Experimental
ONCOLOGY

CYTOGENETIC EFFECTS OF NEUTRON THERAPY IN PATIENTS WITH PAROTID GLAND TUMORS AND RELAPSE OF BREAST CANCER

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Aim: To assess the frequency and spectrum of chromosome aberrations and micronuclei in peripheral blood lymphocytes of patients with parotid salivary gland tumors and relapse of breast cancer during the course of neutron therapy. *Materials and Methods:* Samples of peripheral blood were obtained from 9 patients with parotid salivary gland tumors (T3N0–3M0) and 8 patients with relapse of breast cancer before, after first fraction and at the end of neutron therapy. The treatment course specified 5.5–8.4 Gy (equivalent to 23–44 Gy of photon irradiation) with 1.3–2.2 Gy per fraction for patients with parotid salivary gland tumors and 4,8–8.0 Gy (equivalent to 30–40 Gy of photon irradiation) with 1.6 Gy per fraction for patients with relapse of breast cancer. Control group established for conventional cytogenetic analysis consisted of 15 healthy persons. Assessment of chromosome aberrations frequency was performed on routinely stained metaphase plates. Lymphocytes from the same patients were analyzed by micronucleus test in combination with fluorescent *in situ* hybridization (FISH) using pancentromeric DNA probe. *Results:* Level of chromosome aberrations and micronuclei significantly increased in lymphocytes of patients from both groups during neutron therapy (*P* < 0.05). This increase was mainly due to chromosome-type aberrations and centromere-negative micronuclei. The prevalent types of aberrations are in agreement with theoretical mechanisms of neutron effects on cells. *Conclusion*: Cytogenetic effects of fast neutron therapy in lymphocytes of patients with parotid salivary gland tumors and relapse of breast cancer were observed. A positive dynamics of radiation-induced chromosomal damages formation during the course was denoted in lymphocytes of cancer patients in both groups.
Key Words: chromosome aberrations, micronucleus test, neutron therapy.

World-wide experience of past decades showed that neutron therapy significantly increases the treatment efficacy of patients with radioresistant head and neck cancer and breast cancer due to higher damaging effects of neutrons [1]. However, it is often hard to eliminate the most radioresistant fraction of tumor cells without crossing the border of healthy tissue tolerance [2–4]. This may result in late adverse reactions to neutron therapy and thus, require the development of new effective approaches to assess the radiation damage in healthy tissues of patient during neutron therapy.

Cytogenetic assessment is recommended by World Health Organization, United Nations Scientific Committee on the Effects of Atomic Radiation and International Atomic Energy Agency as accurate and reliable assay to indicate the radiation damage in human cells [5]. Biological effects of ionizing radiation are the sum of absorbed dose, dose distribution throughout the body and individual radiosensitivity of the patient [6]. In addition, the issue of radiation-induced numerical chromosome aberrations in human cells remains open today [8]. Data concerning the aneugenic effect of radiation are insufficient and sometimes contradictory to make a clear conclusion. This requires the assessment of both structural and numerical chro-

mosome abnormalities for complete characterization of radiation-induced effects in cells of cancer patients during the neutron therapy. In addition to conventional cytogenetic analysis of routinely stained metaphase plates cytokinesis-blocked micronucleus test in combination with fluorescent *in situ* hybridization (FISH) is used to assess the levels of both structural and numerical chromosomal abnormalities in human peripheral blood lymphocytes [9, 10]. Thus, the aim of this study was to analyze the level and spectrum of chromosomal aberrations and micronuclei in peripheral blood lymphocytes of patients with parotid salivary gland tumors and relapse of breast cancer during the neutron therapy.

MATERIALS AND METHODS

Patients. Samples of peripheral blood were obtained from 9 patients with parotid salivary gland tumors (T3N0–3M0) and 8 patients with relapse of breast cancer before, 24 h after first fraction and at the end of neutron therapy. Before entering the study all subjects were informed about the aim and the experimental details and gave their signed informed consent. This study was approved by Bioethics Committee of Cancer Research Institute Siberian Branch of the Russian Academy of Medical Sciences.

Therapy using 6.3 MeV fast neutrons was performed on cyclotron U-120 at the Research Institute of Nuclear Physics of Tomsk Polytechnic University. The treatment course specified 5.5–8.4 Gy (equivalent

to 23–44 Gy of photon irradiation) with 1.3–2.2 Gy per fraction for patients with parotid salivary gland tumors and 4.8–8.0 Gy (equivalent to 30–40 Gy of photon irradiation) with 1.6 Gy per fraction for patients with relapse of breast cancer. Two radiation field sizes were used: 6 by 6 cm and 6 by 8 cm.

Control group. Control group established for conventional cytogenetic analysis consisted of 15 healthy persons.

Blood sampling. Samples of venous blood (4 mL per each sampling time) were collected in heparinized vacutainer tubes (Greiner, Austria). Patients were sampled three times throughout the study. Using blood samples collected before therapy, individual baseline values for each test parameter were done. The pre-treatment blood sample was collected two hours prior to the first fraction of radiation. The response of peripheral blood leukocytes to the radiotherapy was evaluated on blood samples taken after the application of the first dose, as well as after the last received radiotherapy dose.

After venipuncture, the blood samples were coded and transferred to laboratory. They were processed immediately after transportation and cell cultures were launched for the analysis of structural chromosomal aberrations and the cytokinesis-block micronucleus (CBMN) assay in combination with FISH following the recommendations by International Atomic Energy Agency and the HUMN project [11–13].

Chromosomal aberrations analysis. Chromosomal aberrations analysis was performed according to International Atomic Energy Agency guidelines [12]. In brief, cultures were incubated *in vitro* for 52 h in RPMI-1640 medium (Sigma, USA) with 10% fetal bovine serum (Thermo Scientific, USA) and stimulated by phytohaemagglutinin (PHA; PanEco Russia). To arrest dividing lymphocytes in metaphase, colchicine was added 2 h prior to the harvest. Preparations were made according to the standard procedure. Slides were stained with 5% Giemsa solution (Sigma, USA). All slides were coded and scored blindly at 1000′ magnification under oil immersion on microscope "Axioskop" (Carl Zeiss, Germany). Structural chromosomal aberrations were classified based on the number of sister chromatids and breakage events involved. Only metaphases containing 45–47 centromeres were analyzed. Three hundred metaphases per sample per each point of irradiation were analyzed.

CBMN assay in combination with FISH. CBMN assay was performed using lymphocyte cultures according to the standard protocol with minor modifications [13]. Lymphocyte cultures were incubated in RPMI-1640 medium for 72 h. Cytochalasin B was added at 44 h in final concentration 5 µg/mL. Slides were made according to the standard procedure.

FISH was performed with the use of pancentromeric DNA probe specific for the pericentromeric satellite DNA sequences of all human chromosomes [7]. A fluorescent label (TAMRA-dUTP) was incorporated into the DNA probes using the standard nick-transla-

tion reaction [14]. The specificity of the resultant DNA probes was estimated in normal human metaphase plates. FISH was performed as described earlier [15].

Criteria proposed by M. Fenech et al. (2007) for MN identification were used [11]. Altogether 1000 binuclear (BN) cells per each sample per point of experiment were scored. Total number of MN and their types were determined, along with the number of micronucleated cells.

The nonparametric Wilcoxon matched pairs test was used to statistically estimate the differences of chromosomal aberrations and micronuclei levels between various time points during radiation therapy.

RESULTS AND DISCUSSION

Frequency of aberrant metaphases before radiation therapy was $0.32 \pm 0.05\%$ in the group of patients with parotid salivary gland tumors and $1.62 \pm 0.72\%$ in the group of patients with relapse of breast cancer. No significant differences were observed in the mean frequency of aberrant metaphases between patients with parotid salivary gland tumors and healthy persons ($0.29 \pm 0.14\%$, $P = 0.69$). However, significant differences were found between patients with relapse of breast cancer and control group ($P = 0.012$).

Frequency of micronuclei in groups of patients with parotid salivary gland tumors ($0.72 \pm 0.05\%$) and with relapse of breast cancer ($1.12 \pm 0.19\%$) was significantly higher than in healthy persons ($0.55 \pm 0.04\%$, $P < 0.05$) [9].

Frequency of all chromosomal aberrations increased in the group of patients with relapse of breast cancer from $2.01 \pm 0.95\%$ before the radiation therapy to $3.13 \pm 0.84\%$ after the first irradiation and to $4.65 \pm 0.91\%$ at the end of treatment course (Table 1). Significant increasing was observed also for the frequencies of aberrant metaphases ($P = 0.046$), chromosome-type aberrations ($P = 0.017$), atypical monocentrics ($P = 0.027$), paired fragments ($P = 0.010$), centromere-negative micronuclei ($P = 0.016$) and sum of all micronuclei ($P = 0.01$) only at the end of radiation therapy in comparison with levels before the treatment.

In the group of patients with parotid salivary gland tumors the frequency of all chromosomal aberrations was significantly higher after the first fraction ($2.00 \pm 0.31\%$) and at the end of neutron therapy ($3.01 \pm 0.35\%$) in comparison with $0.32 \pm 0.15\%$ before the treatment ($P < 0.05$, Table 2). Opposite to the patients with relapse of breast cancer the frequency of all chromosomal aberrations and micronuclei significantly increased after the first fraction of the therapy and subsequently till to the end of treatment course (Table 2).

Chromosome-type aberrations in peripheral blood lymphocytes are known to be a specific biomarker of ionizing radiation exposure [16–18]. As expected, in our study chromosome-type aberrations prevailed among detected abnormalities at the end of the neutron therapy (97 and 95.5% in the groups of patients with salivary gland tumors and with relapse of breast cancer, respectively). In turn, the most frequent ab-

normalities among the chromosome-type aberrations were paired fragments. This is not surprising because of paired fragments formed both due to the lack of DNA double-strand break repair and as a result of misrepair together with chromosome translocations. Moreover, most of detected micronuclei did not contain centromere (centromere-negative micronuclei) and corresponded to acentric chromosomal fragments.

Table 1. Frequencies of chromosomal aberrations and micronuclei in peripheral blood lymphocytes of 8 patients with relapse of breast cancer during neutron therapy (mean ± standard error, %)

Time point	Before treatment	After the 1st fraction	At the end of treatment
Dose range of neutron irradiation	-	1.6 Gy	4.8–6.56 Gy
Equivalent dose range			25–35 Gy
Chromosome aberration analysis (routinely stained metaphase plates)			
Number of analyzed metaphase plates	2260	2440	2290
Aberrant metaphases	1.62 ± 0.72	2.68 ± 0.78	4.16 ± 0.77*
Total number of aberrations	2.01 ± 0.95	3.13 ± 0.84	4.65 ± 0.91
Chromosome-type aberrations	1.81 ± 0.86	3.03 ± 0.74	4.45 ± 0.75*
Paired fragments	1.01 ± 0.41	1.62 ± 0.43	2.74 ± 0.38*
Ring chromosomes	0.16 ± 0.12	0.25 ± 0.13	0.20 ± 0.08
Dicentric chromosomes	0.37 ± 0.22	0.45 ± 0.21	0.54 ± 0.26
Atypical monocentrics	0.08 ± 0.05	0.33 ± 0.14	0.75 ± 0.21*
Double-minute chromosomes	0.16 ± 0.11	0.37 ± 0.13	0.20 ± 0.14
Chromatid-type aberrations	0.21 ± 0.08	0.09 ± 0.09	0.21 ± 0.16
Chromatid breaks	0.16 ± 0.09	0.05 ± 0.05	0.21 ± 0.16
Isochromatid breaks	0.04 ± 0.04	0.04 ± 0.04	0
Micronucleus test (using pancentromeric DNA-probe)			
Total number of micronuclei	1.24 ± 0.19	1.83 ± 0.06	3.48 ± 1.01*
Centromere-negative micronuclei	0.78 ± 0.16	1.44 ± 0.08	2.60 ± 0.88*
Centromere-positive micronuclei	0.46 ± 0.06	0.39 ± 0.08	0.88 ± 0.14
Nucleoplasmatic bridges	0.02 ± 0.01	0.04 ± 0.01	0.09 ± 0.03

Notes: *statistically significant differences in comparison with level before treatment, $P < 0.05$

Table 2. Frequencies of chromosomal aberrations and micronuclei in peripheral blood lymphocytes of 9 patients with parotid salivary gland tumors during neutron therapy (mean ± standard error, %)

Time point	Before treatment	After the 1st fraction	At the end of treatment
Dose range of neutron irradiation	-	1.3–2.2 Gy	5.5–8.4 Gy
Equivalent dose range			23–44 Gy
Chromosome aberration analysis (routinely stained metaphase plates)			
Number of analyzed metaphase plates	2520	2550	2580
Aberrant metaphases	0.32 ± 0.05	1.80 ± 0.26*	2.69 ± 0.23*
Total number of aberrations	0.32 ± 0.15	2.00 ± 0.31*	3.01 ± 0.35*
Chromosome-type aberrations	0.15 ± 0.08	1.89 ± 0.26*	2.86 ± 0.29*
Paired fragments	0.08 ± 0.08	1.33 ± 0.28*	1.71 ± 0.23*
Ring chromosomes	0	0.03 ± 0.03	0.33 ± 0.19
Dicentric chromosomes	0	0.15 ± 0.08	0.15 ± 0.08
Atypical monocentrics	0	0	0.26 ± 0.07*
Double-minute chromosomes	0.07 ± 0.05	0.38 ± 0.20	0.40 ± 0.15
Chromatid-type aberrations	0.17± 0.07	0.11 ± 0.05	0.11 ± 0.06
Chromatid breaks	0.13 ± 0.07	0	0.07 ± 0.05
Isochromatid breaks	0.04 ± 0.04	0.11 ± 0.05	0.07 ± 0.05
Micronucleus test (using pancentromeric DNA-probe)			
Total number of micronuclei	0.72 ± 0.05	1.28 ± 0.08*	1.87 ± 0.26*
Centromere-negative micronuclei	0.43 ± 0.56	0.97 ± 0.07	1.32 ± 0.19*
Centromere-positive micronuclei	0.29 ± 0.03	0.31 ± 0.04	0.55 ± 0.10
Nucleoplasmatic bridges	0	0.07 ± 0.03	0.10 ± 0.04

Notes: *statistically significant differences in comparison with level before treatment, $P < 0.05$

On the contrary to the frequency of centromere-negative micronuclei, the level of centromere-positive micronuclei increased insignificantly after the neutron therapy (Table 1, 2). Centromere-positive micronuclei contain whole chromatid/chromosome(s) or centric chromosome fragments and they are produced as a result of indirect radiation effects expressed less markedly after exposure to high-LET radiation (including neutrons). Therefore, significant increase of the levels of acentric fragments, centromere-negative micronuclei and insignificant increase of centromere-positive micronuclei are in agreement with the nature of fast neutrons. Regarding to the importance of these chromosomal malformations for the cell fate, loss of chromosomal fragments or whole chromosome usually leads to reduced viability of cells and decreased risk of malignant transformation.

Translocations are two-hit chromosome aberrations and could be detected by the analysis of routinely stained metaphase plates as a dicentric chromosome or monocentric chromosome with atypical appearance (atypical monocentrics). Misrepair of radiation-induced damage in the peripheral blood lymphocytes of cancer patients in our study was observed as significant increase of atypical monocentrics at the end of neutron therapy in both groups (Table 1, 2). Also, there was insignificant increase of dicentric chromosomes and corresponding nucleoplasmic bridges during the radiotherapy. Cells with atypical monocentrics are viable and a number of specific chromosome translocations are known to be a cause of leukemia [19]. Increase of stable translocation level in the lymphocytes of cancer patients during neutron therapy could possibly be a risk factor of radiation-induced secondary cancer.

Thus, in our study for the first time we have observed cytogenetic effects of fast neutron therapy in lymphocytes of patients with parotid salivary gland tumors and relapse of breast cancer. It was shown that total increase of cytogenetic damages during the treatment course is mainly due to formation of chromosome-type aberrations. A positive dynamics of the formation of radiation-induced chromosomal damage in the course of neutron therapy was observed in lymphocytes of cancer patients in both groups. Such bioindication of cytogenetic abnormalities are believed to be an important tool to assess the level of damage in irradiated tissues and could be used with the aim of optimization and individualization of the treatment.

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