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Accumulation of the common mitochondrial DNA deletion induced by ionizing radiation

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Abstract **Point mutations and deletions in mitochondrial DNA (mtDNA) accumulate as a result of oxidative stress, including ionizing radiation. As a result, dysfunctional mitochondria suffer from a decline in oxidative phosphorylation and increased release of superoxides and other reactive oxygen species (ROS). Through this mechanism, mitochondria have been implicated in a host of degenerative diseases. Associated with this type of damage, and serving as a marker of total mtDNA mutations and deletions, the accumulation of a specific 4977-bp deletion, known as the common deletion (Δ-mtDNA^{4977}), takes place. The Δ-mtDNA^{4977} has been reported to increase with age and during the progression of mitochondrial degeneration. The purpose of this study was to investigate whether ionizing radiation induces the formation of the common deletion in a variety of human cell lines and to determine if it is associated with cellular radiosensitivity. Cell lines used included eight normal human skin fibroblast lines, a radiosensitive non-transformed and an SV40 transformed ataxia telangiectasia (AT) homozygous fibroblast line, a Kearns Sayre Syndrome (KSS) line known to contain mitochondrial deletions, and five human tumor lines. The Δ-mtDNA^{4977} was assessed by polymerase chain reaction (PCR). Significant levels of Δ-mtDNA^{4977} accumulated 72 h after irradiation doses of 2, 5, 10 or 20 Gy in all of the normal lines with lower response in tumor cell lines, but the absolute amounts of the induced deletion were variable. There was no consistent dose–response relationship. SV40 transformed and non-transformed AT cell lines both showed significant induction of the deletion. However, the five tumor cell lines showed only a modest induction of the deletion, including the one line that was deficient in DNA damage repair. No relationship was found between sensitivity to radiation-induced deletions and sensitivity to cell killing by radiation.**

Keywords: Ionizing radiation; mtDNA; PCR; Mutation and common deletion

1. Introduction

Ionizing radiation (IR) is known to induce multiple cellular and biological effects either by direct interaction with nuclear DNA or through the formation of OH radicals, which leads to additional DNA damage. Although mitochondria undergo regular exposure to oxidative stress, studies have been focused on the role of nuclear DNA as the most important target of radiation induced cell killing. Although this may be true for cell killing, the long-term effects of radiation in surviving cells may lead to abnormal function in cells and tissues that survive. Since mitochondrial degenerative diseases are slow progressing conditions often associated with impaired oxidative phosphorylation, they may serve as interesting models for radiation effects on mitochondria. Yoneda et al. [1] suggested that oxidative stress leads to mtDNA mutations and deletions, which then results in disintegrity in the electron-transport chain, followed by enhanced leakage of reactive oxygen species (ROS). They commented that mtDNA, which is a major target for attack by ROS, could be involved in the mechanism.

Each mitochondrion contains 2–10 copies of mtDNA and there are up to 10^3 mitochondria per human cell. Human mtDNA exists as a double stranded closed circular 16 569 bp molecule, with genes coding for 13 polypeptides involved in respiration and oxidative phosphorylation, two rRNAs and a set of 22 tRNA molecules [2–5]. During mitochondrial oxidative phosphorylation, a significant amount of ROS is generated which can cause mitochondrial and nuclear DNA damage. Since mtDNA lacks the protective effects of histones and since mitochondrial DNA is generally repaired less efficiently than nuclear DNA, the mutation frequency of mitochondrial DNA is 10- to 1000-fold higher [6]. As all genes in the mtDNA are essential for the biogenesis and bioenergetic function of mitochondria, any mutation that leads to altered expression of these genes would be expected to cause a deficiency in energy metabolism and an enhanced production of ROS released as intermediates during oxidative phosphorylation [7,8]. In addition to the mitochondrial degenerative diseases, the life-long accumulation of mtDNA mutations and deletions has been hypothesized as a contributor to the aging process [2,9]. This is supported by evidence of an age-related decrease in oxidative phosphorylation and the accumulation of mtDNA mutations and deletions. As a marker of these

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Abbreviations: mtDNA, mitochondrial DNA; ROS, reactive oxygen species; AT, ataxia telangiectasia; KSS, Kearns Sayre Syndrome; IR, ionizing radiation; SF, survival fraction; PCR, polymerase chain reaction

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processes, both aging and mitochondrial degenerative diseases display an accumulation of a 4977 bp mtDNA deletion, referred to as the "common deletion" [10]. The site of this specific mtDNA deletion (Δ-mtDNA^{4977}) is flanked by two 13 bp direct repeats and it is proposed that DNA damage between these repeats can result in inappropriate pairing during DNA replication, thus causing the deletion. This is referred to as a slip-replication mechanism [11–13] and explains why random DNA damage can cause such a precise deletion. Studies have shown that this deletion can be used as a marker of oxidative damage to mtDNA [14–18], even after very low doses of damage because the lesion is essentially amplified during mtDNA replication. Radiation damage to mtDNA results in many single copy deletions and mutations in mtDNA, which are not practical to detect or quantify. However, the common deletion is more easily detected because of its unique mechanism of formation, making it a very sensitive marker of mtDNA damage. Even though it represents only a fraction of the mtDNA damage following irradiation, it is a representative surrogate for total damage.

The purpose of this study, therefore, was to investigate whether IR induces the accumulation of the common deletion and to determine if it is associated with cellular radiosensitivity in 15 human cell lines, including normal, transformed and tumor cells. The results showed a marked variation among the cell lines in the levels of Δ-mtDNA^{4977} accumulated 72 h after irradiation. There was no apparent relationship between the radiosensitivity of the lines and the levels of induced deletions.

2. Materials and methods

2.1. Cell cultures and lines

Normal fibroblast cell lines were obtained under protocols approved by the institutional review board of the University of Texas, M.D. Anderson Cancer Center (UTMDACC). The lines were established for several years, but the procedures were as follows. Dermal fibroblasts were obtained from patients either by a punch biopsy performed over the gluteal region or by surgical biopsies. Cultures were maintained in α-MEM with 20% serum, L-glutamine, antibiotics and 5% CO_2, as described elsewhere [19]. Ataxia telangiectasia (AT) and Kearns Sayre Syndrome (KSS) lines were purchased from Coriell Institute, Camden, NJ. DNA repair glioblastoma lines, MO59J (DNA-PK deficient) and MO59K (normal DNA-PK proficient) and colon carcinoma lines, SW620 and HT29, were obtained from the American Type Culture Collection, Manassas, VA and a glioblastoma line, U251, was obtained from Dr. P. Tofilon, UTMDACC.

2.2. Radiosensitivity assays (SF2)

Radiosensitivity measurements were performed on all cell lines by at least three independent clonogenic assays according to the procedures described [19,20].

2.3. Irradiation and hydrogen peroxide (H_2O_2) treatment

Cultures were irradiated at room temperature using a ^{137}Cs source at a dose rate of 4.17 Gy/min. Cultures were treated with 200 μM H_2O_2 for 15 min in serum-free medium.

2.4. Isolation of DNA

Following irradiation, cultures were incubated for various times before extracting total cellular DNA using the DNeasy Tissue Kit (Qiagen, Valencia, CA). The extract, containing both nuclear and mtDNA, were used for polymerase chain reaction (PCR) analysis without further purification.

2.5. Primer sets and PCR

Primers were synthesized by Sigma-Genosys, The Woodlands, Houston, TX. To detect Δ-mtDNA^{4977}, primer sites were chosen at regions flanking the 4977 bp and PCR was carried out under conditions that allowed a product to form only if the deletion had occurred. Primers designed to detect non-deleted DNA employ primers across the deletion break point. The mtDNA sites for all primers and their oligonucleotide sequences are shown in Fig. 1. The PCR mixture contained 200 ng cellular DNA as template, 200 M of each dNTPs, 1× reaction buffer and 2.5 U *Taq* polymerase (Roche Applied Science, Indianapolis, IN). Amplification was accomplished by an initial denaturation at 94 °C for 3 min followed by 30 cycles of template denaturation at 94 °C for 1 min, primer-template annealing at 50 °C for 1 min and primer-extension at 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. PCR was performed in a GeneAmp PCR system 9700 (Perkin–Elmer, Boston, MA) using 0.2 ml microcentrifuge tubes. PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. Gels were scanned and densitometric analysis was performed using ImageQuant (Molecular Dynamics Storm 860 system, Sunnyvale, CA).

3. Results and discussion

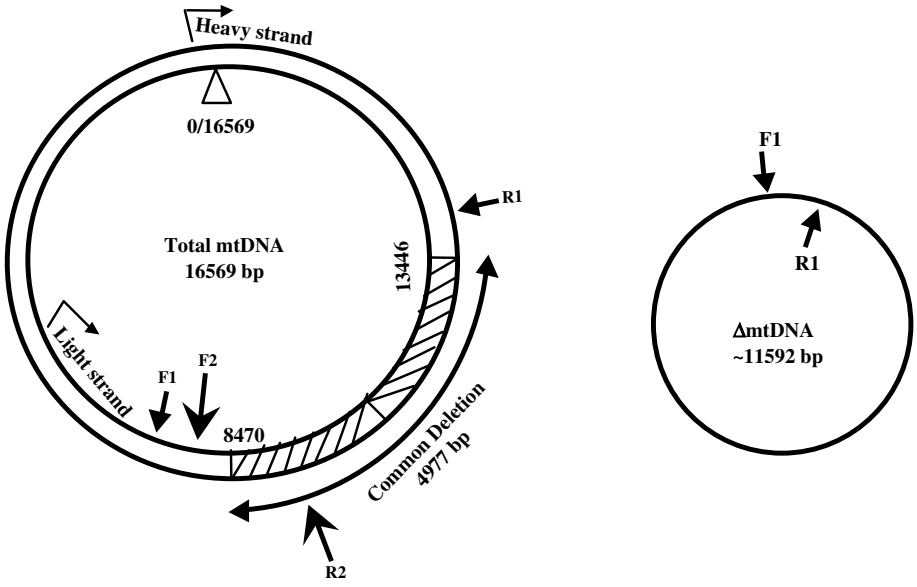
3.1. Cell lines and radiosensitivity measurements

Table 1 summarizes the origins of the 16 different cell lines and the results of radiosensitivity measurements. The end point of radiosensitivity is survival after 2 Gy of irradiation (SF2). The normal human dermal fibroblasts displayed a range of radiosensitivities (SF2 = 0.17–0.41). The non-transformed AT and an SV40 transformed AT fibroblast lines were highly sensitive (SF2 = 0.02 and 0.04, respectively). The three malignant cell lines (HT29, SW620 and U251) were relatively radioresistant (SF2 = 0.73, 0.64 and 0.69, respectively). The glioblastoma wild type line, MO59K, was radioresistant (SF2 = 0.39) and its DNA-PK$^{-/-}$ counterpart, MO59J, was highly radiosensitive (SF2 = 0.05). KSS cells were relatively radioresistant with SF2 value of 0.36. Cell lines derived from KSS patients contain high levels of the common deletion, so they were used as a positive control for identification of the deletion. The specific mechanisms that result in differences in radiosensitivity are not known for all of these cell lines. Sensitivity differences among the normal fibroblast lines are within the normal range of individuals, although line 3 (Table 1) was derived from a cancer patient with a family history of radiosensitivity and cancer proneness [21]. The AT and protein kinase deficient lines (lines 10, 11 and 13 in Table 1) have altered functions that directly or indirectly influence the repair of DNA damage [22,23]. The lines derived from various human tumors (lines 14–16 in Table 1) do not have identified genetic defects related to radiosensitivity.

Since it has been reported that cellular radiosensitivity and the sensitivity to radiation–induction of the common deletion may be correlated [24], we tested for the possibility of a significant correlation between the radiosensitivity of several cell lines and the accumulation of the common deletion. The results do not suggest a correlation, although the range in sensitivities of the different lines is quite large (SF2 = 0.02–0.73).

3.2. Cytotoxic treatment of mtDNA

KSS cells were used to demonstrate that our PCR primers amplify the common deletion in mtDNA and that it can be easily detected by PCR. Increased sensitivity was achieved by using PCR primers to specifically amplify the region flanking the common deletion. Primers were designed separately to amplify mtDNA with and without the specific common deletion as shown in Fig. 1. The results (Fig. 2) show the amplification of the mtDNA from KSS cells and from H_2O_2 treated



Primer Sequence 5' → 3'	Nucleotide position	Reference
F1. ATGGTACTGAACCTACGCGTACACCG	7895 bp	-
R1. AGGGTGGGGTTATTTTCGTTAATG	13641 bp	
F2. GAAATGCCCCAACTAAATACTACCGT	8373 bp	-
R2. AATGAGTGAGGCAGGAGTCCG	8782 bp	
F3. TGAGGCCAAATATCATTCTGAGGGGC	Total mtDNA	Yakes *et al.* 1997
R3. TTTCATCATGCGGAGATGTTGGATGG	1.6 kb	

Fig. 1. Mitochondrial DNA, oligonucleotide positions and sequences of the primers used to amplify the common deletion and the wild type mtDNA sequence by PCR. The large double circle represents full-length wild type mtDNA and the hatched potion indicates the region of the common deletion (Δ-mtDNA^{4977}). The smaller circle on the right represents the size of the mtDNA after the loss of the common deletion sequence. It is 4977 bp smaller than the wild type mtDNA. PCR primers for wild type and Δ-mtDNA are shown in the table.

and untreated C46 control cells. KSS cells have the 438 bp PCR product formed from wild type mtDNA and an 814 bp product that is only formed from a template of Δ-mtDNA. The untreated normal fibroblast line, C46, shows the wild type band, as well as the small band associated with the common deletion. However, C46 cells treated with H_2O_2 show a significant increase in the deletion, which is consistent with reports that oxidative treatment induces formation of the common deletion. This result demonstrates that cytotoxic treatments induce the common deletion in the mtDNA.

Table 1
Characteristics of the donors and tumors from which the dermal fibroblast cell lines were established

No.	Cell line	SF2	Age/Sex
1	KSS	0.36	10/M
2	C29	0.33	45/F
3	C42	0.17	3/F
4	C46	0.32	42/F
5	C49	0.28	54/F
6	C52	0.27	55/F
7	C65	0.3	66/M
8	C80	0.41	27/F
9	S23	0.25	70+/F
10	AT	0.02	M
11	ATsv40	0.04	18/M
12	MO59K	0.39	33/M
13	MO59J	0.05	33/M
14	HT29	0.73	44/F
15	SW620	0.64	51/M
16	U251	0.69	Not known

SF2: Surviving fraction of fibroblasts at 2 Gy.

3.3. *Δ-mtDNA^{4977} accumulation after IR*

As shown in Table 1, a number of cell lines with distinctive characteristics were used. These include primary and malignant cells with a variety of radiosensitivities and known DNA repair defects. After DNA extraction, specific PCR primer sets were used to identify the relative amounts of both

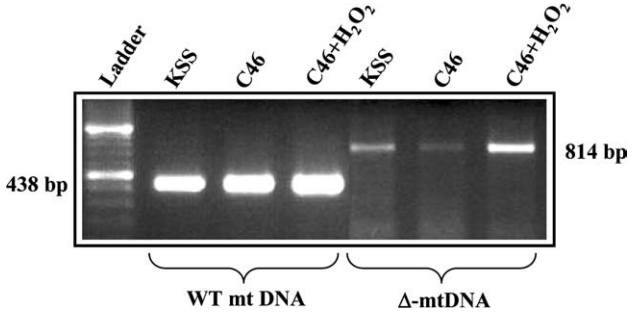


Fig. 2. PCR analysis of mtDNA in KSS and C46 cell lines using primers 1 and 2 from Fig. 1. C46 was treated with H_2O_2 to demonstrate that cytotoxic treatment induces mtDNA damage. KSS and C46 cells show the presence of the common deletion. H_2O_2 treatment of C46 followed by 72 h of incubation shows the accumulation of the common deletion.

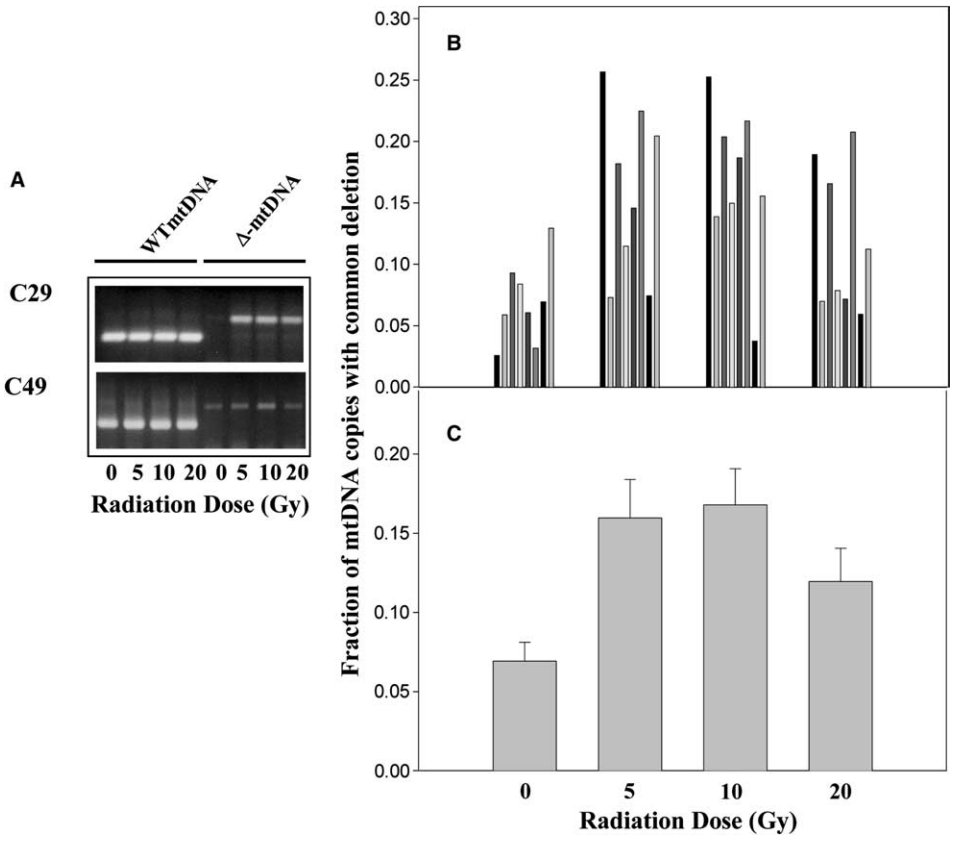


Fig. 3. IR induction of the common deletion in mtDNA in primary human fibroblasts. (A) PCR products showing the accumulation of common deletion with increasing radiation doses 72 h after treatment. (B) Quantitative analysis of the PCR products expressed as the fraction of total mtDNA copies that contain the common deletion in eight normal fibroblast lines. The bars represent cell lines 2–9 listed in Table 1, in order from left to right. (C) Average of all the eight fibroblast lines from B versus radiation dose.

Δ-mtDNA^{4977} and total mtDNA in fibroblasts following irradiation. Of the 16-fibroblast cell lines, representative gels from two normal lines (Fig. 3), AT and ATSV40 lines (Fig. 4), and tumor lines (Fig. 5) are displayed as individual figures. However, the data from all cell lines in that category are summarized in the graphs.

Primary human fibroblasts. Mitochondrial deletions induced by radiation are not immediately present after treatment, because their formation requires mtDNA replication [24]. Therefore, cultures were incubated for 72 h after irradiation with a variety of radiation doses (5, 10 or 20 Gy). Fig. 3 shows the accumulation of the wild type mtDNA and common deletion after irradiation in 2 normal fibroblast lines (C29 and C49). The PCR products (Fig. 3A) corresponding to ~5 kb deletion were visible after irradiation of C29 and C49 fibroblasts. In general, increasing radiation dose has no appreciable effect on the level of wild type mtDNA in all the cell lines studied. C29 has very little to no mtDNA deletion product without radiation, but it has a very strong induction of Δ-mtDNA after IR. There was no evidence of a dose response. On the other hand, C49 had significant levels of the Δ-mtDNA without IR, which is typical in cultures derived from older patients (54 years of age). The Δ-mtDNA in C49 appears to have a dose–response up to 10 Gy, although there was a decrease at 20 Gy. Perhaps, this was due to the loss of cells killed by radiation. Fig. 3B shows the quantitative results from all eight normal human fibroblast lines, expressed as the fraction of total mtDNA copies, which contains the common deletion. It shows variability in the initial levels of the deletion, that the magnitude of response to radiation is highly variable between individual fibroblast lines and that there is a general increase in levels following irradiation. When the results from the eight fibroblast lines were averaged (Fig. 3C), the results show a significant increase in Δ-mtDNA after irradiation, but there is no evidence of a dose-dependent response relationship. There was, however, a statistically significant increase of Δ-mtDNA between 0 and 5 Gy, although it is recognized that the degree of variability among the lines is considerable.

AT fibroblasts. The rationale for examining AT lines is based on a report that induction of the common deletion in mtDNA by radiation depends upon the inherent radiosensitivity of the cells [24]. Two highly radiosensitive AT lines, immortalized and SV40 immortalized, were given radiation doses of 2, 5 and 10 Gy. Both AT and ATSV40 lines showed increased accumulation in the common deletion after radiation doses of 2, 5 and 10 Gy (Fig. 4A). In this case, the increase in deletion appears to be dose-dependent in the transformed cells but not in the non-transformed AT line. The results are shown in Fig. 4B.

AT cells are highly sensitive to IR [25]. The mutated ATM protein appears to modulate radiation sensitivity by mechanisms that include repair, cell cycle checkpoints and genome instability [26]. As reported by Kubota et al. [24], we found an

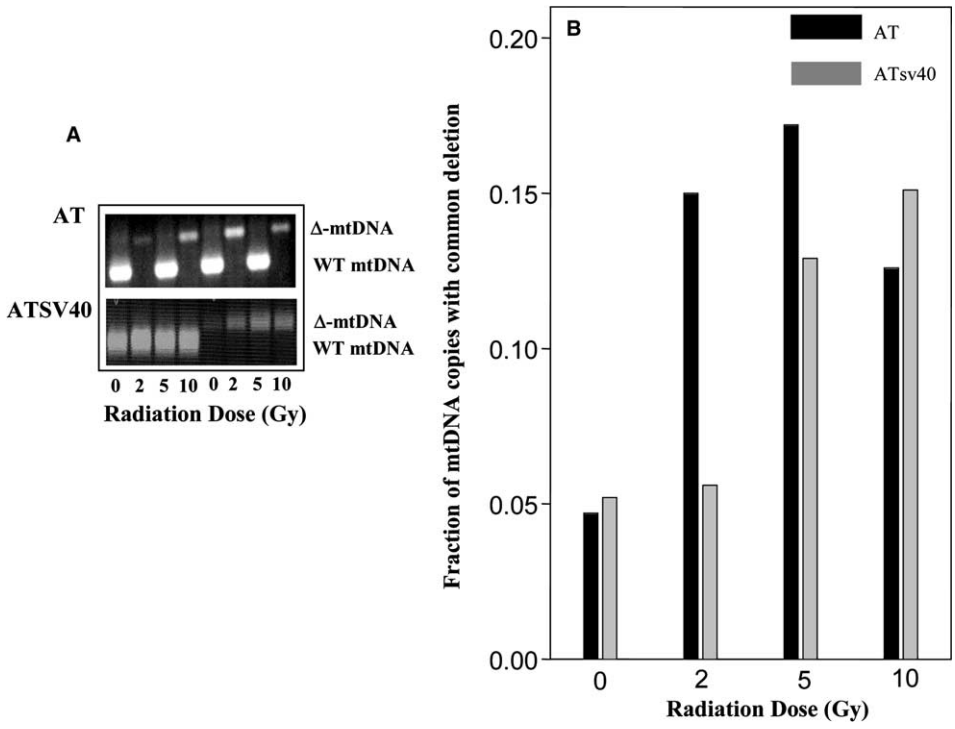


Fig. 4. Increased levels of total mtDNA and common deletion induced by IR in AT and ATSV40 fibroblasts. (A) PCR showing the progressive increase in the accumulation of the common deletion in AT and ATSV40 transformed lines after IR. (B) Quantitative results of both cell lines expressed as the ratio of mtDNA containing the deletion relative wild type mtDNA.

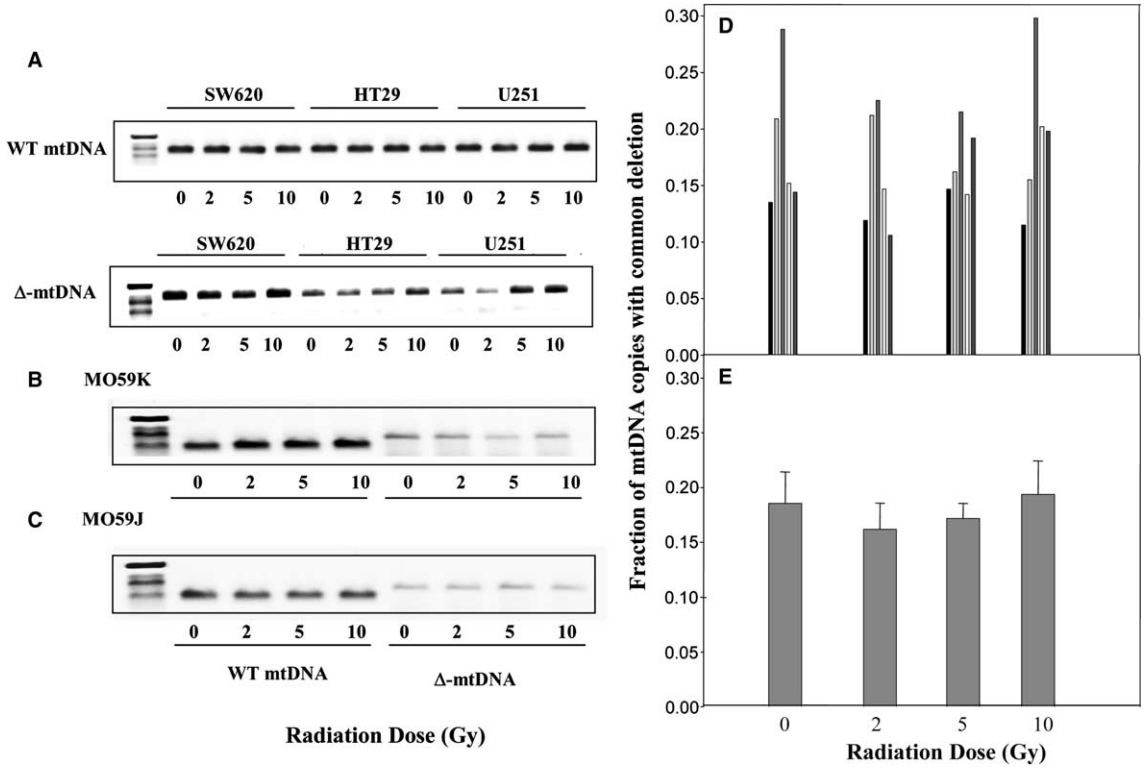


Fig. 5. PCR analysis of the mtDNA common deletion in malignant lines, 72 h after various doses of IR. (A) The top panel shows the levels of total mtDNA and the bottom panel shows the accumulation of the common deletion in SW620, HT29 and U251 lines. (B) Agarose gel showing the PCR products of wild type and Δ-mtDNA in MO59K cells. (C) Wild type and Δ-mtDNA PCR products in MO59J cells. (D) Quantitative analysis. (E) Average of the Δ-mtDNA in all five tumor lines, expressed as fraction of mtDNA containing the deletion.

increase in mtDNA deletions in AT cells, a radiation dose of only 2 Gy, while the increase in other cell types was not observed after doses less than 5 Gy. Kubota et al. [24] also reported that AT cells showed Δ-mtDNA^{4977} accumulation after doses as low as 1 Gy. They hypothesized that nuclear genes involved in the repair of radiation-induced mtDNA damage may be responsible for any relationship between of the sensitivity of human cells to radiation-induced cell killing and the sensitivity to the induction of mitochondrial DNA deletions. Since we found no correlation between sensitivity to cell killing and deletion induction, our results do not support that hypothesis.

Tumor lines. It is known that there is considerable variation in the sensitivity of human tumor cells to killing by radiation [27,28]. Since mtDNA is more prone to oxidative damage than genomic DNA and since studies have suggested that various tissues, such as brain, liver, heart and skeletal muscle, accumulate point mutations and deletions in their mtDNA with age [10,11,29], we examined the accumulation of the Δ-mtDNA^{4977} in malignant and DNA repair deficient cell lines. Fig. 5A shows the PCR amplified products corresponding to the wild type and deletions in SW620, HT29 and U251 malignant cell lines. These lines were relatively radioresistant (Table 1). All three lines had significant baseline levels of the common deletion, in the absence of IR. With IR, a modest, but dose-independent, accumulation of the deletion was observed. Two additional glioblastoma lines, MO59K and MO59J, were examined. Fig. 5B and C show the results. Neither of these lines exhibited an increase in Δ-mtDNA. MO59K is DNA-PK proficient and relatively resistant to IR, while MO59J is DNA-PK deficient, repair deficient and highly radiosensitive. This is further evidence that radiosensitivity to cell killing and sensitivity for the induction of mitochondrial DNA deletions are independent. Quantitative analysis of all the five tumor lines is shown in Fig. 5D. The average data of the five malignant cell lines are shown in Fig. 5E and that confirms the absence of significant deletion induction.

In summary, these studies suggest that IR induces an increase in mtDNA deletions. This increase is (1) radiation dose independent, (2) requires 72 h to accumulate to detectable levels, (3) occurs in normal and in tumor cell lines, (4) the extent of the induction is highly variable in magnitude, and (5) there is no correlation with radiosensitivity.

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