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

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Abstract

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

Full Text

Preamble

Alterations of mtDNA Copy Number and 4977 bp Deletion Induced by Ionizing Radiation in Human Peripheral Blood

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Abstract

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

Key Words: Ionizing radiation, Human peripheral blood, Mitochondrial DNA, 4977 bp common deletion, TaqMan real-time PCR

Introduction

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

Chemical reagents and radiation produce more lesions in mtDNA than in comparable nuclear DNA fragments in cells. Shieh et al. reported that N,N-dimethylformamide damaged mtDNA and increased the proportion of CD in human blood [10]. UV radiation can increase the accumulation of CD in human skin [11,12].

Using a nested PCR approach, Gattermann and coworkers first detected the CD in human blood [13]. Improvements in PCR conditions allowed detection of

deleted mtDNA in blood from younger and older individuals without employing two different primer pairs [14]. Using in situ PCR technology, a dose-dependent increase in the frequency of human peripheral blood lymphocytes (HPBL) with CD was observed in cultured HPBL in interphase when exposed to gamma radiation between 0.5 and 2.0 Gy [15].

In our previous study, higher CD levels were observed in mtDNA samples from six healthy donors' blood exposed to 1–5 Gy ^{60}Co gamma rays compared with samples exposed to 0 Gy at 2 h after exposure by real-time PCR, but both CD levels and exposure doses were not obviously correlated [16]. Recently, Duan et al. [17] detected the CD in human peripheral blood exposed to ^{60}Co gamma rays using relatively quantitative real-time PCR and found that the relative amount of CD showed a certain dose-effect relationship in the dose range from 0–8 Gy at 2 h after exposure. However, it remains unknown whether the deletion would exhibit a dose-effect relationship over a wider time range after irradiation in human peripheral whole blood by real-time PCR.

Furthermore, 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 in mtDNA copy number was also found in mtDNA samples from human peripheral blood nucleated cells exposed to 1–5 Gy ^{60}Co gamma rays 2 h after irradiation in our previous study [16]. However, changes in mtDNA copy number in genomic DNAs of human peripheral blood exposed to ionizing radiation or over a wider time range after irradiation remain unclear in vitro.

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

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 to each subject, and written informed consent was obtained. The Ethics Committee of HIOM approved all experiments. All subjects were healthy and had no history of chronic disease, substance abuse, or toxic chemical exposure. No radiation exposure or viral infection was documented during the months preceding the study. Twenty-four milliliters of peripheral blood samples were collected from each donor by venipuncture into vacutainers containing EDTA and divided into six equal aliquots for dose-effect relationship analysis.

2.2 In Vitro Irradiation

Single fraction irradiation was carried out on a gamma-installation containing a ^{60}Co source at a dose rate of 0.620 Gy/min in the 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 each donor were exposed to different doses of ^{60}Co gamma rays (0 Gy, 1 Gy, 2 Gy, 3 Gy, 4 Gy, and 5 Gy) at 25°C. 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 incubated for 2 h or 24 h at 37°C in an incubator prior to collection for DNA extraction.

2.3 Extraction of Total DNA

After incubation, red blood cells in the samples were discarded first as previously described [20]. The total DNAs from the remaining white blood cells (including mitochondrial and nuclear DNA) were 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 L TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total DNA quality and quantity were tested by separating aliquots of the DNA on 1.0% agarose gels stained with ethidium bromide, and the signal intensity was compared to known amounts of DL 2000 DNA Marker (Takara, Dalian, China) using a gel imaging system (Syngene, Cambridge, UK). The remaining DNA sample was stored at −20°C.

2.4 TaqMan Real-Time PCR

PCR primers for 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 relatively conserved region spanned the deletion junction. Another 151-bp region was also used to represent CD. The forward primers (12S rRNA: 5'-AAATCCACCTTCGACCCTTAAGT-3'; CD: 5'-ACCCCCATACTCCTTACACTATTCCT-3'), reverse primers (12SrRNA: 5'-AACCCTGATGAAGGCTACAAAGTAA-3'; CD: 5'-CGGTTTCGATGATGTGGTCTTT-3'), and TaqMan hybridization probes (12S rRNA: 5'-FCCATTTCTTGCCACCTCATGGGCTACP-3'; CD: 5'-FCCACCTACCTCCCTCACCATTGGCAP-3') were synthesized by GeneCore 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 a total volume of 20 L containing 100 ng total DNA template, 200 nM primer, and 200 nM TaqMan probe. After an initial denaturation step at 95°C for 20 seconds,

40 cycles of amplification were performed. Real-time PCR of all samples and standards were performed in triplicate. 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 ratio of CD molecules to total mtDNA molecules (CD/mtDNA_{total}). All statistical analyses were conducted using SPSS version 15.0 (SPSS, Chicago, USA). Data were expressed as mean (±SD) values. 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. Pearson's correlation test was used to explore correlation between mtDNA/CD levels and irradiated dose. $P<0.05$ was considered statistically significant. All reported P values were two-sided.

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 targeted 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 CD copy number in the samples. The standard curve equation for total mtDNA copy number was $y=40.8-3.34\log x$ ($r^2=0.998$), and for CD copy number was $y=42.9-3.34\log x$ ($r^2=0.995$) (Fig. 1). This demonstrated that the employed TaqMan assay was sensitive enough to detect single molecules of CD and showed high linearity 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 generated high sensitivity and could provide reliable data for this study.

[Figure 1: see original paper]

3.2 Copy Number of CD in the Irradiated Samples

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

[Figure 2: see original paper]

3.3 Copy Number of mtDNA in the Irradiated Samples

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

[Figure 3: see original paper]

3.4 Rate of CD in the Irradiated Samples

The rate of CD in genomic DNA was calculated and analyzed as described in Materials and Methods. The mean CD ratio in each sample ranged 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 2 h after irradiation, but showed no obvious difference among groups with elevated dose at 24 h after irradiation (F=1.373, P=0.404) as shown in Fig. 4B.

[Figure 4: see original paper]

4 Discussion

It has been reported that ionizing radiation induces the CD in different human cell lines and human peripheral blood. Using polymerase chain reaction (PCR), the CD could be detected in genomic DNAs of differently radiosensitive cells (radiosensitive AT cells, radiosensitive squamous cell carcinoma cells, or radioresistant squamous cell carcinoma cells) after X-ray radiation doses of 1, 2, or 10 Gy, demonstrating that ionizing radiation induces the CD in human cells and that the radiation doses required to induce the deletion reflect cellular sensitivity to radiation [21]. 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 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 developed PCR or Nest-PCR methods at 2 h after irradiation with 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 in 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 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 CD in genomic DNAs of human peripheral blood exposed to ^{60}Co gamma rays showed a certain dose-effect relationship between 0 and 8 Gy at 2 h after exposure [17]. Using TaqMan real-time PCR, we found that both CD copy number and CD ratio showed a dose-dependent increase at 2 h 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 CD [27]. In mtDNA samples from human peripheral blood nucleated cells, a dose-dependent increase was not observed 2 h after irradiation (0–5 Gy) in our previous study. The mean CD copy number was between $10.84\$\pm7.10 and 46.57\pm51.16, or the CD ratio between 0.000360\pm0.000169 and 0.000840\pm\$0.000976$ [16], which was lower than that in genomic DNA samples in this study (Table 1). This may be related to the quality of mtDNA samples, which might contain components that affected the efficiency of real-time PCR in the previous study [16]. Moreover, the basal level of CD was very low, and none of the peripheral whole-blood genomic DNAs exhibited age-dependent accumulation of deletion or any difference between genders [25,26,28], which can meet the basic needs for constructing irradiation biodosimetry. Therefore, alterations of the CD may serve as an early biomarker that reflects irradiation doses after exposure.

The increase in mtDNA copy number was observed in peripheral blood cells from 21 breast cancer patients after chemoradiotherapy by real-time PCR, and it is presumed that mtDNA lesions would induce biosynthesis of mtDNA copies during chemoradiotherapy, which may represent a compensatory reaction in peripheral blood cells as a consequence of damaged energy biogenesis [18]. Recently, obvious accumulation of mtDNA copies was observed in human peripheral blood lymphocytes from 26 acute lymphoblastic leukemia patients 24 h after total body irradiation (TBI) by real-time PCR, and it was thought that the content of mtDNA copies may be considered a predictive factor for irradiation toxicity [19]. Using real-time PCR, an increase in mtDNA copies was detected in peripheral blood cells from mice at 1 h after TBI exposure to 1 Gy X-ray, and it was thought that the increase may also result from a compensatory reaction developed in mice in response to irradiation damage to a portion of mtDNA molecules [29]. We obtained similar results in this study using TaqMan real-time PCR, but the increase in mtDNA copies occurred at 24 h rather than at 2 h after exposure. This may be explained by the compensatory reaction as discussed previously [29], which was delayed in vitro. These studies suggest that alterations in mtDNA copy number induced by ionizing radiation may also serve as a candidate 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

a dose-dependent increase in CD ratio and CD copy number at 2 h, and mtDNA copy number accumulation at 24 h in genomic DNAs of human peripheral blood after irradiation. These parameters may be considered as molecular biomarkers for application in constructing a new biodosimeter.

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