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A Preliminary Study on DNA Damage in Peripheral Blood of Cancer Patients After Radiotherapy with Comet Assay

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AbstractThe aim of this study was to look at DNA damage studied in cancer patients during radiotherapy using the comet test. Subjects were from patients diagnosed with different solid tumors who received radiotherapy and control groups adjusted for age, sex. Blood samples were collected from patients with several types of cancer after receiving cumulative radiation doses of 10, 12 and 14 Gy. The level of DNA damage was evaluated using the alkaline comet test on peripheral blood lymphocytes. Samples consisting of 3 ml of whole blood were collected by venipuncture. Isolation of lymphocytes was done using gradient centrifugation method. Evaluation on the capability of this compound in suppressing DNA damage was done by using alkaline Comet assay and The frequency of DNA cells was evaluated by calculating a total of 50 cells per slide. data analysis was done using CaspLab program. The comet test results showed that the average tail length, tail DNA, and tail moments of radiotherapy patients were higher than the control group and significantly different between both group (P <0.01). There is a positive correlation between radiation doses received by patient with DNA damage. The conclusion is that exposure to ionizing radiation leads to an increase in the level of DNA damage in the patient's peripheral blood lymphocytes. Meanwhile, it was found that there was inter-individual variability in response to radiotherapy among patients.

Keywords: DNA damage, cancer, radioteraphy, comet assays

I. INTRODUCTION

Cancer is a degenerative disease characterized by uncontrolled cell division (proliferation) and can cause the ability of cells to migrate to other body tissues through blood circulation or the lymphatic system (metastasis) in pathology. Cancer is a major health problem worldwide and the second biggest killer disease after cardiovascular disease. In developing countries cancer is one disease with very many sufferers. Radiation therapy is an efficient treatment for cancer [1].

Radiotherapy is a treatment aimed at shrinking mass tumors or removing residual tumor cells by exposing the tumor to ionizing radiation. Radiotherapy mostly uses X and gamma ray radiation [1,2]. Radiation besides affecting tumors also affects healthy cells around it. Ionizing radiation causes DNA damage in both direct and indirect effects. Radiation causes the formation of ROS (reactive oxygen species) which is indirectly involved in DNA damage (3,4). Radiosensitivity is the susceptibility of cells or tissues to ionizing radiation. Some patients may be more sensitive to radiation. Sensitivity results from the toxic effects of radiotherapy which results in lesions in normal tissue patients (5).

The latest literature in the field of clinical oncology and radiotherapy still does not adequately explain the ratio of damage to non-tumor cells and tissues after radiation treatment. Ionizing radiation is a proven mutagen, besides damaging tumor tissue, it also causes damage to genomes in other cell (3,4,6). Peripheral blood lymphocytes are most often monitored to see genome damage caused by radiation or other mutagens. It is important to monitor lymphocyte cells in radiotherapy patients to studying the DNA repair process in various cytogenetic biomarkers. This can indicate the individual's sensitivity to radiotherapy and the potential risk for the appearance of secondary tumors (7). Thus, cytogenetic and molecular-biological tests need to be carried out in oncological patients. This is to monitor the increase in the frequency of chromosomal aberrations and the increase in the number of micronucleus and the level of DNA damage in peripheral blood lymphocytes in oncological patients treated with radiation (6).

Comet Test is a simple, fast and sensitive visual method for measuring and analyzing DNA damage Single strand break and Double strand break. This technique is carried out at the individual cell level and requires only a small number of cells in each sample. The comet test is an electrophoresis method that estimates damage by measuring the migration of DNA fragments (8). Comet alkaline tests are reported to detect DNA damage at radiation dose limits of up to 0.6 cGy. This method provides important information about the risk of diseases associated with oxidative stress. Over the

past decade Comet alkali testing has become a popular way of detecting various types of DNA damage and its use in clinical practice has also increase (9–11).

This study, is an initial study of alkali comet test applied to survey the level of DNA damage in peripheral blood lymphocytes. Samples were collected from 10 patients of various types of cancer who were receiving radiotherapy treatment. The aim of our investigation is also to study the dynamics of radiotherapy to induce DNA lesions in non-target cells and estimate the potential value of the alkali comet test as a possible predictor of response to treatment.

II. MATERIAL AND METHOD

A. Blood Sampling.

The study was performed on peripheral blood samples obtained from ten healthy people as control and 10 cancer patient. The control donors are never exposed to ionizing radiation. The research included ten patients (four female and six male; aged 35–79 years) with solid tumors of the head and neck, Humeri+Anterabrachi, Coli/Submandibula Sinister, breasts, Detailed patient data is quoted in Table 1. Three mL venous blood were collected under sterile conditions in vacutainer tubes (Becton Dickinson, NJ, USA) containing lithium heparin as anticoagulant. After blood is taken, lymphocyte isolation is carried out using the centrifugation method.

B. Lymphocyte Isolation

Lymphocytes are separated from it using Histopaque-1077. Briefly, blood is diluted 1:1 with PBS and layered over 3 ml Histopaque and centrifuged at 1500 RPM for 30 minutes. The 'buffy' coat is aspirated into 3-5 ml of PBS and centrifuged at 1000 RPM for 15 minutes to pellet the lymphocytes. The pellet is resuspended in ~1 ml of IRPMI and counted over a Haemocytometer. Nearly 2 X 104 cells per 100 µL of medium are taken for each dose of the test material (12).

C. The Comet Assay

About 10^4 cells per 100 µL of medium was taken from each treatment for Comet assay by following the standard procedure with slightly modification. Microscopic slides were prepared. Each slide was covered with 1% Normal Melting Point (NMP) agarose (Sigma). After solidification, the slides were then coated with 0.6% NMP agarose. A Low Melting Point (LMP) agarose was melted and stabilized in a waterbath (RTE10) at 37°C. For each sample and control, 5 µL of cell homogenate was mixed with 100 µl of 1% LMP agarose and placed on the slides. After 10 minutes of solidification on ice, the slides were covered with 0.5% LMP agarose. The slideswere then immersed in a pre-chilled lysis solution ((2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl, adjust until pH 10 with NaOH (Sigma) and added 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Sigma) and kept in at 4°C for 60 minutes. The slides were placed horizontally in a humidity chamber at 37 °C for 30 minutes. All slides were then immersed in an alkali solution (0.3 M NaOH, 1 mM Na2EDTA; pH 12.1) for 40 minutes. Electrophoresis in a pre-chilled alkali solution (0.3 M NaOH, 1 mM Na2EDTA; pH 13) at 1 V/cm was done for 20 minutes in refrigerator (4°C). After electrophoresis, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove excess alkali and detergents. Each slide was fixed with methanol. Slides were stored at C in sealed boxes until analysis (12) .

D. Staining and Microscope Analysis

The stained samples with ethidium bromide were observed using a Nikon flourescence microscope. A total of 50 randomly captured comets from each slide were examined at 250x magnification using an epifluorescence microscope that connected through computerized to an image analysis. Cells were piled not counted. The image of comet was digitally analyzed using *CASPLab comet assay software* (13).

III. RESULTS.

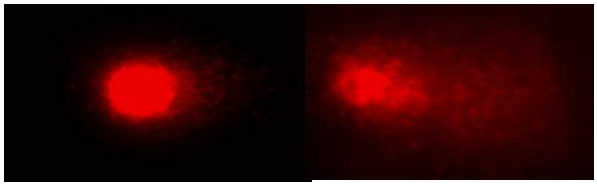
This research is a preliminary study to look at DNA damage from cancer patients after radiotherapy. Blood samples from patients with various types of cancer with different therapeutic doses. Individual data from sufferers of several types of cancer Individual data on DNA damage recorded in peripheral blood leukocytes are reported in Table 1.

TABLE 1. ANAMNESTIC AND CLINICAL DATA ON CANCER PATIENTS INVOLVED IN THE STUDY

No	Age (Year)	Gender	Cancer	Total Doses (Gy)	DNA migration/ Tail Length) (µm)
PSJ4	29	L	Sarcoma extermity	10	21,74
PSJ5	51	L	Submandibul a Sinister	14	31,32
PSJ7	21	L	Parotis	12	26,24
PSJ11	48	P	Breast	12	31,1
PSJ12	61	L	Nasopharynx	12	25,4
PSj15	68	P	Tongue	14	34,16
PSJ16	61	P	Parotis	12	29,86
PSJ17	45	P	Breast	14	31,06
PSJ22	57	P	Humeri+Ante rabrachi	10	30,02
PSJ24	48	P	Tongue	10	31,06

DNA damage in patients after radiation will be compared with DNA from control samples.

After the comet assay is carried out then an observation is carried out under the fluorescent microscope. In this research, the DNA damage of irradiated lymphocytes was assessed by comet assay by staining the cells with ethidium bromide and the comet that mainly consist of single stranded DNA can be seen with a fluorescence microscope as presented in Figure 1.



A B

FIGURE 1. VISUALIZATION OF THE RESULTS COMET ASSAYS OF DNA LYMPHOCYTE OF CONTROL (A) AND PATIENT (B).

Results of visualization showed that lymphocyte cells patient formed tail of comet than control due to DNA damage in the form of breaking one DNA strand (single strand break/SSB) and the rupture of both strands of DNA at the opposite position (double strand breaks/DSB) (Fig. 1). Denatured DNA fragments that migrate out of the cell nucleus during the electrophoresis process. The migration will form a comet's tail while the non-deposition area will form a comet head. (6,11). The DNA damage can be estimated by measuring the length of the comet tail using image analysis software.

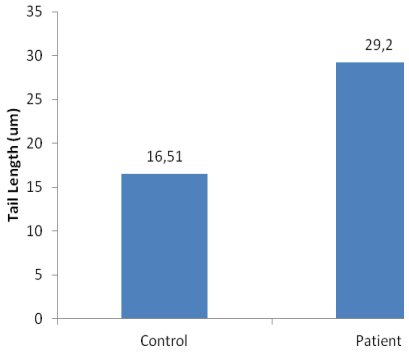


FIGURE 2. DNA MIGRATION (TAIL LENGTH) OF THE COMET ASSAY RESULTS ON CONTROL AND PATIENT SAMPLE.

The results of calculation with the Casplab software that's of the length of the comet tail (DNA migration) is controls 8.02 - 37.86 with an average value of 16.51 ± 8.81. DNA migration of patients ranged from 21.74 - 34.16 with an average of 29.2 ± 3.5. There was a significant difference between the two groups at $p < 0.05$.(Figure 2)

In this study based on the type of organ in the cancer are grouped into 3 namely, Breast, Head and neck (Submandibules Sinister, Nasopharynx, Parotid, Tongue), and extremity (Sarcoma extremity, Humeri+Anterabrachi). The results of calculation with the Casplab software that's of range and average the length of the comet tail (DNA migration) as follow, the breast cancer is 31.06 – 31 and 31.08±0,03 , Head & neck is 25,4 - 34,16 and 29,67±3,3 , extremity is 21,74 - 30,02 and 25,88±5,8 (Figure 3).

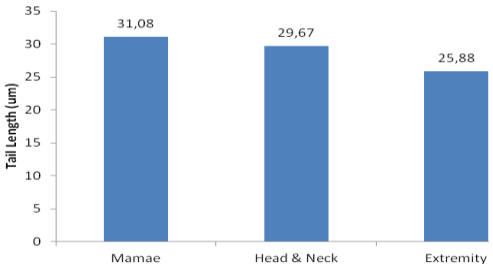


FIGURE 3. THE MEAN MIGRATION OF DNA (TAIL LENGTH) FROM THE SAMPLE COMET TEST RESULTS IN DIFFERENT CANCER GROUPS.

Post-therapy DNA damage in peripheral blood leukocytes in cancer patients shows differences between groups that the mean DNA migration is highest in breast cancer patients and the lowest in extremity. DNA migration in groups may be affected by large doses. The dose of radiotherapy in the extremity group was 10 Gy, in the head and neck group ranged 10-14 Gy and the dosage range in the breast group was 12-14 Gy. This shows that DNA migration in groups can be affected by large doses of radiotherapy

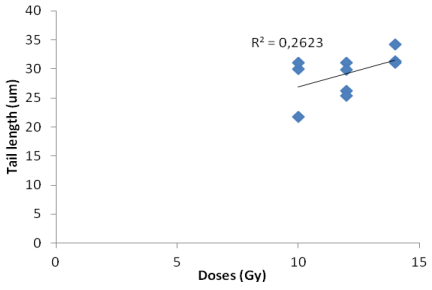


FIGURE 4. THE RELATIONSHIP BETWEEN DOSES RECIEVED PATIENT WITH DNA DAMAGE WITH CORRELATION COEFICIENT (R2).

The fractionation dose range that's received by patients is 10-14 Gy. The effect of doses on DNA damage is shown on the correlation curve between doses with a tail length value (Figure 4). The curve shows a positive correlation between the doses and DNA damage with a value of R2 = 0.2623

The curve relations curve between doses with a tail length value shows a positive correlation. This shows that the greater the radiation dose received by the patient will result in increased DNA damage in the patient.

IV. DISCUSSION

As observed in our study, radiotherapy critically affected the level of DNA damage in cancer patients, as detected by the alkali comet test. In the results of the study above the DNA damage patients was significantly higher than the control. This shows DNA damage in patients receiving radiotherapy. The same study was also carried out by Gamulin et al. in 10 patients with different types of cancer. Showed a significant increase in TL values in 6 patients after radiotherapy (14). Higher radiation received the greater the DNA damage that occurs. DNA damage in patients receiving the same dose shows varying DNA (TL) migration values. This shows that there is an inter-individual variation of course related to age and some lifestyle factors (especially smoking habits and alcohol consumption), as well as previous medical measures, or related to inherited biological factors.

Radiotherapy is a major part of the treatment of cancer patients. This can be used as the main therapy, but often combined with surgery and chemotherapy. Radiotherapy works by damaging the cancer cells of DNA, therapeutic interventions cause inevitable exposure of non-target cells to patients (3,14,15). Because only a portion of the population treated will develop secondary cancer so that the patient's biomonitoring after therapy becomes very important (1,9,16).

V. CONCLUSION.

Radiotherapy is accompanied by significant DNA damage in peripheral blood lymphocytes. DNA damage has a positive correlation with the dose received by the patient.

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