RESEARCH ARTICLE

Relationship between Malignant Melanoma and Chromosome Damage in Human Peripheral Blood Lymphocytes

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Abstract

The incidence of malignant melanoma increases with age. One significiant effect of aging processes is an accumulation of oxidative damage in the genetical material. In this study, the relationship between malignant melanoma and damage in chromosomes and proliferative effectiveness of human peripheral lymphocytes were investigated by the micronucleus (MN) technique. A total of 15 malignant melanoma patients and appropriately matching 15 healthy controls were involved in the study. MN frequencies and proliferative indexes (PI) after non toxic levels of hydrogen peroxide treatment were also measured to determine damaging effect of oxidative stress in genome in addition to measuring the spontenous levels of micronuclei and PI. The patient group had a significantly higher rate of spontaneous MN than the control group ($p<0.01$). After treatment with H_2O_2, MN frequencies in the patient group was significantly decreased ($p<0.01$) although there was no difference between the treated and untreated results of control group ($p=0.29$). There was also difference ($p<0.01$) between the MN frequencies of the patient and the control group either in the spontaneous levels or in the H_2O_2 treated groups. The same significant difference persisted when the PI values were compared between patient and control groups. Increase in the MN frequency in patients could mean the alterations in the chromosomal structure which may lead to the chromosome instability and therefore genetic susceptibility to cancer. This increased number of micronuclei can also be used for cytological marker in identifying high risk cases for malignant melanoma.

Keywords: Cancer - malignant melanoma - micronuclei - lymphocytes - aging

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Introduction

Most of deaths from skin cancer is responsible for malignant melanoma and it developes from malignant transformation of melanocytes which are pigment producing cells. Epidemiological studies have revealed that the risk of getting melanoma increases with an increasing age (Helfand et al., 2001; Diepgen et al., 2002; Cho et al., 2005; Mensink et al., 2011; McLean et al., 2012). The one of the significiant effect of aging process is an accumulation of oxidative damage occuring in macromolecules, particularly in the genetical material in the length of lifetime (Oresajo et al., 2012).

Damage in the genetic material may then lead to an activation of protooncogens and inactivation of tumor suppressor genes resulting in cancer susceptibility (Miguel et al., 1980; Hagmar et al., 1994; Knowles et al., 2005). Susceptibility to cancer can be associated with structural chromosome alterations (Boveri et al., 1914, Nowell et al., 1960). Cytogenetic alterations, such as CAs may reflect genotoxic exposure. These alterations can determine the individual sensitivity for the particular genotoxic agent and this can be used as a marker in a correlation to cancer. Some epidemiological studies have shown that the individuals with high levels of CAs in their peripheral blood lymphocytes (PBL) have an increased risk of cancer (Bonassi et al., 2004; Kolusayın et al., 2005; Vodicka et al., 2010a; 2010b; Bonassi et al., 2011). This association between a high level of CAs in peripheral blood lymphocytes and the risk of devoloping cancer for an healthy individual is also supported with many other studies (Rossner et al., 2005; Norppa et al., 2006; Boffetta et al., 2007; Bonassi et al., 2008). So the presence of high levels of CAs could be considered as a marker of cancer risk.

The MN assay is the one of the in vitro genotoxicity techniques which measures CAs and evaluates the effects of genotoxic exposures in human (Geller et al., 2002). In this technique, scoring micronuclei in binucleate cells is easier and faster than scoring CAs in metaphase cells. Therefore scoring thousands of cells with using the MN assay enables more statistical certainty (Norppa et al., 2003). Micronuclei are formation of additional nuclei that appeares at mitosis. They may consist of a whole chromosome or an acentric chromosome which are not associated with main nucleus. They have similar shape, structure and painting features with the main nucleus but they are smaller. In the studies with the MN technique, it has been observed that the level of cytogenetic disorder as being measured with an excess amount of micronuclei

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increase with an increasing age in human peripheral blood lymphocytes (Vanparys et al., 1990; Bolognesi et al., 1997; Cardoso et al., 2001; Iarmarcovai et al., 2008). This relationship is also hold between MN frequency and the risk of cancer. The increased amount of micronuclei has been shown to be a marker of chromosome damage, gene instability, genetic susceptibility and mutations (Hagmar et al., 1994; Iarmarcovai et al., 2008).

In this study with using human PBL, we have investigated a possible relationship between CAs and malignant melanoma by using the MN assay in order to determine if this MN method could be used as being a cytological marker in the diagnosis of the disease besides the classical diagnosing methods. Chromosamal damage and proliferative efficiency of cells were also measured after treating cells with the non-toxic levels of H_2O_2 in addition to measuring the spontaneous levels of micronuclei and proliferative indexes.

Materials and Methods

Cell culture

In this study, we have measured micronuclei in the peripheral blood lymphocytes in the group of 15 patients with malignant melanoma and in the group of 15 healthy controls. Control individuals matched the similar gender and age range as in the patient group. This work was approved by the ethical committee of the Cerrahpaşa Medical Faculty of the Istanbul University. Volunteers were properly informed and signed consent documents.

Peripheral blood samples were collected by venous puncture from both the patients and the controls. Blood samples were drawn into two separate 4, 5 ml sterilized lithium-heparin tubes. One tube was left as a control for spontaneous MN frequencies and the other was treated with hydrogene peroxide (H_2O_2). 0.5 ml of whole blood was added to culture medium containing 15μg/ml of phytohemagglutinin (Sigma), 0.5 ml of Newborne Calf Serum and 4 ml of RPMI-1640 with glutamine (Sigma) supplemented with 100 μg/ml streptomycin and 100 IU/ml penicilin. The cultures were incubated at 37 °C for 44 hours. After adding cytochalasine-B (Sigma: 6 μg/ml) solution, cells were left for incubation for another 28 hours. At the end of 72 and hours cells were centrifuged at 200g for 10 minutes, then treated with hypotonic KCL solution (0.075M). 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.

Scoring

1000 binucleated cells were analysed per person and MN frequencies were evaulated according to the criteria described by Fenech (Fenech et al., 2003). MN scoring in binucleate cells was made using light microscope at a magnification of 400. Also 1000 magnification (immersion objectives) was used for the comfirmation of MN. Additionally, for each individual, cells including 1,2,3 or 4 nuclei were analyzed for PI.

Statistical analysis

Spontaneous and H_2O_2 induced MN frequencies and PI for individuals in both malignant melanoma group and matched control group were compared using t-test.

Results

BetPatient group

The patients consisted of 9 women and 6 men. Their ages ranged from 36-75 with the mean of 53. The type of malignancies in the group of melanoma patients were superficial spreading melanoma (in 8 patients), nodular melanoma (in 3 patients), lentigo maligna melanoma (in 2 patients) and acral-lentiginous melanoma (in 2 patients). Eight patients had grade-1 tumor, six patients had grade-2 tumor and one patient had grade-3 tumor.

The spontaneous MN frequencies of patient group were calculated for each individuals in at least 1000 binucleated cells per culture (Table 1). The results for patients showed that mean value of spontaneous MN frequency was 0.029±0.022 (mean±standart deviation) within the range from 0.009-0.084. After H_2O_2 treatment was applied to cultures of patient groups, MN frequencies decreased significantly, ranging from 0.006-0.059 with the mean value of 0.019±0.014 (mean±standart deviation).

When these MN results before and after H_2O_2 treatment were compared by paired t test in the patient group, there was a significiant difference ($p<0.01$). Spontaneous ($p=0.82$) and H_2O_2 induced ($p=0.65$) MN frequencies were not different between the patients with grade-1 tumor and the patients with grade-2 tumor.

Additionally, cells with one, two, three and four nuclei (M1, M2, M3, M4 respectively) were scored for calculating PI for each individuals as; $PI=(M1+2(M2)+3(M3)+4(M4))/N$, N represents total number of cells. Spontaneous PI for patients were found to be within the range from 1.390-1.920 with the mean value of 1.690±0.045 (±standart deviation). After H_2O_2 treatment was applied to the same group, PI values decreased (mean value:1.503±0.187, range: 1.222-1.820). When we compared the results between spontaneous and H_2O_2 treated patient groups by paired t test, we obtained very significiant difference ($p<0.0001$).

Control group

The control group contained 15 healthy individuals who had similar age and gender with patients. Nine of them were women and six of them were men. Their ages were between 36 and 76. Similar applications were applied to their blood samples as in the patient group.

The results for controls showed that the mean value of spontaneous MN frequency was 0.009±0.003 (mean±standart deviation) and MN distribution ranged from 0.005-0.015. After treating control individuals with H_2O_2 MN frequencies were distributed between 0.004 and 0.013 with the mean MN frequency of 0.008±0.003 (Table 2). When we compared the results between the two groups by paired t test, there was no difference ($p=0.29$) between the two.

The mean value for the spontaneous PI for controls was found to be 1.931±0.324 ranging from 1.303-2.340.

Table 1. Results of MN and PI Frequencies in the Malignant Melanoma Patients

Patient No.	Age/Gender	MN/BN		PI	
		Spontaneous	H_2O_2 induced	Spontaneous	H_2O_2 induced
1	36/M	0.028	0.011	1,658	1,766
2	37/M	0.071	0.007	1,390	1,265
3	38/F	0.040	0.015	1,682	1,562
4	39/F	0.040	0.009	1,730	1,607
5	39/F	0.009	0.006	1,905	1,501
6	47/F	0.028	0.012	1,920	1,719
7	50/F	0.020	0.023	1,444	1,222
8	51/M	0.024	0.015	1,589	1,593
9	54/M	0.013	0.019	1,578	1,312
10	56/F	0.024	0.018	1,775	1,591
11	64/F	0.013	0.012	1,441	1,401
12	68/F	0.010	0.015	1,907	1,820
13	72/M	0.084	0.039	1,723	1,340
14	75/F	0.020	0.059	1,796	1,529
15	76/M	0.017	0.019	1,817	1,315

Table 2. Results of MN and PI Frequencies in the Control Group

Patient No.	Age/Gender	MN/BN		PI	
		Spontaneous	H_2O_2 induced	Spontaneous	H_2O_2 induced
1	36/M	0.015	0.009	1,547	1,255
2	36/M	0.010	0.006	2,261	1,944
3	38/F	0.006	0.010	2,055	1,925
4	39/F	0.011	0.009	1,829	2,018
5	43/F	0.005	0.010	2,213	2,009
6	50/F	0.011	0.013	1,923	1,903
7	50/F	0.013	0.009	2,340	2,081
8	52/M	0.007	0.004	2,282	2,034
9	54/M	0.008	0.007	2,046	2,033
10	56/F	0.009	0.012	1,303	1,241
11	65/F	0.015	0.013	1,723	1,639
12	69/F	0.007	0.008	1,934	1,635
13	72/M	0.013	0.008	1,383	1,491
14	75/F	0.005	0.004	1,945	1,666
15	76/M	0.006	0.005	2,190	1,593

†MN: Micronucleus, ‡BN: Binucleate, §PI: Proliferative index

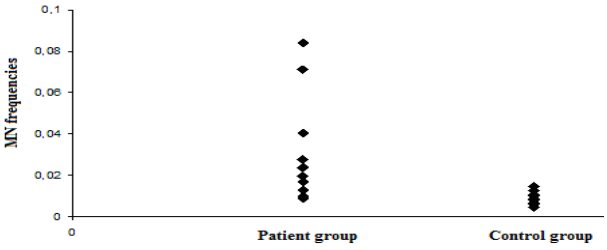

Figure 1. Comparison of MN frequencies between patient and control group.

After H_2O_2 treatment was applied to the same group, PI values decreased ranging between 1.240 and 2.081 with the mean value of 1.764±0.284. When the comparision was made between the treated and untreated samples, the difference was extremely significiant ($p<0.0001$).

Comparisions between the two groups

The findings of patients and mated healthy individuals were compared by using unpaired t-test. Spontaneous MN frequencies of the patient group were higher than the frequencies of the control group. The significant difference ($p<0.01$) was observed by applying unpaired t-test. This difference ($p<0.01$) remained the same between the H_2O_2 treated samples of the two groups.

When the same statistical test was applied to compare the PI values between patient and control groups, significiant differences were found for the spontaneous values ($p<0.05$) and H_2O_2 treated values ($p<0.01$).

Discussion

Chromosomal damages which can be measured as translocations, acentric fragments, telomere shortening, nondisjunction, loss of chromosomal material, uneuploidy and micronuclei increases with an increasing age (Countryman et al., 1976; Rossner et al., 2005; Kazimirova et al., 2009). Epidemiological studies have reported that the risk of cancer increases in individiuls who had high level of CAs in their PBL (Bonassi et al., 2004; Vodicka et al., 2010). Studies with MN assay showed increases in the MN frequency in PBL and fibroblast cultures of individuals with familial cutaneous malignant melanoma (Weichenthal et al., 1989; Berg-Drewniok et al., 1997). On the other hand, Widel et al investigated radiosensitivity of 25 malignant melanoma patients with MN assay and found an association between MN sensitivity and radiation responsive tumor cells (Widel et al., 1997).

In this study, we have investigated spontaneous and H_2O_2 induced MN frequencies and proliferative indexes in the peripheral blood lymphocyte cultures of 15 malignant melanoma patients and appropriately matching 15 healthy controls. Their ages ranged from 36-76 years. In the patient group there was no influence of either sex or having an immunotherapy on the induction of the MN frequencies. We have observed a significant decrease ($p<0.01$) in the MN frequencies of the patients lymphocytes after H_2O_2 treatment. But H_2O_2 treatment didn't change the MN frequencies in healthy individuals giving no difference between the two ($p=0.29$). Application of H_2O_2 to the cultures resulted in the decreases of the PI values in both the patient group ($p<0.0001$) and the control group ($p<0.01$).

When we compared the groups in terms of MN frequencies, there was a significiant difference ($p<0.01$) between the patient and control groups. Higher MN frequencies in the patients compared to the healthy individuals in the control group can be associated with the patients having individual sensitivity factors which could lead to cancer as a result of the long term exposure to genotoxic carcinogens (Figure 1). The difference ($p<0.01$) between the groups was also observed between the H_2O_2 teated cultures. This difference is due to the decreases in the micronuclei which occured particularly in the patient group after application of H_2O_2. Because H_2O_2 application didn't alter the MN frequency in the control groups.

In this study, increases in the MN frequencies were obtained in the malignant melanoma patients compared to the MN frequencies in the healthy individuals (Figure 1). Increasing amount of damage to DNA that is observed with the increasing amounts of MN frequencies in malignant melanoma patients can be associated with the genetic instability leading to the chromosome instability which may be important in cancer induction and aging

processes. This increased number of micronuclei can also be used for cytological marker in identifying malignant melanoma.

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References

Berg-Drewniok B, Weichenthal M, Ehlert U, et al (1997). Increased spontaneous formation of micronuclei in cultured fibroblasts of first-degree relatives of familial melanoma patients. *Cancer Genetics and Cytogenetics*, **97**, 106-110.

Boffetta P, Hel OV, Norppa H, et al (2007). Chromosomal aberrations and cancer risk: results of a cohort study from Central Europe. *Am J Epidemiol*, **165**, 36-43.

Bolognesi C, Abbondandolo A, Barale R, et al (1997). Age related increase of baseline frequencies of sister chromatid exchanges, chromosome aberrations, and micronuclei in human lymphocytes. *Cancer Epidemiol Biomarkers Prev*, **6**, 249-56.

Bonassi S, El-Zein R, Bolognesi C, Fenech M (2011). Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis*, **26**, 93-100.

Bonassi S, Norppa H, Ceppi M, et al (2008). Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries. *Carcinogenesis*, **29**, 1178-83.

Bonassi S, Znaor A, Norppa H, Hagmar L (2004). Chromosomal aberrations and risk of cancer in humans: an epidemiologic perspective. *Cytogenet Genome Res*, *104*, 376-82.

Boveri T, Fisher JG (1914). Zur Frage der Entstehung maligner Tumoren. *Science*, **40**, 857-59.

Cardoso RS, Takahashi-Hyodo S, Peitl P Jr, Ghilardi-Neto T, Sakamoto- Hojo ET (2001). Evaluation of chromosomal aberrations,micronuclei and sister chromatid exchanges in hospital workers chronically exposed to ionizing radiation. *Teratog Carcinog Mutagen*, **21**, 431-39.

Cho E, Rosner BA, Feskanich D, Colditz GA (2005). Risk factors and individual probabilities of melanoma for Whites. *J Clin Oncol*, In press.

Countryman PI, Heddle JA (1976). The Production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat Res*, **41**, 321-32.

Diepgen TL, Mahler V (2002). The epidemiology of skin cancer. *Br J Dermatol*, **146**, 1-6.

Fenech M, Chang WP, Kirsch-Volders M, et al (2003). HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res*, **534**, 65-75.

Geller AC, Miller DR, Annas GD, Demierre MF, Gilchrest BA (2002). Melanoma incidence and mortality among US Whites, 1969-1999. *JAMA*, **288**, 1719-20.

Hagmar L, Brogger A, Hansteen IL, et al (1994). Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes. Nordic Study Group on the Health Risk of Chromosome Damage. *Cancer Res*, **54**, 2919-22.

Helfand M, Mohon SM, Eden KB, Frame PS, Orleans CT (2001). Secreening for skin cancer. *Am J Prev Med*, **20**, 47-58.

Iarmarcovai G, Ceppi M, Botta A, Orsiere T (2008). Micronüclei frequency in peripheral blood lymphocytes of cancer patients. A meta mnalysis. *Mutat Res*, **659**, 274-83.

Kazimirova A, Barancokova M, Dzupinkova Z, Wsolova L, Dusinska M (2009). Micronuclei and chromosomal aberrations, important markers of ageing: possible association with XPC and XPD polymorphisms. *Mutat Res*, **661**, 35-40.

Knowles MA, Selby PJ (2005). Introduction to the cellular and molecular biology of cancer. In 'Oncogenes ', Ed Knowles MA. 4th ed. *Oxford University Pres*, Oxford (NY) **Vol ???** 117-134.

Kolusayın Ozar MÖ, Orta T (2005). The use of chromosome aberrations in predicting breast cancer risk. *J Exp. Clin Cancer Res*, **24**, 217-222.

McLean DI, Phillips N, Zhou Y, Gallagher R, Lee TK (2012). Year Trends in Skin Cancer in British Columbia, Canada, 1973 to 2003. *J Cutan Med Surg*, **16**, 83-91.

Mensink HW, Vaarwater J, Keizer RJW et al (2011). Chromosomal aberrations in iris melanomas. *Br J Ophthalmol*, **95**, 424-428.

Miguel J, Economos AC, Fleming J, Johnson JE (1980) Mitochondrial role in cell aging. Exp Gerontol, 15, 575-591.

Norppa H, Bonassi S, Hansteen IL, et al (2006). Chromosomal aberrations and SCEs as biomarkers of cancer risk. *Mutat Res*, **600**, 37-45.

Norppa H, Falck GC (2003). What do human micronuclei contain? *Mutagenesis*, **18**, 221-233.

Nowell PC, Hungerford DA (1960). A minute chromosome in human granulocytic leukemia. *Science*, **132**, 1488-1501.

Oresajo C, Pillai S, Manco M, Yatskayer M, McDaniel D (2012). Antioxidants and the skin: U nderstanding formulation and efficacy. *Dermatologic Therapy*, **25**, 252-9.

Rossner P, Boffetta P, Ceppi M, et al (2005). Chromosomal aberrations in lymphocytes of healthy subjects and risk of cancer. *Environ Hlth Perspect*, **113**, 517-20.

Vanparys P, Vermeiren F, Sysmans M, Temmerman R (1990). The Micronucleus assay as a test for the detection of aneugenic activity. *Mutat Res*, **244**, 95-103.

Vodicka P, Polivkova Z, Sytarova S, et al (2010). Chromosomal damage in peripheral blood lymphocytes of newly diagnosed cancer patients and healthy controls. *Carcinogenesis*, **31**, 1238-41.

Weichenthal M, Roser M, Ehlert U, et al (1989). Increased numbers of spontaneous micronuclei in blood lymphocytes and cultures fibroblasts of individuals with familial cutaneous malignant melanoma. *J Cancer Res Clin Oncol*, **115**, 264-8.

Widel M, Dobrut M, Maka B, Lubecka B, Pluciennik A (1997). The Radiosensitivity of human malignant melanomas evaluated by cytokinesis-block micronucleus assay. *Neoplasma*, **44**, 09-16.