Can micronucleus technique predict the risk of lung cancer in smokers?

Ebru GÜREL[1], Tuncay ORTA[1], Süreyya GÜNEBAKAN[1], Ayfer UTKUSAVAŞ[2], M. Özlem KOLUSAYIN OZAR[1]

[1] İstanbul Üniversitesi Fen Fakültesi, Biyoloji Bölümü,
[2] İstanbul Üniversitesi İstanbul Tıp Fakültesi, Göğüs Hastalıkları Anabilim Dalı, İstanbul.

ÖZET

Mikronükleus tekniği sigara içen bireylerde akciğer kanseri riskini önceden belirleyebilir mi?

Akciğer kanserinin karsinogenez sürecinde ana etyolojik faktör, sigara içimidir. Sigara içenlerde oksidaz enzimi seviyelerindeki artış veya kromozom aberasyonları gibi tanımlı genetik faktörlerin, akciğer kanserine daha yüksek derecede yakalanma olasılığı ile ilişkide olduğu gösterilmiştir. Bu çalışmada, uzun süreli sigara tüketen akciğer kanseri tanısı almış olan ve uzun süreli sigara tüketen ancak akciğer kanseri olmayan bireylerin periferal kan lenfositlerindeki, radyasyon sonrası mikronükleus (MN) tekniği ile ölçülen kromozom aberasyonları araştırılmıştır. Amacımız, sigara içicileri arasında kanser gelişimi olabilecek, kansere yakalanacak bireylerde MN skorlamasının rolünü araştırmaktır. Oniki akciğer kanseri hastası ve bunlara uygun 10 sağlıklı birey değerlendirildi. Spontan ve radyasyon indüklü bireylerin MN frekansları değerlendirildi. Spontan frekanslara bakıldığında hem hasta hem de sağlıklı bireylerde 3 Gy radyasyon sonrası MN miktarlarında artış gözlendi. Radyosensitivitenin belirleyicisi olan mutlak MN frekansları, 3 Gy radyasyonda oluşan MN frekanslarından, spontan MN frekansları çıkarılarak hesaplandı. Akciğer kanseri hastalarında değerler, 0.0116 ve 0.3883 arasında, 0.1114 ± 0.0390 (SE) ile dağılım gösterirken, kontrollerde bu değerlerin, 0.0216 ve 0.2291 arasında ve 0.1410 ± 0.0234 (SE) ile dağıldığı görüldü. Her iki grubun mutlak MN frekansları arasında karşılaştırma yapıldığında, iki grup arasında anlamlı (p= 0.159) fark saptanmadı. Sonuç olarak, çalışmamızda, uzun süreli sigara içicilerinin periferal kan lenfositlerindeki MN skorları, akciğer kanser riskini önceden belirleyememektedir.

Anahtar Kelimeler: *Mikronükleus, akciğer kanseri, karsinojenik ajanlar, radyasyon, lenfositler.*

SUMMARY

Can micronucleus technique predict the risk of lung cancer in smokers?

Gurel E, Orta T, Gunebakan S, Utkusavas A, Kolusayin Ozar MO

Department of Biology, Faculty of Science, Istanbul University, Istanbul, Turkey.

Smoking is the main etiological factor in the carcinogenesis process of lung cancer. But genetically defined factors such as increased levels of oxidase enzymes or chromosome aberrations have been shown to correlate with the higher possibility of contracting lung cancer among smokers. In this study, chromosome aberrations measured by micronucleus (MN) tech-

Yazışma Adresi (Address for Correspondence):
Ebru GÜREL, İstanbul Üniversitesi Fen Fakültesi, Biyoloji Bölümü, Vezneciler, İSTANBUL - TURKEY
e-mail: ebrugurel@email.com

nique following in vitro irradiation were investigated in peripheral blood lymphocytes of long term smokers with or without lung cancer. Our aim is to establish the role of MN scores in identifying the individuals who might develop cancer among smokers. Twelve lung cancer patients and appropriately matching 10 healthy controls were evaluated. Spontaneous and radiation induced MN frequencies were evaluated in the two groups. An increase in the amounts of MN after 3 Gy irradiation was observed in the patient and control group when compared to spontaneous frequencies. Absolute MN frequencies as a determinant of radiosensitivity were calculated by subtraction of spontaneous aberration frequencies from the frequencies that were obtained following 3 Gy of irradiation. Absolute MN frequency range was between 0.0116 and 0.3883 with the average value of 0.1114 ± 0.0390 (SE) for the lung cancer patients, and was between 0.0216 and 0.2291 with the average value of 0.1410 ± 0.0234 (SE) for the controls. When the comparision was made between the absolute MN frequencies of both groups, there was no difference (p=0.159) between the two groups. In our study, it can be concluded that radiation induced MN scores in peripheral blood lymphocytes of long term smokers do not predict the risk of lung cancer.

Key Words: *Micronucleus, lung cancer, carcinogenic agents, irradiation, lymphocytes.*

Lung cancer is the most common type of all cancers in men and also in women, exceeding the rate of deaths due to breast cancer in some countries (1,2). Age is the most important factor in countries where the incidence of lung cancer is rather high (2,3). Environmental pollution and occupational hazardous conditions play a certain role in the development of lung cancer. But the main etiological factor in developing lung cancer is cigarette smoking (4). Smoking is 90% responsible for the whole lung cancer cases. The age of starting to smoke cigarettes, the amount of cigarettes smoked during a day and total amount of period spent by smoking cigarettes increase the risk of cancer. Lung cancer death rates in smokers may vary between 15-20 folds depending on the total amount of cigarettes smoked (2). Therefore lung cancer is largely considered as a preventable disease.

Damages to the DNA molecule by carcinogenic substances and/or reactive intermediate agents due to various metabolic processes within the lung cells during carcinogenesis are considered important. More than 4000 chemical substances such as acetone, benzene, benzopyrene, cyanamid, methane that are found in cigarettes are carcinogenic (2). Levels of nitrate proteins are higher in patients with lung disease when compared with healthy controls (5). Increased levels of aryl hydrocarbon hydroxylase (AHH) and its metabolic phenotype are related with the risk of lung cancer (6,7). By determining the genetic variations that designate the differences in response to carcinogenic agents because of alterations in the human metabolism, it may now be possible to determine the risk of lung cancer in individuals earlier (8). The levels of oxydase enzymes and the levels of chromosome damage in long term smokers with lung cancer have been shown to be higher than the levels in long term smokers without lung cancer (5). Chromosomal anomalies have also been shown to be by far more frequent in smoking lung cancer patients (9).

The aim of this study was to investigate the differences in the amounts of chromosome damage after 3 Gy of in vitro irradiation in peripheral blood lymphocytes of both lung cancer patients and healthy individuals with a long term smoking history. Chromosome damage was evaluated by using micronucleus (MN) assay (10). MN can be identified as a chromosome fragment without a centromere or a whole chromosome which is not properly segregated during mitosis.

MATERIALS and METHODS

Twelve patients with lung cancer diagnosis and 10 non-cancer individuals (control group) who had history of 40 package/year cigarettes were involved in this study. Written concent was taken for each patient. Peripheral blood samples were collected by venous puncture from both patients and controls before starting prescribed therapy. For each patient and control individual, samples were drawn into two seperate 4.5 mL sterilized lithium-heparin tubes. One tube was left unirradiated (control) and the other one was irradiated with 3 Gy of Co-60 gamma irradiation, performed by using Alcyon II Co-60 teletherapy machine at 1.17 Gy/min.

Microculture technique was applied to blood lymphocytes (11). Briefly, 0.5 mL of either non-irradiated or irradiated whole blood was added to culture medium containing 15 µg/mL phytohemagglutinin (Sigma), 1 mL Newborn Calf Serum and 4 mL of RPMI-1640 with glutamine (Sigma) supplemented with 100 µg/mL streptomycin and 100 IU/mL penicillin, and incubated at 37°C for 45 hours. After adding cytochalasin-B (Sigma; 6 µg/mL) solution, cells were left for incubation for another 24 hours. At the end of the 69th hour, cells were treated with hypotonic KCl solution (0.075 M). They were then washed and fixed three times with methanol/acetic acid (7/1) solution. Cell suspension that was left at the end of the third wash was dropped on glass microscope slides which were in turn stained by 5% Giemsa solution (prepared in phosphate buffer) for 10 minutes. Binucleate cells containing two identical nuclei were considered for evaluation, by examining under the light microscope at 400 magnification. Any detached nuclei with no bigger than 1/3 in diameter of a parent nucleus were scored as micronucleus. In the patient group, 11950 BN cells for 0 Gy and 7638 BN cells for 3 Gy, and in the control group, 9465 BN cells for 0 Gy and 9472 BN cells for 3 Gy were counted.

Paired t-test was applied for the comparision of spontaneous and radiation induced micronucleus frequencies for each individual in both patient and control groups. In order to compare two groups, non-parametric Mann Whitney U-test was used.

RESULTS

The spontaneous, 3 Gy radiation induced and absolute micronucleus frequencies in lymphocytes of lung cancer patients and controls are given in Table 1. Absolute MN frequency as a determinant of radiation sensitivity was calculated by subtraction of non-irradiated MN scores (0 Gy) from 3 Gy irradiated MN scores.

The mean frequency (± SE) of spontaneous micronuclei was 0.0131 ± 0.0024 (ranged between 0.0045 and 0.0288) in the patient group while the mean (± SE) in the control group was 0.0291 ± 0.0096 (with the range of 0.0071-0.0927). Increases in MN scores were observed after 3 Gy in vitro radiation. These increases were statistically significant in the patient group with $p= 0.016$ and in the control group with $p< 0.001$ compared to their relevant spontaneous frequencies. Absolute MN frequencies which determine the cellular radiosensitivity ranged between 0.0116 and 0.3883 with the average value of 0.1114 ± 0.039 (SE) for lung cancer patients and ranged between 0.0216 and 0.2291 with the average value of 0.1410 ± 0.0234 (SE) for the controls.

Radiosensitivity ranges of two groups were compared in Figure 1, in terms of MN induction. When absolute MN frequencies of both groups were compared with each other using the Mann-Whitney U-test, it was observed that there was no significant difference between the two groups ($p= 0.159$).

Observed binucleate cells and micronuclei are shown in the following photographs (Figure 2-4).

DISCUSSION

Metabolism of carcinogenic agents is important in cigarette smoke induced lung cancer. Oxidase enzymes that are genetically determined are an essential part of this metabolism. There are differences in the levels of oxidase enzymes between long term smokers with lung cancer and long term smokers without lung cancer. Because of this genetically determined variation, only about %20 of long term smokers contract lung cancer. Oxidant agents that are present in cigarettes and/or that form in the lungs of smokers can initiate carcinogenesis action in cells due to oxidative and nitrative damages in DNA and in cellular combinations (12). Cigarette smoking accelerates oxidative stress and therefore, the formation of reactive nitrogen derivations during the development of lung cancer can result in a nitration and oxidation process of plasma proteins. Namely, the levels of oxidized proteins are higher in smokers when compared with non-smokers.

Homeostatic factors determining these genetic variations may also be effective in a variable response to radiation-induced damage. In this study, therefore, MN assay was used as a biomarker of chromosomal damage in PBL of long term smokers in order to identify cancer phenotype. Scoring micronuclei in binucleate cells is easy to

Table 1. Micronucleus frequencies belonging to the patients and controls.

	MN frequencies (MN/BN)		
	Spontaneous (0 Gy)	**3 Gy induced**	**Absolute (3-0 Gy)**
Patient group			
1.	0.0288	0.0545	0.0257
2.	0.0045	0.0244	0.0199
3.	0.0235	0.0626	0.0391
4.	0.0141	0.4024	0.3883
5.	0.0120	0.1824	0.1704
6.	0.0051	0.0170	0.0119
7.	0.0127	0.0500	0.0373
8.	0.0092	0.0788	0.0696
9.	0.0092	0.0208	0.0116
10.	0.0088	0.0369	0.0281
11.	0.0246	0.3836	0.3590
12.	0.0048	0.1806	0.1758
Control group			
1.	0.0132	0.0837	0.0705
2.	0.0156	0.1420	0.1264
3.	0.0071	0.2362	0.2291
4.	0.0105	0.2361	0.2256
5.	0.0767	0.2164	0.1397
6.	0.0276	0.0492	0.0216
7.	0.0927	0.2552	0.1625
8.	0.0073	0.0559	0.0486
9.	0.0165	0.1961	0.1796
10.	0.0235	0.2303	0.2068

MN: Micronucleus, BN: Binucleate cell.

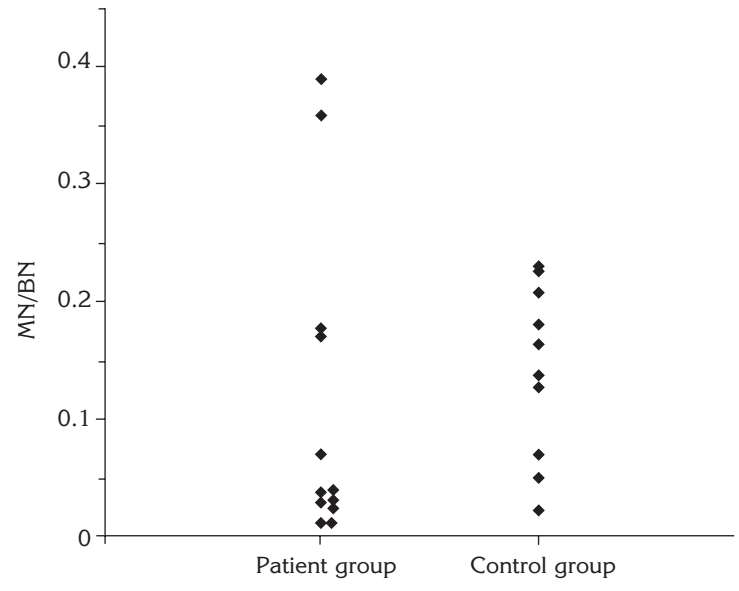

Figure 1. Comparison of absolute MN frequencies between the patient and control group.

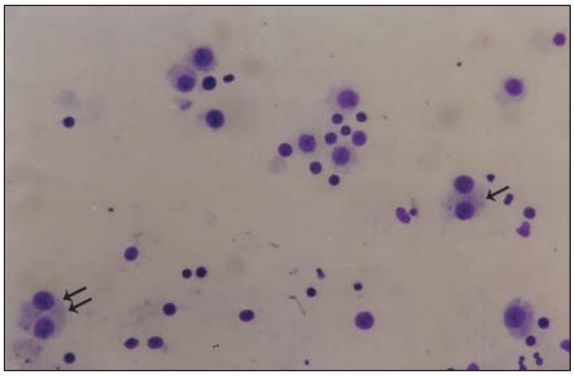

Figure 2. A group of binucleate cells (↑), some containing MN formation (↑↑) x600.

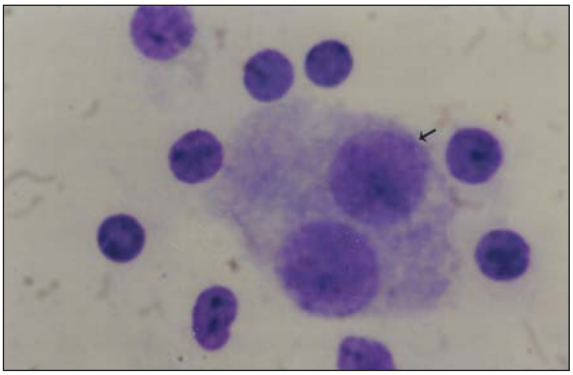

Figure 3. A binucleate cell (↑) x1500.

perform and therefore widely used in the studies concerning radiation cytogenetics and cytogenetical monitoring of chemical agents (13-15).

Spontaneous and radiation induced micronuclei in binucleate cells obtained from lung cancer patients and matching controls were scored. MN frequencies induced by 3 Gy irradiation in both groups showed a significant level of increases when compared to spontaneous aberrations in non irradiated lymphocytes (Table 1).

Two out of 12 patients were female (numbered 8 and 11). MN frequencies of these female patients remained in the related frequency intervals of the patient group. Therefore female individuals were evaluated as a usual member of the patient group. When the two groups were compared about spontaneous MN frequency, it was observed that MN frequency in the patient group (mean: 0.0131) was not significantly different (p= 0.147) from the control group (mean: 0.0291) (Table 1).

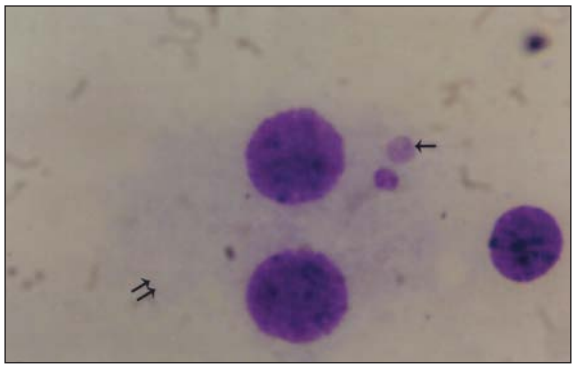

Figure 4. Two MN (↑) in a binucleate cell (↑↑) x1500.

It may be suggested that, spontaneous levels of MN formations may give false results as a consequence of differences in the exposure times and the processes of genotoxic agents in each individual. Therefore, giving a stress inducing agent in a controlled environment may differentiate individual's response depending on differences in their genomic structural integrity and/or DNA repair mechanisms. Radiation was used as a stress inducing agent in this study. But there was also no difference (p= 0.159) between absolute MN frequencies as a determinant of radiosensitivity in the two groups (Figure 1). This indicates that there were quantitative similarities in the radiosensitivity range measured by MN assay.

As a result, our study suggest that MN technique applied to lymphocytes did not appear to be a predictive approach to determine genetic variations between lung cancer patients and control individuals with both having a long term cigarette smoking history.

Acknowledgements: *This work was supported by the Research Found of Istanbul University (Project No: ÖR-121/17072000).*

REFERENCES

1. *Franks LM, Teicht NM. Cancer. In: Franks LM, Teicht NM (eds). Introduction to the Cellular and Molecular Biology of Cancer. New York: Oxford University Press, 1986: 63.*
2. *Ece T, Kılıçaslan Z. Lung cancer. In: Arseven O (ed). Lung Diseases. İstanbul: Nobel Medical Bookstore, 2001: 303-15 (in Turkish).*
3. *Sethi T. Lung cancer. Thorax 2002; 57: 992-3.*

4. *Mitchell G, Mitchell C. Lung cancer. Aust Fam Physician 2004; 33: 321-5.*
5. *Pignatelli B, Li CQ, Bofetta P, et al. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. Can Res 2001; 61: 778-84.*
6. *Yoshikawa M, Arashidani K, Kawamoto T, Kodama Y. Aryl hydrocarbon hydroxylase activity in human lung tissue: in relation to cigarette smoking and lung cancer. Environ Res 1994; 65: 1-11.*
7. *Kiyohara C, Ohno Y. Role of metabolic polymorphisms in lung carcinogenesis. Nippon Koshu Eisei Zasshi 1999; 46: 241-9.*
8. *Au W, Oh HY, Grady J, et al. Usefulness of genetic susceptibility and biomarkers for evaluation of environmental health risk. Environ Mol Mutagen 2001; 37: 215-25.*
9. *Helal SM, Mohamed HA, Shaymaa HA, et al. The effect of smoking on human chromosomes. In: Seed Journal Publishes, 2001.*
10. *Fenech M. The cytokinesis-block micronucleus technique. Mutation Research 1993; 285: 35-44.*
11. *Moorhead PS, Nowell PC, Mellmann WJ, et al. Chromosome preparations of leukocytes cultured from human peripheral blood. Exp Cell Res 1960; 20: 613-6.*
12. *Slonina D, Gasinska A. Intrinsic radiosensitivity of healthy donors and cancer patients as determined by the lymphocyte micronucleus assay. Int J Radiat Biol 1997; 72: 693-701.*
13. *Muller WU, Streffer C. Micronucleus assays. In: Obe G (ed). Advances in Mutagenesis Research. Berlin: Springer-Verlag, 1994: 1-134.*
14. *Fenech M. Important variables that influence base-line micronucleus frequency in cytokinesis-blocked lymphocytes-a biomarker for DNA damage in human populations. Mutat Res 1998; 404: 155-65.*
15. *Kirsch-Volders M, Sofuni T, Aardema M, et al. Report from the in vitro Micronucleus Assay Working Group. Environ Mol Mutagen 2000; 35: 167-72.*