



Assessment of Correlation between Chromosomal Radiosensitivity of Peripheral Blood Lymphocytes after *In vitro* Irradiation and Normal Tissue Side Effects for Cancer Patients Undergoing Radiotherapy

Kamilė Guogytė, Aista Plieskienė¹, Rima Ladygienė, Žygimantas Vaisiūnas, Olga Sevriukova, Vinsas Janušonis¹, Julius Žiliukas

Radiation Protection Centre, Vilnius, ¹Klaipėda University Faculty of Health Science, Klaipėda University Hospital, Klaipėda, Lithuania

ABSTRACT

Patients receiving identical radiation treatments experience different effects, from undetectable to severe, on normal tissues. A crucial factor of radiotherapy related side effects is individual radiosensitivity. It is difficult to spare surrounding normal tissues delivering radiation to cancer cells during radiotherapy. Therefore, it may be useful to develop a simple routine cytogenetic assay which would allow the screening of a large number of individuals for radiosensitivity optimizing tumor control rates and minimizing severe radiotherapy effects with possibility to predict risk level for developing more severe early normal tissue adverse events after irradiation. This study was conducted to assess the correlation between *in vitro* radiosensitivity of peripheral blood lymphocytes from cancer patients who are undergoing radiotherapy using the cytokinesis-block micronucleus (CBMN), G2 chromosomal radiosensitivity assays, and normal tissue acute side effects. The CBMN and G2 chromosomal radiosensitivity assays were performed on blood samples taken from cancer patients before radiotherapy, after first fractionation, and after radiotherapy. Acute normal tissue reactions were graded according to the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer. This study suggests that there is a correlation between higher frequency of micronuclei after *in vitro* irradiation of blood samples and higher degree of normal tissue reactions. In addition, higher number of chromatid breaks was observed in patients with more severe normal tissue reactions. This pilot study included only 5 cancer patients, and therefore, further studies with a bigger cohort are required to identify radiosensitive patients.

Key words: Cytokinesis-block micronucleus assay, G2 radiosensitivity assay, individual radiosensitivity, radiotherapy

Introduction

Radiotherapy is the use of precisely targeted X-rays to destroy cancer cells while reducing the impact of radiation on healthy cells. It may be difficult to spare normal tissues relative to adjacent targets because the process of delivering radiation to the cancer cells will result in radiation passing through surrounding normal structures.^[1] Multiple studies have shown that cancer patients receiving identical radiation treatment experience different effects, from undetectable to severe, on normal tissues, which may depend on individual radiosensitivity (IRS).^[2,3] About 5% of cancer patients suffer from severe side effects due to radiotherapy.^[4]

Repair of radiation-induced DNA damage is crucial for patients' susceptibility to side effects and enhanced cytogenetic effects in single individuals might refer to enhanced tissue effects.^[5,6] Many studies show a correlation between chromosomal radiosensitivity and increased susceptibility to cancer. Radiosensitive individuals have increased risk of secondary cancer.^[7] Higher sensitivity to ionizing radiation is detectable not only among patients with cancer prone syndromes such as ataxia-telangiectasia (AT) but also among many other cancer-prone conditions including Down's syndrome, Li-Fraumeni syndrome, Wilms' tumor,^[8] and systemic lupus erythematosus.^[9] Current techniques allow detecting even small differences in radiosensitivity. Identification of radiosensitive cancer patients before radiotherapy can be helpful in the clinical management. Patients that are more

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Address for correspondence:

Dr. Kamilė Guogytė, Chief Specialist, Radiation Protection Centre, Kalvarijų Street 153, Vilnius, Lithuania.
E-mail: kamile.guogyte@rsc.lt

sensitive could be excluded from dose intensification, and relatively radioresistant patients might profit from dose escalation.^[10] Chromosome aberration frequency could be used as a good indicator of normal tissue radiosensitivity.

Higher yield of chromatid breaks the following irradiation at G2 phase of cell cycle could be one of the markers for radiosensitivity. Pantelias and Terzoudi^[11] proposed a standardized G2-assay for the prediction of IRS. In the cell-cycle-based G2 radiosensitivity assay, human peripheral blood lymphocytes are irradiated *in vitro*. Colcemid, used in cytogenetics, is a microtubule-depolymerizing drug such as vinblastine. It blocks mitotic cycle of the cell to visualize and quantify the chromatid breaks at metaphase stage. For each individual, two cultures are set up after *in vitro* irradiation - one represents the maximum yield of chromatid breaks when G2 checkpoint is abrogated with caffeine, and another yield of breaks is formed without this chemical. Caffeine induces G2-M checkpoint arrest, and the chromatid breaks produced simulate high radiosensitivity level obtained for AT patients.

Cytokinesis-block micronucleus (CBMN) assay is one of the most commonly used techniques for the assessment of radiosensitivity in human cells. Characterization of DNA repair in lymphocytes through micronuclei could be suitable approach to evaluate IRS *in vitro*.^[3] The CBMN assay developed by Fenech and Morley in 1985^[12] determines the frequency of the radiation-induced MN in peripheral blood lymphocytes. Ionizing radiation is one of the genotoxic agents which induces the formation of micronuclei.^[13,14] CBMN assay could be a promising method for evaluating normal tissue morbidity in cancer patients during radiotherapy because of its reliability and easy performance, reproducibility, and ease of automation using microscopy.^[15,16]

Our research work is focused on the assessment of correlation between the frequency of chromatid breaks, micronuclei in peripheral blood lymphocytes, and normal tissue acute side effects in cancer patients who are undergoing radiotherapy.

Materials and Methods

Patients, treatment, and normal tissue reactions

This pilot study included 5 patients – 4 with prostate cancer (Stage II–III) and 1 with rhabdomyosarcoma of the uterus (Stage II). The mean age was 64.5 (range 57–77 years). They all received external pelvic radiotherapy with a linear accelerator. Modern three-dimensional conformal radiation therapy (3D-CRT) (1 case) or volumetric modulated arc therapy (VMAT) technique (4 cases) was applied. One 3D-CRT plan was calculated using 18-MV, and five VMAT plans were calculated using 6-MV photons with a maximum variable dose rate of 600 MU/min. The planning target volume included tumor or tumor bed after surgery and pelvic lymph nodes. Bladder, small bowel, rectum, and femur heads were contoured as organs at risk. The total dose delivered was 50–76 Gy with 2 Gy daily fractions, given five sessions per week. The patients included had no history of the previous toxic treatment or exposure. Patients were seen by

a radiation oncologist at least weekly during the radiotherapy. Follow-up visits were arranged each month after the completion of treatment and every 3–6 months for the next years. Acute normal tissue reactions were graded according to the toxicity criteria of the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC).^[17] Gastrointestinal (GI) and genitourinary (GU) side effects were observed and registered. Bioethical approval for our study was obtained from the Lithuanian Bioethics Committee.

Blood sample and cell culture

Peripheral blood samples were collected from each patient into Li-heparin vacutainers. Blood lymphocytes were cultured adding 0.5 ml of whole blood to 4.5 ml of F-10 medium supplemented with 13% fetal bovine serum, 2% L-glutamine, 2% phytohaemagglutinin and antibiotics (penicillin: 100 U/ml, streptomycin: 100 µg/ml). Cultures were incubated at 37°C in a humidified incubator in 5% CO₂.^[13]

G2 chromosomal radiosensitivity assay

Blood samples were taken from cancer patients before radiotherapy, after the first fraction, and after radiotherapy. All cell cultures were exposed *in vitro* to 1 Gy of X-rays at room temperature (23°C ± 2°C), 48 h after culture initiation. Each culture was divided into two parts after irradiation: One part was supplemented by caffeine solution (4 mM), (G2 caffeine yield), and another part remained without caffeine (G2 yield). Cell cultures incubated for 20 min at 37°C in a humidified incubator in 5% CO₂. Colcemid was subsequently added to both cell cultures for 1 h. Lymphocytes were collected by centrifugation, treated in 75 mM KCl solution for 15 min at 37°C, and fixed in methanol: glacial acetic acid (3:1). Cells were spread on wet slides, air dried, and stained with 5% Giemsa stain. Metaphases were captured using a Zeiss Axio Imager Z2 microscope equipped with the high-resolution camera and Metafer 4 software (Metasystems, Altlußheim, Germany), image acquisition was done using metaphase finder MSearch and AutoCapt software (Metasystems, Altlußheim, Germany). Approximately 50 cells per sample were scored. Chromatid breaks were analyzed in well-spread metaphases. IRS calculated as a percentage of the high radiosensitivity level of AT patients using formula $IRS = (G2/G2 \text{ caffeine}) \times 100\%$.^[11]

Cytokinesis-block micronucleus assay

Blood samples were taken from cancer patients before radiotherapy, after the first fraction, and after radiotherapy. Each blood sample taken before radiotherapy was divided into two parts. One part was *in vitro* exposed to 2 Gy of X-rays at room temperature (23°C ± 2°C), and another part remained without irradiation. Blood samples taken after the first fraction and after radiotherapy were not *in vitro* exposed. Peripheral blood lymphocytes were cultured for 72 h, and the cytokinesis was blocked by the application of cytochalasin B (6 µg/ml) after 24 h. Lymphocytes were collected by centrifugation, treated in cold (4°C) 75 mM KCl solution, and fixed one time in methanol, glacial acetic acid, sodium chloride (4:1:5) solution, and then twice

in methanol, glacial acetic acid (4:1). After this procedure, cells were spread on wet slides, air dried, and stained with fluorescent stain 4',6-diamidino-2-phenylindole. Images were captured using the Zeiss Axio Imager Z2 microscope. Automated MN scoring was performed using MN software module specifically developed by Metasystems for the Metafer 4 platform (Metasystems, Germany).

Micronuclei were scored using criteria proposed by Fenech and Morley^[12] For each patient, 1000 binucleated cells were analyzed. The mean frequency of lymphocytes containing MN/1000 binucleated cells (cells with MN/1000) and the mean frequency of the MN/1000 binucleated cells (MN/1000) was calculated per sample for cancer patients. For these parameters, the spontaneous frequency, the frequency after *in vitro* irradiation with 2 Gy, frequency after first radiotherapy session, and frequency after radiotherapy were calculated.

Results and Discussion

The grades (RTOG/EORTC) of observed acute normal tissue side effects in 5 cancer patients are shown in Table 1. There were no acute Grades 3–4 events and Grade 2 GU side effects were recorded in 1 patient. Patients with Grade 1 GI toxicity complained of increased frequency of rectal discomfort not requiring analgesics, and patients with Grade 1 GU toxicity complained of dysuria, polyuria, and nocturia. Out of 5 patients, 2 did not develop any side effects.

In this study, frequencies of MN/1000 and cells with MN/1000 before radiotherapy, after first radiotherapy session, and after radiotherapy vary significantly [Table 2].

Cancer patients were divided into groups according to their acute normal tissues side effects grades (RTOG/EORTC). When different side effects in one patient occurred, the highest score from all recorded reactions was selected for evaluation of the data. The results of cytogenetic assays were compared between these groups.

Figure 1 shows the values of MN/1000 cells, and Figure 2 shows the values of cells with MN frequency in 1000 binucleated cells before radiotherapy, after *in vitro* irradiation, after first radiotherapy session, and after radiotherapy compared with each group.

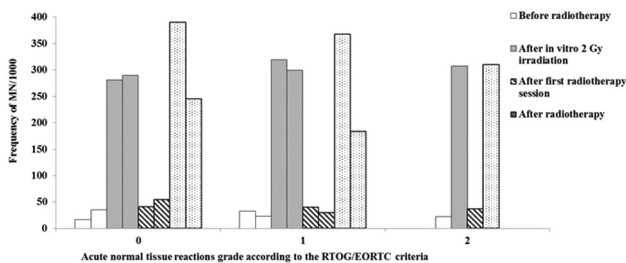


Figure 1: Frequency of micronucleus/1000 before radiotherapy, after *in vitro* irradiation, after first radiotherapy session, and after radiotherapy compared with acute normal tissue reactions grade according to recommendations of Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer in 5 cancer patients

According to the results of the study, the higher frequency of MN/1000 cells is determined after, *in vitro* irradiation of blood samples, the greater is the degree of the development of normal tissue reactions. As shown here, the frequency of MN/1000 cells after *in vitro* irradiation between group of patients without side effects and with Grade 1 side effects increases by 8%, the frequency of MN/1000 between group of patients without side effects and the patient with Grade 2 side effects increases by 9%, the frequency of MN/1000 cells between groups of patients with Grade 1 side effects and the patient with Grade 2 side effects increases by 1%.

Table 1: Acute normal tissue side effects observed in 5 cancer patients graded according to recommendations of Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer criteria

Patients	GU toxicity grade	GI toxicity grade
A	2	1
B	0	0
C	0	0
D	1	1
E	1	0

GU: Genitourinary, GI: Gastrointestinal

Table 2: Frequency of micronucleus/1000 cells and cells with micronucleus/1000 cells in lymphocytes of cancer patients before radiotherapy, after *in vitro* irradiation, after first radiotherapy session, and after radiotherapy

Patients	Frequency before radiotherapy		Frequency after <i>in vitro</i> irradiation		Frequency after first radiotherapy session		Frequency after radiotherapy	
	MN	Cells with MN	MN	Cells with MN	MN	Cells with MN	MN	Cells with MN
A	22	21	307	247	37	33	310	224
B	17	16	281	224	41	38	390	320
C	35	34	290	229	55	50	245	175
D	33	31	319	261	40	37	368	217
E	23	22	299	256	30	28	184	153

MN: Micronucleus

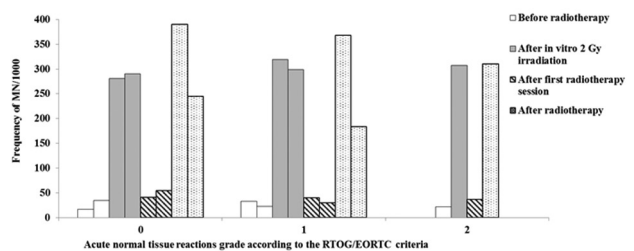


Figure 2: Frequency of cells with micronucleus/1000 before radiotherapy, after *in vitro* irradiation, after first radiotherapy session and after radiotherapy compared with acute normal tissue reactions grade according to recommendations of Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer in 5 cancer patients

As demonstrated by the results of this study, the frequency of MN/1000 cells increased gradually, whereas frequency of cells with MN/1000 cells after *in vitro* 2-Gy irradiation increased between group of patients without side effects and the patient with Grade 1 side effects about 13%, but between group of patients with Grade 1 side effects and the patient with Grade 2 side effects, the frequency decreased 3%.

G2 chromosomal radiosensitivity assay was performed for 2 patients without acute normal tissue reactions and for 1 patient with Grade 2 acute normal tissue reactions (RTOG/EORTC). The value of IRS (percent of AT radiosensitivity level) before radiotherapy, after first radiotherapy session, and after radiotherapy compared with each group [Table 3].

Figure 3 shows that patient with Grade 2 has the highest percent of IRS in comparison with patients group with Grade 1. According the radiosensitivity, thresholds to cancer patients as described in Pantelias and Terzoudi,^[11] patient with Grade 2 side effects can be included in the radiosensitive group while the patient group with Grade 1 side effects is in the normal group and cannot be included in the radiosensitive group.

Conclusions

Data presented here indicate that the higher frequency of MN/1000 cells after *in vitro* irradiation of blood samples correlate with the development of higher degree of normal tissue reactions. Higher number of chromatid breaks was observed in patients with more severe normal tissue reactions and these patients can be considered as radiosensitive. Since this is a pilot study involving five patients, caution should be

exercised in arriving at the conclusions. Further investigations are required involving both radiotherapy patients as well as healthy cohort.

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Conflicts of interest

There are no conflicts of interest.

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Table 3: Individual radiosensitivity (percent of ataxia-telangiectasia radiosensitivity level)

Patients	Before radiotherapy	After first radiotherapy session	After radiotherapy
A	53.2	59.3	63.9
B	52.3	44.4	44.8
C	46.4	40.8	43.5

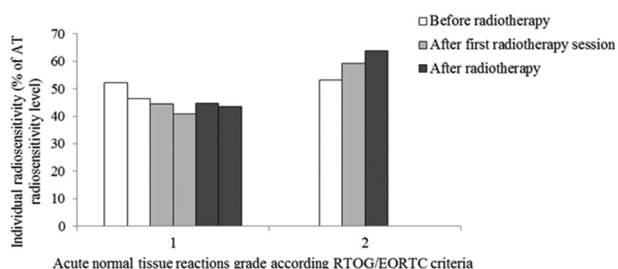


Figure 3: Individual radiosensitivity (percent of ataxia-telangiectasia radiosensitivity level) before radiotherapy, after first radiotherapy session and after radiotherapy compared with acute normal tissue reactions grade according to recommendations of Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer in 5 cancer patients

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