irradiation doses of 2 Gy, 5 Gy, 10 Gy or 20 Gy in genomic DNAs of eight normal human skin fibroblast lines, but the absolute amounts of the induced deletion were variable and no consistent dose-response relationship was found[22]. In genomic DNAs of human peripheral blood, the CD was detected using the developed PCR or Nest-PCR method at 2h after irradiation to 1–5 Gy ^{60}Co gamma rays, but not before irradiation[23,24]. The CD was observed in genomic DNAs of cultured HPBL exposed to ionizing radiation by in situ PCR, and the CD ratio exhibited a dose-dependent increase with the dose range from 0 to 2.0 Gy[15]. These results indicated that PCR, nested PCR and in situ PCR were applied for detecting the CD induced by irradiation, but the detection efficiency was low[25]. Real-time quantitative PCR assay is a reliable, sensitive and convenient method to quantify the CD[25,26]. Using real-time PCR in relative quantification, it has been demonstrated that the relative amount of the CD in genomic DNAs of human peripheral blood exposed to ^{60}Co gamma ray showed certain dose-effect relation between 0 and 8 Gy at 2 h after exposure[17]. Using TaqMan real-time PCR, we found that both the CD copy number and the CD ratio showed a dose-dependent increase at 2h after gamma ray radiation doses from 0–5 Gy, but not at 24 h after irradiation, which is due to irradiation-induced cell death with the CD[27]. In the mtDNA samples from human peripheral blood nucleated cells, the dose-dependent increase was not observed 2h after irradiation (0–5 Gy) in our previous study. And the mean of the CD copy number was between 10.84±7.10 and 46.57±51.16 or the CD ratio between 0.000360± 0.000169 and 0.000840±0.000976[16], and was lower than that in the genomic DNA samples in this study (Table 1). This may have some connection with the quality of the mtDNA samples, which might contain some component that affected the efficiency of real-time PCR in the previous study[16]. Moreover, the basal level of the CD was very low, and none of genomic DNAs of the peripheral whole-blood

exhibited an age-dependent accumulation of the deletion and any difference in both genders[25,26,28], which can meet the basic needs for constructing irradiation boidosimetry. Therefore, Alterations of the CD may be as an early biomarker, which reflects irradiation doses, after exposure.

The increase of mtDNA copy number was observed in peripheral blood cells from 21 breast patients after chemoradiotherapy by real-time PCR, and it is presumed that mtDNA lesion would induce the biosynthesis of mtDNA copies in the course of chemoradiotherapy, which may be a compensatory reaction in peripheral blood cells as a consequence of a damaged energy biogenesis[18]. Recently, the obvious accumulation of mtDNA copies was observed in human peripheral lymphocytes from 26 acute lymphoblastic leukemia patients 24 h after total body irradiation (TBI) by real-time PCR, and it was thought that content of the mtDNA copies may be considered as a predictive factor to irradiation toxicity[19]. Using real-time PCR, the increase in mtDNA copies was detected in the peripheral blood cells from mice at 1h after their TBI exposure to 1 Gy X-ray, and it was thought that the increase may also be as a result of a compensatory reaction, which was developed in the mice in response to the irradiation damage with a part of mtDNA molecules[29]. We got the similar results in this study by TaqMan real-time PCR, but the increase in the mtDNA copies occurred at 24 h rather than at 2h after exposure. This may have its explanation in the compensatory reaction as the described discussion[29], which was delayed *in vitro*. These studies suggest that alterations of mtDNA copy number induced by ionizing radiation may also be as a candidate of molecular biomarker associated with irradiation in genomic DNAs of human peripheral blood.

5 Conclusion

This study describes the development of a rapid, sensitive, and practical real-time PCR assay to quantify the CD and mtDNA copy number in genomic DNAs of human peripheral blood. Our results suggest that ionizing radiation induced the CD ratio and the CD copy number increase 2 h with a dose-dependent, and mtDNA copy number accumulation 24h in genomic DNAs of human peripheral blood after irradiation. The parameters may be considered as molecular biomarkers to applying construction of new biodosimeter.

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