The Cytogenetic Effects on Peripheral Blood Lymphocytes in Cancer Patients After Radiation Therapy: Chromosome Aberrations and Micronuclei

Yanti Lusiyanti1*, Harry Nugroho Eko Surniyantoro1, Nastiti Rahajeng1, Viria Agesti Suvifan1, Sofiati Purnami1, Lina Choridah2

1Center for Technology of Safety and Radiation Metrology, National Nuclear Energy Agency, Indonesia
2Department of Radiology, Dr Sardjito General Hospital, Yogyakarta, Indonesia
*Corresponding Author: k_lusiyanti@batan.go.id

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Abstract. Individual responses to radiotherapy are often observed whether or not regimes with identical treatments were applied. Patient-related factors, the therapeutic process, and therefore the intrinsic factors of individual radiosensitivity are considered to be causing the variability of side effects. A preliminary evaluation was done on cytogenetic biomarkers found in cancer patients. The purpose of this present study was to assess the individual response of patients with cancers after radiation therapy. The sample obtained from 11 patients with different types of cancer as a case group and 12 people as a control group from a healthy volunteer. Blood samples were stimulated by an in vitro culture using phytohemagglutinin, and the cultures were assessed by using the Dicentric and Cytokinesis-Block Micronucleus (CBMN-) assay. These two methods were compared. The results showed that the overall dicentric chromosome and micronuclei in binucleate cells (MN/BNC) have a significantly higher frequency in the breast, head, and neck compared to extremity cancer. A high frequency of micronuclei in lymphocyte patients was seen after radiotherapy treatment but relatively not much higher compared to the range of micronuclei backgrounds in healthy people. The CBMN is the most effective assay for evaluation of the cytogenetic studies in cancer patients because it is more radiosensitive to study individual responses. By evaluating the effects of radiotherapy based on DNA damage, the severity of radiation exposure can be studied. This study can be useful for researchers and related stakeholders in the application of radiotherapy.

Key words: cytogenetic biomarkers; micronuclei; dicentric; response; cancer patients

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INTRODUCTION

Ionizing radiation is the most commonly used in cancer radiotherapy. The detrimental effects of mutagenic agents, including ionizing radiation on exposed-people, increase a serious concern in the world. The major impact in cells initiated by ionizing radiation is DNA breaks, either a single strand or double strands, base damage, sugar damage, and apyrimidinic site. The double-strand breaks (DSBs) damage in the DNA are believed to be much more important biologically because these may generate the formation of cancer cells, and determining the extent of DSBs may help to detect the development of cancer cells earlier. (Lomax, Folkes, & Neill, 2013). Different responses to radiotherapy can be assessed even if a regimen with the same treatment is applied. The variability of the detrimental effects can be influenced by many factors, such as the radiosensitivity of patients, the treatment process of radiotherapy, and another patient-related factors, including different genetic predispositions in the mechanisms of DNA repair (Ginsberg, et al., 2011).

In the biomonitoring of human populations to assess genotoxic risks during radiotherapy, the cytogenetic biomarkers widely used are cytogenetic assays such as chromosome aberration (CA), sister chromatid exchanges (SCE), and micronucleus (MN). (Pajic et al., 2015; Roch-lefe et al., 2010). Determination of chromosome aberration by using peripheral blood lymphocytes is a more sensitive assay for detecting the effect of exposure to natural radiation and a biomarker of cancer risk and also to predict patient’s radiosensitivity after radiotherapy treatment (Saberi, Salari, & Latifi, 2013; Schuster et al., 2018). Dicentric is a type of chromosomal aberration that forms a complex event, one that needs DSB in at least two different chromosomes in close proximity to each other and is considered an indicator of radiation-induced injury.

In addition, the micronucleus (MN) assay that reflects chromosomal injury is useful as a biomarker for monitoring environmental effects on the genetic material in human cells (Perumal et al., 2015). The two methods have also been used in previous research...
to evaluate the cytogenetic impact of chronically exposed to low dose ionizing radiation hospital medical workers (Lusiyanti, et al., 2017). The previous study reported that cancer patients indicated different sensitivities and showed increased chromosomal instability compared with healthy individuals (Garaj-vrhovac & Kopjar, 2018). Moreover, another study reported that a significant increase of chromosomal damage was observed in a patient undergoing radiochemotherapy parallel to increasing radiation doses, but independent of the chemotherapy applied (Wolff et al., 2011).

Chromosome aberration and micronucleus occurrence are important biomarkers as results of a response to the environment including diet and ionizing radiation exposure. Micronucleus measurements in radiation-working populations or residents living in areas with high natural radiation exposure can be used to determine the impact of exposure to DNA damage. The purpose of the present research was to assess the response sensitivity in patients with different types of cancer after radiotherapy and to compare between the two methods, Dicentric and Cytokinesis-B lock Micronucleus (CBMN-) assay. By evaluating the effects of radiotherapy based on DNA damage, the severity of side effects of radiation exposure can be studied.

METHODS

Sample subject

This study is meant to be a case-control study. Subjects consisted of 11 nonsmoker patients (4 men and 7 women) with various types of cancer in the radiotherapy installation at Dr. Sarjito Yogyakarta General Hospital, Indonesia, are used as a case study of radiation. Twelve people from healthy volunteer were as the control group. Questionnaires have been given to compile comprehensive records about sex, age, and the full history of each illness. Each subject was briefed about the protocol, with particular information given about the cytogenetic test, the aim of the study, and the signed consent form. The age range of the patients varied between 25 to 58 years, with an average of 46.8 years. The cancer regions in this study were grouped according to accepted fractionation doses: breast cancer (10-14 Gy), head and neck cancer (12-14 Gy), and extremity cancer (10 Gy). The study was approved by the Ethics Committee of the National Institute of Health Study and Development, Indonesian Ministry of Health, with the decree number LB.02.01/5.2.KE.051/2017. Details of patient data, such as age, sex, and cancer type, are presented in Table 1.

Table 1. Description of patient and control subject

| Patient Code | Sex | Age (Years) | Type of Cancer | Dose Delivered (Gy) | Control Code | Sex | Age (Years) |
|--------------|-----|-------------|----------------|---------------------|--------------|-----|--------------|
| PN1          | L   | 29          | Extremity Sarcoma | 10                  | C1           | P   | 53           |
| PN2          | L   | 51          | Soft Tissue Sarcoma | 14                  | C2           | L   | 25           |
| PN3          | L   | 21          | Parotid         | 12                  | C3           | C   | 33           |
| PN4          | P   | 48          | Breast          | 12                  | C4           | L   | 49           |
| PN5          | L   | 61          | Nasopharyngeal  | 12                  | C5           | P   | 53           |
| PN6          | P   | 68          | Tongue          | 14                  | C6           | P   | 51           |
| PN7          | P   | 61          | Parotid         | 12                  | C7           | P   | 20           |
| PN8          | P   | 45          | Breast          | 14                  | C8           | P   | 49           |
| PN9          | P   | 57          | Humerus         | 10                  | C9           | L   | 27           |
| PN10         | P   | 71          | Breast          | 10                  | C10          | L   | 52           |
| PN11         | P   | 48          | Tongue          | 10                  | C11          | P   | 53           |
|              |     |             |                 |                     | C12          | P   | 37           |

Blood sampling

Blood samples obtained from venous blood, consisted of 5 mL, were collected and stored in heparinized vacutainer tubes (BD Vacutainer Systems, Bellvue dustrial Estate, Plymouth, UK) after radiation therapy treatment. The blood samples were labeled and transported to the cytogenetic laboratory at the Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency of Indonesia, Jakarta, for the next analysis.

Lymphocyte culture and chromosome analysis

Detection of the chromosome was performed according to the (Anonymous, Cytogenetic Dosimetry, 2011) a standard protocol with some modification as previously published paper by Lusiyanti et al., (2013). Each whole blood sample was cultured and grown for 48 hours at 37°C with an enriched medium consisting of RPMI 1960-medium.
supplemented with 10% fetal bovine serum (Sigma Chemical Co., St Louis, MO, USA), phytohemagglutinin, and streptomycin. Colchicine (Sigma Chemical Co., St Louis, MO, USA) at a final concentration of 0.1 mL was added for the last 3 hours of culture to arrest cells in metaphase. After a total culture of 48 hours, cells were collected by centrifugation and briefly treated with a hypotonic solution of 0.075M KCl, a freshly prepared incubation in a water bath at 37°C for 25 minutes, and repeated with fixative Carnoy’s solution (methanol and acetic acid 3:1). Slide preparation was done by dripping cell suspensions onto clean slides then submitted to 5% buffered Giemsa solution (Sigma Chemical Co., St Louis, MO, USA). Scoring of chromosome aberrations was performed using a light microscope at 100x magnification based on the criteria of Chromosome structural chromosome-type aberrations (e.g. dicentric chromosomes, acentric fragments, and ring chromosome) for each patient in up to 200 metaphases.

Micronucleus analysis

The micronucleus assay was performed as described by (Fenech & Natarajan, 2011) with some modifications as previous publish on Yanti Lusiyanti, et al (2016). Heparinized whole blood samples were cultured in the same conditions as the analysis of the structural chromosome aberrations. Cytochalasin-B (Sigma Chemical Co., St Louis, MO, USA) at a final concentration of 15 µL/mL was added to each sample at 44 hours, and the cells were harvested after a further incubation of 28 hours. Furthermore after a total culture of 72 hours, cells were collected by centrifugation and briefly treated with a hypotonic solution of 0.075M KCl, cold freshly and repeated fixation cells with Ringer's solution and a fixative Carnoy solution (mixture of methanol and acetic acid 10:1).

Permanent preparations were obtained by administering a few drops of the cell suspension onto clean slides. Slides were air-dried overnight at room temperature and stained with 5% Giemsa solution (Sigma Chemical Co., St Louis, MO, USA) at pH 6.8 for 10 minutes. The scoring of micronuclei with a frequency of MN was evaluated by scoring 1000 binucleated cells for each staining technique.

The data analysis statistically by using the Med Calc 2013 program for Windows. All of the data were displayed as mean ± SD. An independent sample T-test was used to test a significant relationship between micronuclei and dicentric at various dose levels at P <0.05. For statistical analysis, the total number of MN per 1000 binucleated cells was used (MN/BNC). Differences between the frequencies of MN and the dicentric for each type of cancer patient and between the dose-level fractionation determined in patients with the same therapy protocol were analyzed using the one-tailed independent samples T-test.

RESULTS AND DISCUSSION

Cytogenetic damage after therapy

In the present study, there are 11 patients with six different types of cancers: Parotid, Breast, Thyroid, Nasopharyngeal, Sinonasal, Basal cell, Tongue., and Humerus cancers. The numbers of each cancer are shown in Table 1. All of the patient data were obtained from Dr. Sardjito General Hospital, Yogyakarta. In this study, the number of various chromosomal aberrations (CAs) such as Dicentric (DC), Acentric Fragment (AF), ring (R) (which were categorized as aberrant cells [AC]), and micronuclei in binucleate cells (MN/BNC) was noted in each cancer patient. The MN frequency results were reported as the total number of micronucleus in binucleated (BNC). Whereas for the dicentric, chromosome aberration was reported as a complex event that needs DSB in at least two different chromosomes in close proximity during metaphase. The distribution of various chromosomal aberrations and micronuclei in the peripheral blood of cancer patients and healthy subjects (control) are given in Table 2. Image of various chromosomal aberrations and MN are showing in Figure 1.

It can be seen in Table 2 that, the different sensitivities and interindividual responses in the aberration chromosome (dicentric, acentric fragment, and ring) and MN frequencies found in patient cancer after radiotherapy treatment. The DC frequencies in the range of 1% to 21%, AF in the range of 2% to 11%, and Ring in the range of 0% to 1.5%. Whereas the mean of DC and AF were relatively the same 5.9 ± 2.19, and AF 5.9 ± 1.59 while for ring type 0.31 ± 0.14. Furthermore, the total frequency for aberrant cells varies from 1 % to 33.4 % with a mean of 11.07.94 ± 3.5247. Meanwhile, for the control subject, there was neither chromosome aberration nor aberrant cell. Furthermore, the MN frequency in patients varies in the range of 3% to 24% with a mean of 11.91 ± 2.76% while for control subject in ranges of 0.1% to 1.1% with a mean of 0.73 ± 0.16. Significantly different from the patient at the level of confidence p <0.05. Similar research from (Saha et al., 2017) reporting the frequency of aberrant cells varied from 5% to 69% in leucocytes of breast cancer patients, meanwhile in the healthy peoples no CAs
were found. While the previous result by Surniyantoro HNE., et al (2019) showed that micronuclei frequency was significantly higher in cancer patients compared to controls. Based on the location of the cancer type in this study, the cancers were divided into 3 groups while collected with approved fractionation doses: for breast cancer, the dosage range was 10-14 Gy, 12-14 Gy for head and neck cancer, and 10 Gy for extremity cancer. The comparison percentage of the aberrant cell (AC) and MN with respect to applied dose are presented in Figure 1. Meanwhile, a comparison between DC alone and MN/BNC frequencies based on cancer type is presented in Figure 2.

![Image](A.png) ![Image](B.png) ![Image](C.png) ![Image](D.png)

**Figure 1.** Image of chromosome in metaphase cells with normal chromosome (A), metaphase cells with dicentric and fragment (1), metaphase cells with 2 dicentric and fragment (2), and metaphase cells with ring (3) (B), binucleated cells (normal) (C). Binucleated cells with 1MN (1), 2 MN (2) 3 MN (3) and 4 MN (4) (D)

**Table 2.** Distribution of various chromosomal aberration and MN observed in the peripheral blood of cancer patient and healthy subject (Control).

| Patient Code | DC % | AF % | Ring % | AC % | MN % | Control Code | DC % | MN % |
|--------------|------|------|--------|------|------|--------------|------|------|
| PN1          | 1    | 2.5  | 0      | 3.5  | CF   | C1           | 0    | 1.1  |
| PN2          | 15   | 11   | 1      | 27   | CF   | C2           | 0    | 0.2  |
| PN3          | 1    | ND   | 0      | 1    | 3    | C3           | 0    | 0.7  |
| PN4          | 21   | 11   | 1.4    | 33.4 | 24   | C4           | 0    | 0.5  |
| PN5          | ND   | 1.9  | 0      | 1.9  | CF   | C5           | 1    | 1.1  |
| PN6          | 8    | 16   | 0.5    | 24.5 | 13   | C6           | 0    | 0.7  |
| PN7          | 3    | 3    | 0.5    | 6.5  | 5    | C7           | 0    | 0.5  |
| PN8          | 1    | 2.5  | 0      | 3.5  | 18   | C8           | 0    | 2.2  |
| PN9          | 2    | 2    | 0      | 4    | 8    | C9           | 0    | 0.1  |
| PN10         | 1    | 2.5  | 0      | 3.5  | 9    | C10          | 0    | 0.4  |
| PN11         | 6    | 7    | 0      | 13   | 18   | C11          | 0    | 0.5  |
|              |      |      |        |      |      | C12          | 0    | 0.8  |

**Mean** 5.9±2.19 5.9±1.59 0.31±0.14 11.07±3.52 11.91±2.76 0.73±0.16

DC; dicentric chromosome; AF; acentric fragment; AC; aberrant cells; BNC; binucleat cells; MN, micronuclei; ND, not detected; CF, culture failed.
Figure 2. Comparison of chromosome aberration with respect to applied dose (Gy), % Aberrant Cell (AC) (A), and % Micronuclei in Binucleated Cells (B)

According to the fractionation dose given to cancer patients, in general, the frequency of aberrant cells and micronuclei shows that higher doses lead to higher frequencies as shown in Figure 2. The result indicated that the percentage of aberrant cells for a dose of 10 Gy had the lowest aberrant cells compared to the dose of 14 Gy and 20 Gy. Meanwhile, the micronuclei showed slightly similar results with aberrant cells. The varying frequency is related to the process of repair and the different immune systems of each individual. Overall it can be seen in Figure 2 that the aberrant cell (AC) and micronuclei in the binucleated cell (MN/BNC) increased proportionally to the level of radiation doses. Previous research (Wolff et al., 2011) stated that an increase in chromosome damage equal to increasing radiation doses was determined using DC assay as well as at the CBMN assay.

The response of each individual to dicentric alone and micronuclei for each type of cancer is shown in Figure 3. Breast cancer had the highest dicentric and micronuclei frequencies, followed by cancer of the head and neck, and the lowest frequencies were found on extremity cancer. A previous study by Santos & Cla, (2010) found higher micronuclei frequencies observed in breast cancer patients. According to the results obtained in the present study, the results of cytogenetic endpoints often conflict due to different amounts of doses and different degrees of exposure, different individual sensitivities, and repair ability to the cytogenetic damage. However, in our study, CA and MN frequencies were higher in patients undergoing heavier treatments. Based on the methods compared separately at the individual dose levels, the single comparisons between each pair dose levels of DC or MN are described in Table 3.
Comparison of chromosome aberration in accordance with the previous studies has been linked with the various type of cancers. Most of these studies have examined the role of radiotherapy in healthy people but not in cancer patients who treated with radiotherapy. Thus, as far as we know, this is the first study of radiotherapy effects and susceptibility in patients with cancer in Indonesia. This study can be useful for researchers and related stakeholders in the application of radiotherapy.

**CONCLUSION**

The overall dicentric chromosome and micronuclei in binucleated cells have a significantly higher frequency in various patient cancer compare to healthy people. The rate of cytogenetic damage in peripheral blood lymphocytes of patients may additionally be influenced by DNA-repair ability, it is likely that neoplastic disorders themselves are correlated with increased DNA damage or that a patient’s DNA is more fragile than that of healthy people. A high frequency of micronuclei in lymphocyte patients was seen after radiotherapy treatment but relatively not much higher compare to the range of micronuclei backgrounds in healthy people. The CBMN is the most effective assay for an evaluation of the cytogenetic studies in cancer patients because it is more radiosensitive to study individual responses.

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**Table 3. Comparison of chromosome aberration DC/Cells and MN/BNC Separately for the Different Dose Levels**

| Dose (Gy) | Compare Methods | DC and MN | P |
|-----------|----------------|-----------|---|
| 10        | MN Assay       | 0.08      | 0.09 |
|           | DC Assay       | 0.015     | 0.01  | 0.06 | 0.39 |
| 12        | DC Assay       | 0.04      | 0.24 | 0.6  |
|           | MN Assay       | 0.02      | 0.21 |
| 14        | DC Assay       | 0.13      | 0.18 |
|           |                | 0.08      | 0.01  | 0.15 | 0.28 |

According to the comparison of methods in this study, we found that there was no statistically significant comparison of DC assay and MN assay in a dose range of 10-14 either in the breast, head, neck, or extremity cancer when using a T-test (p < 0.05 and p < 0.03, respectively) (Gamulin et al., 2008) reported a positive correlation between the results of the CBMN assay and the level of chromosomal damage by used DC assay for oropharyngeal cancer patients. The data showed a significant difference between the six different dates of the blood samples examined in the frequencies of DC and MN. Another researcher also studied the relationship between the results of the CBMN assay and the DC assay after in vitro irradiation of healthy individuals. The results showed similar patterns of cytogenetic biomarker distribution between donors, but differences in the response of some donors at some doses (Pajic, et al, 2015).

The evaluation study of patient samples by Garaj-vrhovac & Kopjar, (2018) demonstrated that despite the limited number of samples and variations in the patients studied, the results showed that both DC and MN assays can be used as sensitive and appropriate biomarkers in monitoring and determining the exposure to genotoxic materials as a consequence of received exposure. That research also found interindividual variability response among the cancer patients, which in accordance with the previous research. As stated by different authors in the research literature, patients with cancer often show an increased chromosomal instability compared to healthy people as shown in Table 3.

The rate of DNA damage in patients with cancers may additionally be affected by DNA repairability, it is likely that neoplastic disorders themselves are correlated with increased DNA damage. The structure of DNA in patients is more fragile than healthy people. In this study, there were also limitations to the fractionation dosages received in different types of cancer. As a result, we cannot fully determine the response of the individual to the therapeutic dose of fractionation received. To better understand this, there should be more studies done on patients who have been given the same fractionation dose and who have the same type of cancer.

The novelty of the present study is the assessment of the DNA damage response by using both Dicentric and Cytokinesis-Block Micronucleus (CBMN-assay) to measure the micronucleus frequencies and the number of chromosome aberrations as biomarkers of DNA damage response. The data were combined and were linked with the various type of cancers. Most of the previous studies have examined the role of radiotherapy in healthy people but not in cancer patients who treated with radiotherapy. Thus, as far as we know, this is the first study of radiotherapy effects and susceptibility in patients with cancer in Indonesia. This study can be useful for researchers and related stakeholders in the application of radiotherapy.
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