ARAŞTIRMA

F.Ü.Sağ.Bil.Tıp Derg.
2009: 23 (1): 15 - 20
http://www.fusabil.org

The Frequency of Micronuclei and Morphological Effects on White Blood Cells Following Radiotherapy

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In this study, we tried to define the frequency of micronuclei (MN) and morphological damages in peripheral *blood samples* of cancer patients exposed to radiotherapy (RT) for *lung* tumors. For this purpose, we used in vivo cumulative dose-effect relationship, and correlated these data with statistical parameters. Cytological analyses were performed on white blood cells (WBC) of patients exposed to γ–radiation. MN assay was used as a biomarker of radiation damage. Besides, the effect of different factors like smoking status and age was investigated by MN assay. Scoring of MN was performed on mononucleated lymphocytes. Peripheral *blood smears* were *prepared* for *morphological* characterization and the number of WBC. The results indicated that, morphological damages and MN frequency in WBC were significantly higher in cancer patients exposed to γ–radiation than in the controls, and difference was statistically significant (P<0.01). The morphological changes such as vacuolization, membrane defects and increase in cytoplasmic granulation were detected in WBC. Moreover, an increase in the frequency of MN and a decrease in the numbers of WBC were observed depending on radiation dose. In conclusion, it was showed that morphological damages and MN frequency are very sensitive and useful biomarkers for the investigation of the effects of RT.

***Anahtar Kelimeler*:** *Gamma radiation, lung cancer, micronuclei, morphological damage, radiotherapy.*

Radyoterapiyi Takiben Beyaz Kan Hücrelerindeki Morfolojik Etkiler ve Mikronukleus Sıklığı

Bu çalışmada, akciğer tümörleri nedeniyle radyoterapiye (RT) maruz kalan kanser hastalarının periferal kan örneklerindeki morfolojik hasarları ve mikronukleus (MN) sıklığını belirlemeye çalıştık. Bu amaçla, in vivo kümülatif doz-etki ilişkisini kullandık ve bu verileri istatistiksel parametreler ile ilişkilendirdik. Sitolojik analizler, γ–radyasyonuna maruz kalan hastaların beyaz kan hücrelerinde (WBC) gerçekleştirildi. MN testi, radyasyon hasarının bir belirteci olarak kullanıldı. Ayrıca, sigara alışkanlığı ve yaş gibi farklı faktörlerin etkileride MN testi kullanılarak araştırıldı. MN sayımı, tek nükleuslu lenfositlerde gerçekleştirildi. WBC'nin sayısı ve morfolojik özelliklerini belirlemek amacıyla periferal kan yayma preparatları hazırlandı. Sonuçlar, γ–radyasyonuna maruz kalan kanser hastalarının WBC'deki MN sıklığı ve morfolojik hasarların, kontrol grubundakilerden oldukça yüksek olduğunu gösterdi ve fark istatistiksel olarak önemliydi (P<0.01). WBC'de voküolizasyon, membran kusurları ve sitoplazmik granülasyonda artış gibi morfolojik değişmeler belirlendi. Ayrıca, radyasyon dozuna bağlı olarak MN sıklığında bir artış ve WBC'nin sayısında ise bir azalma gözlendi. Sonuç olarak, morfolojik hasarlar ve MN sıklığının, RT 'nin etkilerini araştırmak için çok hassas ve kullanışlı biyolojik belirteçler olduğu gösterildi.

Key Words: *Gama radyasyonu, akciğer kanseri, mikronukleus, morfolojik hasar, radyoterapi.*

Introduction

Radiation has many applications in medicine, communication, agriculture and domestic life (1). Therefore, biological effects of radiation have been investigated for a long time by a large team of scientists. Some biological effects of radiation are cell death, changes in cell function, delayed mitosis, disruptions in cell growth, membrane permeability changes and organelle damage (2). These effects of radiation are basically similar for different kinds and doses of ionizing radiation (3–5). WBC are the most sensitive biological indicator of radiation exposure. Nevertheless, these sensitivities have been confirmed for many cell types, including ovary cells, bone cells, fibroblasts, cartilage cells and nerve cells (6).

One well-understood effect of radiation is related to MN formation. MN is originated from acentric chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during metaphase or anaphase phase of cell division (7). They reflect chromosome damage and may thus provide a marker of early-stage carcinogenesis (8–10). Moreover, MN test can be used to show both clastogenic and aneugenic effects. A number of studies have been designed to evaluate the potential influence of factors such as gender, age, smoking habit and radiation on the MN frequency (9). It has been shown that factors such as age, smoking habit, alcohol, genotoxic agents, chemical substances and radiation had a remarkable effect on the frequency of MN (11). MN test can be performed for different cell types such as

Geliş Tarihi : 03.11.2008
Kabul Tarihi : 05.01.2009

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lymphocytes, fibroblast and epithelial cells (12). Consequently, MN formation is a reliable biomarker of exposure to radiation (13–15).

The aim of the present study was to evaluate the effects of γ–radiation on morphology of WBC and MN formation in patients with lung cancer receiving RT.

Materials and Methods

***Patients and Treatment*:** The present study was carried out on 20 patients treated for lung cancer from November 2004 to July 2006 in Ankara University Andicen Polyclinic of Dr. Abdurrahman Yurtarslan Research Hospital. The patients were randomly selected among smokers. Of these patients, seventeen were males and three were females. The mean age of the patients was 53.5 ± 2.8 years (range 45–60). All patients who were heavy smokers smoked more than 20 cigarettes per day. *All* 20 *patients had smoked* for at *least* 20 *years* without interruption. Besides, sixteen patients were current cigarette smokers. Patients did not receive any chemotherapeutic drugs *during* the 5–week *period* of RT. Besides, sixteen male and four female healty non-smoker persons were examined as control group. Table 1 shows the features of subjects used in this study.

Table 1. Cancer types in subjects included in the study

Lung cancer types	Male patients	Female patients
Small cell Lung cancer	10	1
Adenocarcinoma	4	0
Large cell lung cancer	1	2
Squamous cell lung cancer	2	0
Healthy controls	14	6

***Ethical standards*:** In this study, the methods and techniques applied to patients were carried out according to ethical standards of the local ethical committee of Abdurrahman Yurtarslan Research Hospital (Protocol date: 27.10.2005) and favorable to the guidelines set by the World Health Organization (Geneva, Switzerland). Each patient read and signed an informed–consent form before their participation in the study. This permission is always for the analysis and collection of blood samples from patients, and is not used for purposes other than those for which consent was originally given.

***Radiation Treatment*:** Radiation procedure was carried out using "ATC Cobalt 60 SSD=80 cm" instrument. The dose equivalents *were calculated* and compared to the doses recommended by International Commission on Radiation Protection (ICRP). Totally 20 patients were treated with 10 Gy γ**–**radiation for five weeks in 50 Gy total dose. Radiation was applied to thorax area during 30 min. Cytogenetical analyses were performed on WBC of patients exposed to γ**–**radiation.

***Collection of Blood Samples*:** Peripheral blood samples were collected from 20 patients and 20 healthy donors before and after RT. Blood samples were taken from one of the veins of arm of each individual. Three milliliters (3.0 ml) of venous blood was obtained from each individual, evacuated into sterile heparinized tubes and transported within 3 h to the laboratory.

***MN Assay*:** In the absence of cytochalasin B, mononucleated cells were analyzed for the presence of MN. Peripheral WBC were isolated from heparinized blood tubes. 5 µl drop of blood was spread on a clean glass slide, then cells on slide were fixed with 70% methanol (10 min.) and stained with the May-Grünwald Giemsa stain (5%). From each donor, 1000 cells were examined under a binocular light microscope (Japan, Nicon Elipse E600) at X100 magnification and MN cells were photographed at a magnification of X500. For the scoring of MN the following criteria were adopted from Fenech et al (16). i: The diameter of the MN should be less than one-third of the main nucleus, ii: MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary, iii: MN should have similar staining as the main nucleus.

***Peripheral* Blood Smear *Test*:** Blood smear slides have been prepared for provide information about the number of WBC and determine of the morphological damages in WBC of patients. Slides were formed from a small drop of blood (5µl). Then slides were fixed in 70% ethanol (10 min.) and stained by the May-Grünwald Giemsa stain (5%) for 30 min. Slides were air dried over night at room temperature (25°C). Dried slides were examined using the same microscope (Japan, Nicon Elipse E600).

***Statistical Analysis*:** "Paired Samples T-Test" was used while comparing the morphological damages and the frequency of MN between patients exposed to γ**–** radiation and the controls. *Differences* were *considered significant* if the *P value was* less than 0.01.

Results

Small cell lung cancer was diagnosed in 11 (55%) patients, adenocarcinoma in 4 (20%), large cell lung cancer in 3 (15%) and squamous cell lung cancer – in 2 (10%) patients. Stage I cancer was diagnosed in 2 (10%), stage II in 5 (25%), and stage III and IV in 13 (65%) patients.

In this study, a significant difference was observed in the morphologies of WBC of cancer patients receiving RT when compared to the controls. Detailed information about the morphological alterations in WBC and the MN frequency following RT was showed in Figure 1–5 and Table 2. The morphological changes such as dense-vacuolization and deformity (Figure 1a,b), membrane defects (Figure 2a,b), increase of cytoplasmic granulation (Figure 3a,b), dead cells (Figure 4a,b) and MN formation (Figure 5a,b) were observed in WBC. Moreover, the MN frequency was compared with WBC counts. There were a significant increase in the *frequency* of MN and a significantly reduction in peripheral blood WBC count *during RT* (Table 2). In all patients, the results showed that the highest frequency of

MN was observed at the end of the 5th week of RT and *the lowest frequency* of MN was observed before the initiation of RT (Table 2). We also found a significant increase in the MN frequency in lung cancer patients after and before RT compared with the controls.

Table 2. The frequency of *MN* and *WBC* in the peripheral blood of lung cancer patients receiving *RT*

Parameters	Before RT	First week	Second week	Third week	Fourth week	Fifth week
MN	3.65	8.70	13.25	17.15	19.35	22.80
SD	±2.94	±4.19	±4.77	±5.01	±5.62	±6.29
P	+	+	+	+	+	+
WBC	5247.25	4992.55	4767.75	4601.25	4426.50	4290.20
SD	±311	±286	±274	±235	±286	±267
P	–	–	+	+	+	+

*(+) P<0.01 significant, (–) insignificant.

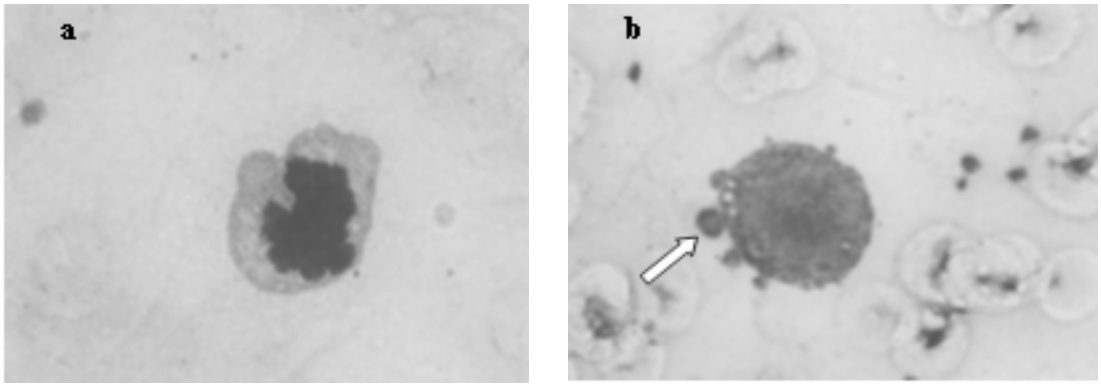

Figure 1. The appearance of deformation (a) vacuole formation (b) in leukocyte cells (X500)

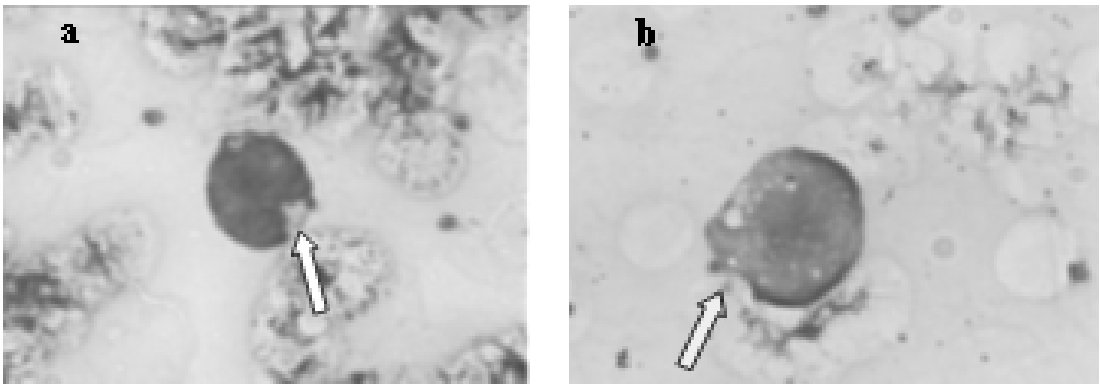

Figure 2. The appearance of cell membrane damages in leucocytes (a,b X500)

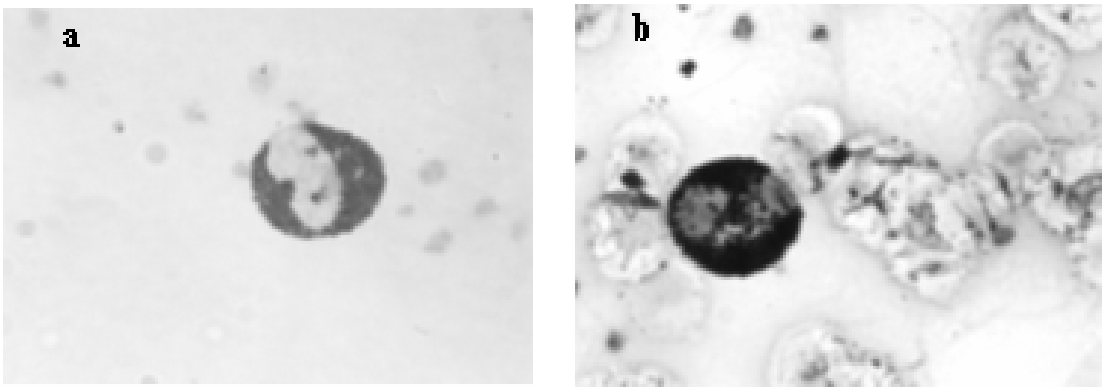

Figure 3. The appearance of dense granulation in leukocyte cytoplasm (a,b X500)

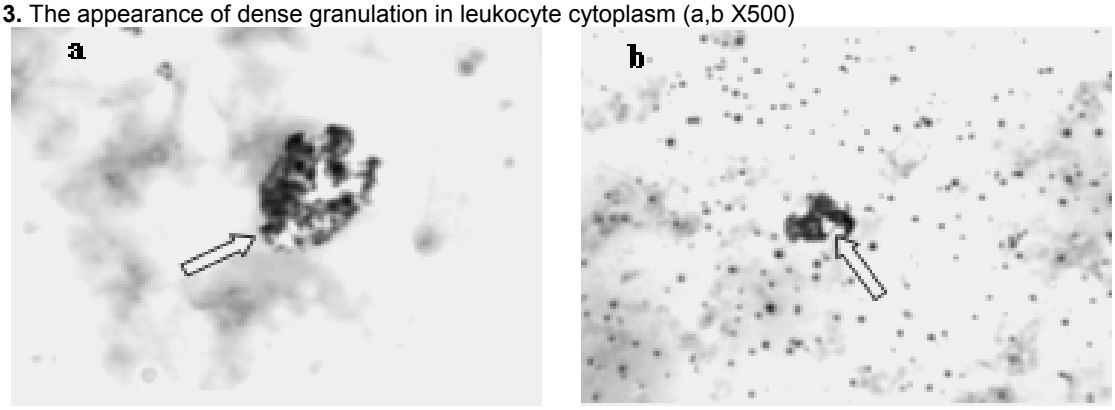

Figure 4. The appearance of dead cells (a,b X500)

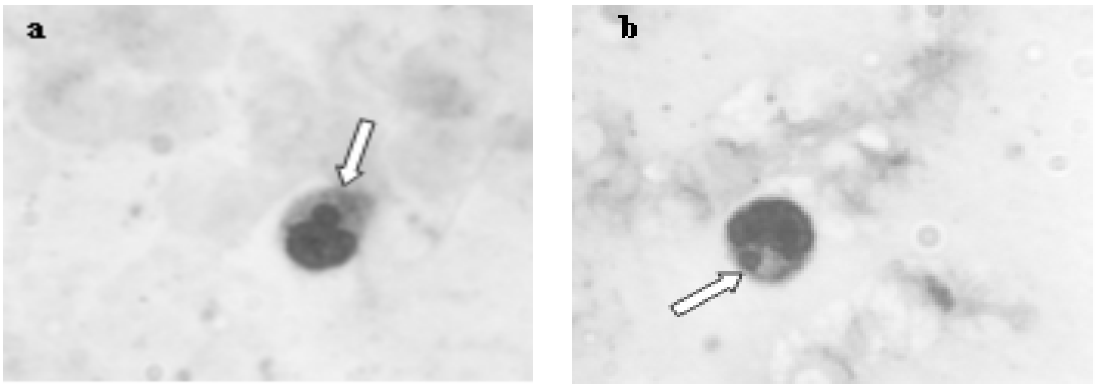

Figure 5. The appearance of micronucleus (a,b X 500)

Discussion

In this study, we investigated the morphological alterations in WBC of patients with lung cancer receiving RT. Histologically, small cell lung cancer was predominant (11 cases). Because, small cell lung cancer comprises 15–20% of all malignant lung tumors and in the majority of cases the tumor has disseminated or is locally advanced at the time of diagnosis and thus the rate of small cell cancer type is higher than the other lung cancer types**.** The exposure to γ–radiation caused some damages in WBC. These damages were observed in WBC, whereas such changes were not observed in erythrocyte and thrombocyte cells with light microscope. These damages might be due to direct exposure to radiation of cell or free radicals generated by γ**–**radiation. As a result, these free radicals cause breakage of chemical bonds, cross-linking and conformational changes in cellular components as lipid, protein and enzyme. Thus, these changes may affect the molecule's biological function and structure. Moreover, this structural

damage can be explained by elevated temperature produced by radiation (17).

In our study, the MN frequency was also compared with WBC count. The MN frequency showed an increase during RT and WBC counts decreased significantly during RT. In all patients, the results showed that the highest frequency of MN was observed at the end of 5^{th} week of RT and the lowest frequency of MN was observed before the initiation of RT. Moreover, the MN frequency in WBC was also elevated in cancer patients before RT when compared with the controls, and difference was statistically significant. Although these patients were not exposed to γ–radiation before RT, MN was observed in prepared slides. This result may be related to the *patients*' age, chemotherapeutic drugs taken before RT and cigarette smoking in particular. The effect of cigarette smoking on MN frequency was reported in many of the biomonitoring studies (18–20). Besides, MN level was showed greater frequency in the elderly control group (non-smokers) with a mean age of 52.6 ± 2.9 years than the young controls with a mean age of 23.5 ± 1.3 years (non-smokers). MN formation was not observed in the young controls, but a low frequency of MN (0.05%) was determined in the elderly controls. The observation of MN in these healthy persons without RT and chemotherapy clearly indicates that MN formation is related to donors' age. These findings suggest that age can influence the formation of MN in WBC. The effect of aging on spontaneous MN frequency has been reported by various authors (11,12,14,21–23). Higher frequencies of MN have previously been reported in females than in males (24). However, the effect of *sex difference* was *observed* in our study.

If we consider the similar studies, our results are found to be in close agreement with previously published data. For example, Anna et al. (25) investigated the level of cytogenetic damage in peripheral blood lymphocytes of patients undergoing chemotherapy. As a result, they showed that the highest level of cytogenetic damage was observed at the end of therapy. They determined the frequencies of increased MN during the first half of therapy and declined thereafter. Moreover, they observed the leukocyte count strongly decreased at the beginning of therapy with an upward trend at the end. Boreham et al. (26) reported the relationship between radiation dose and radiation-induced the apoptosis and MN formation. They found that apoptosis and the MN frequency decreased in low dose rate of radiation, but apoptosis and the MN frequency in binuclear cells increased with increasing of applied radiation dose. Hubert et al. (27) used MN test to determine the effects of radiation on 99 workers studied in Belgium Doel Nuclear Center. They reported an increase in the frequency of MN with increase in the annual exposure to radiation. In another study, MN formation and cell proliferation in human lymphocytes exposed to 50 Hz magnetic fields for 72 h was investigated. As a result, 50 Hz magnetic fields have no effect on MN formation, and a significant increase in *cell proliferation was not observed* (28). Widel et al. (29) investigated the frequency of MN in peripheral blood samples were taken before and after RT in patients with cervical cancer. As a result, they reported a significant increase in the MN frequency compared with the controls. Maes et al. (3) reported effect of 2450 MHz microwave on the MN frequency in human blood lymphocytes in vitro. They determined an increase in the MN frequency with increasing of exposure duration. Rosin and Gilbert (30) investigated the modulation of genotoxic effects of some chemical agents in humans by using MN test as a biomarker. Besides, Stich and Rosin (31) reported the MN in exfoliated human cells as a tool for studies in cancer risk and intervention.

In conclusion, RT has a lethal effect on cancer cell. However, it may cause severe structural and genotoxic damages on healthy cells and tissues such as blood. These damages may also induce other disease such as leukemia and anemia (32). Therefore, effects of RT applications on healthy cells and tissues must be minimized or alternative methods should be developed.

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