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Biological effects of human lung cells MRC-5 in CBCT positioning for image-guided radiotherapy

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Abstract Image-guided radiotherapy (IGRT) provides precise positioning for the tumor target, but it may bring extra irradiation dose in the target positioning with a cone beam CT (CBCT) which has been increasingly used in IGRT. In this work, we focused on biological effects of the low-dose irradiation in IGRT, which have not been considered so far. Primary human fibroblasts cells from the lung and MRC-5 were irradiated by a CBCT. DNA double-strand breaks (γ-H2AX foci) and micronucleus frequency of the irradiated samples were analyzed. Compared to the control, the γ-H2AX foci yields of the samples irradiated to 16 mGy increased significantly, and the micronuclei rate of the samples irradiated for 3 days increased notably. The dose by imaging guidance device can be genotoxic to normal tissue cells, suggesting a potential risk of a secondary cancer. The effects, if confirmed by clinical studies, should be considered prudentially in designing IGRT treatment plans for the radiosensitive population, especially for children.

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1 Introduction

Diagnostic X-rays are the largest man-made source of radiation exposure for general population, contributing about 14% of total worldwide exposure from man-made and natural sources [1, 2]. Although diagnostic X-rays provide great benefits, the risk of developing cancer from their use is of general concern. It is difficult to study a small risk in epidemiological research. However, the risk from diagnostic X-rays can be estimated by extrapolating risk estimations from a certain population exposed to a range of dose, such as the Japanese atomic bomb survivors exposed at 0–4 Gy [1–3]. Peto [1] estimated that about 0.5% of cancer deaths were attributable to diagnostic X-rays in the USA. Berrington and De Gonzalez [3] showed that about 0.6% of the cumulative cancer risk to 75 years old could be attributable to diagnostic X-rays in the UK. Brenner and colleagues estimated that the cumulative risk of cancer mortality from CT examinations in the USA is about 800 radiation-induced cancer deaths per million examinations in children younger than 15 years old [4].

Accurate radiotherapy such as intensity-modulated radiotherapy (IMRT) has become common in clinic [4, 5], where precise radiation dose to the target is needed. Image-guided radiotherapy (IGRT) is one kind of accurate radiotherapy, in which imaging is performed within the treatment room prior to irradiation to improve geometric irradiation accuracy [6, 7]. To improve target images, a CBCT with a KV-source and a flat panel detector mounted

orthogonally to the MV beam is mostly used. The imaging technique of IGRT enables physicians to adjust the radiation beam based on the position of target tumor and critical organs, while the patient is in the treatment position [8]. Concomitant dose during external-beam radiotherapy includes external linac head leakage and scatter, internal direct and scattered therapy dose outside the target volume, and nontherapeutic doses from imaging for planning and delivery. The total dose increases steadily with imaging procedures. Despite the promises, one major concern often raised on the imaging radiation daily is that the diagnostic dose may potentially increase the risk of radiation-induced carcinogenesis, especially for long-term survivors [9].

However, no systematic research has been carried out to evaluate the radiobiological impact of the imaging dose adopted by IGRT. This work was aimed at studying whether DNA damage was induced to human cells exposed to CBCT radiation in vitro. Both DSBs (DNA double-strand breaks) and cytokinesis-block micronucleus (CBMN) were detected. The results revealed that even single CBCT imaging radiation could increase the foci of γ-H2AX in vitro, and 3 days after the CBCT radiation, an escalation was observed for the cytokinesis-block micronucleus frequency.

2 Materials and methods

2.1 Cell line and cell culture conditions

Exponentially growing human lung MRC-5 cells were subcultured 1 day before clinical IGRT CBCT irradiation. The MRC-5 cell line was donated by Professor Lijun WU of Hefei Institutes of Physical Science, Chinese Academy of Sciences. The cells were cultured in Eagle's minimum essential containing 10% fetal bovine serum and antibiotics (100 units/mL each penicillin and streptomycin). This medium was used throughout the experiments. The cells were followed the schedule of IGRT through the CBCT irradiation. Foci of γ-H2AX were calculated to assay the DNA double-strand. For cytokinesis-block micronucleus assay, cells were divided into four groups: single group received 1-day CBCT irradiation, the 3-day CBCT group was exposed to CBCT for 3 days, 0.5 Gy group received MV X-ray irradiation, and the control group was not irradiated.

2.2 Irradiation schedule

Irradiation was carried out in Anhui provincial hospital on the Elekta Synergy^{TM} which is a new breed of radiotherapy linear accelerators specifically designed for IGRT. It equips a kilovoltage X-ray source and opposing amorphous silicon flat panel imagers, mounted at 90° to the treatment head for acquisition of X-ray projection images for radiography and fluoroscopy.

The cells were irradiated according to the clinical technique settings: 120 kV X-rays, 648 frames, and 1036.8 mAs. Based on the CBDI measurements, the Synergy was matched to the Siemens Somatom, DRH, CR conventional scanner. Using the adopted imaging beam settings, the doses estimated by Impact calculator were 16 mGy (nominal scan dose), which was in good agreement with the dose measured using TLDs in Rando.

The sample exposure time was 90 s. The 0.5-Gy group was irradiated with 6 MV X-rays, both at the Synergy machine. Solid water phantom material (1.5 cm) covered the culture dish to make the dose compensation. The cover was 100 cm away from the X-ray source.

2.3 γ-H2AX foci assay

Using a fluorescent antibody specific for the phosphorylated form of H2AX (γ-H2AX), discrete nuclear foci can be visualized at sites of DSBs [10]. A single radiation track can produce this kind of damage. One of the earliest steps in the cellular response to DSBs is the phosphorylation of serine 139 of H2AX. A typical CBCT imaging protocol for the pelvis would result in a patient surface dose of 30 mGy per scan, while DSBs could be detected at doses as low as 1 mGy [10–12].

For the immuno-staining, the fixed cells were permeabilized in TNBS solution (PBS supplemented with 0.1% Triton X-100 and 1% FBS), followed by exposing cells to anti-γ-H2AX primary antibody (Upstate Biotechnology, Lake Placid, New York, USA) for 30 min. Then, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (ZHONGSHAN goldenbridge biotechnology, Beijing, China) for 1 h. After washing with TNBS for 3 × 5 min, the cells were counterstained with 5 mg/mL Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA). Immuno-fluorescent images were captured by confocal laser scanning microscope (ZEISS LSM710 NLO). For quantitative analysis, the cells with at least one γ-H2AX focus were regarded as the positive cells and the fraction of positive cells was calculated. At least 700 cells in each sample were counted, and statistical analyses were performed on the data averaged from at least three independent experiments.

2.4 CBMN assay

A cytological consequence of the induction of chromosome aberrations is the formation of micronuclei (MN) that are observed in interphase cells. As scored in the cytokinesis-block micronucleus (CBMN) assay, the cells

that have completed one nuclear division are recognized by their binucleated appearance and MN, which occurs in cells that have completed at least one mitosis, are scored in these binucleated cells [13].

The cytokinesis-block micronucleus assay was modified from Refs. [14, 15]. After irradiation, cells were incubated for 2 h, the growth medium was replaced by the fresh medium containing 3 μg/mL cytochalasin B (CB, Sigma) and further incubated for 50 h. After that, the cells were rinsed in PBS twice, fixed in a fixing solution (methanol: acetic acid = 9:1) for 20 min, and then stained with 0.01% (wt/vol) acridine orange for 5 min before observation. Micronucleated cells in the binucleated (BN) cells were assayed under a fluorescence microscope (Olympus 1X71, Tokyo, Japan) and identified morphologically using the criteria of Fenech. At least 1000 BN cells were scored, and the frequency of micronucleated cells per 1000 BN cells was calculated. Blinded analysis was carried out by one investigator.

Mean and standard deviations were presented from three independent experiments, which contained at least two replicate dishes. Significance levels were assessed using Student's t test. A p value of 0.05 between groups was deemed significant.

3 Results

3.1 γ-H2AX focus yield after CBCT

In previous studies, the DSB formation reached a maximum 30 min after irradiation [11]. Consequently, in the present studies, we chose 30 min as the post-irradiation incubation time to analyze the dose effect on DSB formation. Figure 1 shows that, the γ-H2AX focus yields (0.09 focus per cell ± 0.02) after CBCT irradiation with a mean dose of 16 mGy were higher ($p < 0.01$) than those of the control group (0.07 focus per cell ± 0.02).

3.2 Cytokinesis-block micronucleus after CBCT

The average values of the cytokinesis-block micronucleus assay are given in Table 1. No significant difference was found between the single CBCT and control groups. The average rates of micronucleus in the 3-day CBCT group and the 0.5-Gy group, without notable difference between them, were significantly higher than in the control group ($p < 0.01$).

4 Discussion and conclusion

Image guidance in IGRT is performed in the treatment room prior to radiotherapy to improve the toxicity profile and to allow for a safe dose escalation. A CBCT approach with a diagnostic KV-source and a flat panel detector mounted orthogonally to the MV beam is mostly used, such as Elekta (XVI) and Varian (On-board Imager). As reported, CBCT was recommended for daily use, at the first three fractions and at least at the 10th, 20th, and 30th treatment fractions, according to an action limit protocol. Despite the precise patient setup of CBCT, the imaging dose in IGRT cannot be neglected. For example, a prostate patient received dose can be as high as 35 mGy, and the average patient dose from verification images is typically 20–40 mGy per pair of images [16].

In this study, the single CBCT irradiation dose was 16 mGy, which is in a good agreement with dose measured using TLDs in Rando. If daily IGRT positioning is acquired for setup over 30 fractions, the total concomitant dose can be bigger than the limit for background dose from the beam and can increase the therapeutic dose by several

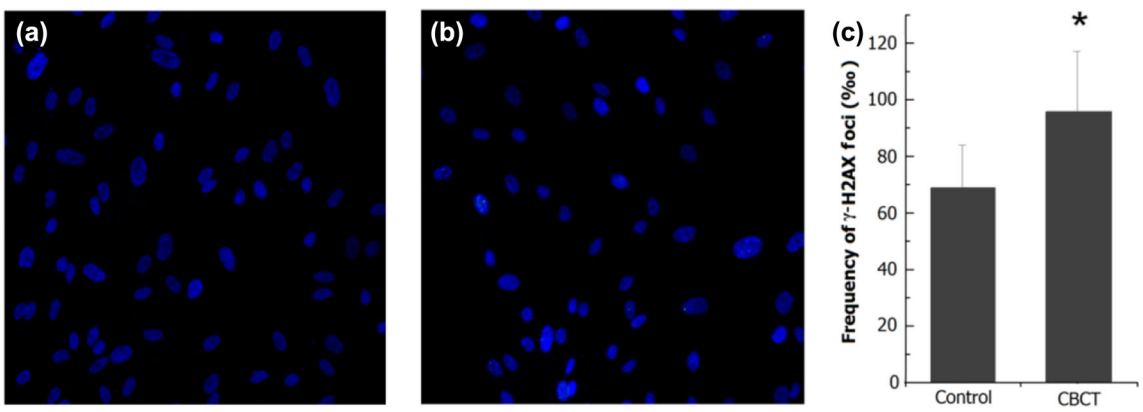

Fig. 1 DSB induction in primary human fibroblasts (MRC-5). **a** γ-H2AX foci (*blue*) in MRC-5 cells; **b** nuclei stained with Hoechst 33342 (*blue*); **c** mean number of foci (‰) in irradiated MRC-5 cells. *Asterisk* significant genotoxicity (t test with $p < 0.01$) compared to the control group. (Color figure online)

Table 1 Number of micronucleus in CB cells after CBCT radiation of culturing time 50 h ($n = 3$)

Groups	Frequency of micronuclei (‰) (mean ± SD)
The control	48.78 ± 0.44
Single CBCT	48.89 ± 2.50
Three-day CBCT	69.56 ± 6.74
0.5 Gy	71.00 ± 2.65

percent. The cumulative extra-target dose has a negative biological effect even within the context of radiotherapy; it is important that the radiation therapy community assess its cost and benefit.

However, to the best of our knowledge, the radiobiological effects caused by imaging irradiation from IGRT have been rarely reported. In this study, we compared γ-H2AX focus yields and the MN rate after CBCT irradiation according to the clinical procedures. The γ-H2AX focus yields after CBCT with a mean dose of 16 mGy were significantly higher ($p < 0.01$) than those of the control group. It may arise from that MN assay method is not sensitive enough to detect very low radiation damage, such as single CBCT imaging radiation. However, the DSBs can be seen in our results. The micronucleus of the 3-day CBCT irradiation group and the 0.5-Gy single irradiation group was significantly greater than that of the control ($p < 0.01$).

What we should pay attention to is that if taken with 3-day CBCT positioning, the damage to cells in vitro could be close to that of the 0.5-Gy irradiation group. This could be explained by the high relative biological effectiveness (RBE) of low-energy X-rays [17] or the low-dose hyper-radiosensitivity [18, 19]. More experiments shall be performed to verify the point.

From this study, we can draw some clues that the imaging radiation adopted in IGRT may bring more harm to the patients who already received high prescription radiation dose especially with a daily position. With a higher RBE value, the CBCT imaging dose may cause some additional effects. Even though a radiotherapy patient is exposed to localized high dose radiation, the additional dose from imaging will add an associated risk and should be kept as low as possible.

More consideration is required when contemplating repeated or serial use of CBCT, and the risk of secondary malignancy should be considered when choosing the optimal treatment technique and delivery system, especially in adolescents. It is reported that children are ten times radiation sensitive than adults, and girls are more sensitive than boys [20, 21]. Radiotherapy to children is frequently associated with severe side effects, such as growth and musculoskeletal abnormalities, endocrine and secondary malignancies. So children should be paid even more attention when be irradiated.

Dose minimization, however, must be within a context of relative cost versus benefit which varies from patient to patient. Further studies are needed to find out an optimization between the imaging quality and the extra radiation dose. Therefore, imaging dose should be managed on a case-by-case basis.

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