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LARYNGOLOGY

Micronucleus, nucleoplasmic bridge and nuclear bud frequencies in patients with laryngeal carcinoma

Micronucleo, ponte nucleoplasmatico e frequenze di gemme nucleari in pazienti con carcinoma laringeo

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SUMMARY

The aim of the study is to determine and compare micronucleus (MN), nucleoplasmic bridge (NPB) and nuclear bud (NBUD) frequencies in patients with laryngeal carcinoma and healthy controls. The study was conducted in the School of Medicine of Onsekiz Mart University. A total of 102 volunteers, 51 of whom had laryngeal carcinoma and 51 of whom were healthy control subjects, participated in this study. The Cytokinesis-Block Micronucleus Assay (CBMN) was applied to peripheral blood lymphocytes taken from patients and controls. We evaluated MN, NPBs and NBUDs frequencies in patients with laryngeal carcinoma and compared the results with those in the control group. The frequencies of MN, NPBs and NBUDs of patients with laryngeal carcinoma were found significantly higher than those in the control group (P = 0.01, P = 0.004, P = 0.01, respectively). MN, NPB and NBUD frequencies were also compared in the patients with and without pesticide exposure, and the means of all frequencies was higher in patients with pesticide exposure (P = 0.001, P = 0.02 respectively). The MN, NPBs and NBUDs frequencies of the patients with laryngeal cancer were significantly higher than those of the control group, and pesticide exposure might be a risk factor that increases genomic instability and risk of laryngeal cancer.

KEY WORDS: micronucleus, nucleoplasmic bridge, nuclear bud, larynx cancer, frequency

RIASSUNTO

Lo scopo dello studio è quello di valutare e confrontare le frequenze relative alla presenza di micronucleo (MN), ponte nucleoplasmatico (NPB) e gemma nucleare (NBUD) in pazienti con carcinoma laringeo e popolazione sana. Lo studio è stato condotto presso un istituto universitario di medicina. Sono stati arruolati 102 volontari, 51 dei quali con carcinoma laringeo e 51 controlli sani. Il metodo di Cytokinesis-Block Micronucleus Assay (CBMN) è stato applicato ai linfociti del sangue periferico dei casi e dei controlli. Abbiamo valutato le frequenze di MN, NPB e NBUD nel paziente con carcinoma laringeo e confrontato i risultati con il gruppo dei controlli. Le frequenze MN, NPB e NBUD dei pazienti con carcinoma laringeo sono risultate significativamente più alte rispetto al gruppo di controllo (P = 0,01, P = 0,004, P = 0,01, rispettivamente). Nello studio, le frequenze MN, NPB e NBUD sono state confrontate in pazienti con e senza esposizione ai pesticidi. I risultati hanno mostrato che la media delle frequenze di NBP, MN e NBUD era significativamente più alta nei pazienti con esposizione ai pesticidi (P = 0,001, P = 0,02 rispettivamente). Il presente studio ha indicato che un aumento delle frequenze di MN, NPB e NBUD nei linfociti periferici umani potrebbe essere utilizzato per prevedere il rischio individuale di sviluppare un carcinoma laringeo. Inoltre, i nostri risultati indicano che l'esposizione ai pesticidi potrebbe aumentare il rischio di carcinoma laringeo.

PAROLE CHIAVE: *micronucleo, ponte nucleoplasmatico, gemma nucleare, cancro alla laringe, frequenza*

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Conflict of interest
The Authors declare no conflict of interest.

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Introduction

Laryngeal carcinoma (LC) comprises 30% of all head and neck malignancies [1]. The incidence of LC is 5 times higher in males than in females, although the incidence of LC in female patients has increased in recent years. This is related to the increasing smoking rates in females and to the increases in participation in work life which also elevates toxic exposure [2]. Cancer rates increase after the fifth decade of life and reache the highest rates in the seventh or eighth decade of life. The incidence is 1% before the third decade [3]. Squamous cell carcinoma comprises 85-90% of laryngeal malignancies [4]. To increase the disease-free life span, survival and organ protection rates in laryngeal carcinoma, better understanding of behaviours is needed.

Tumour stage, anatomical localisation, histopathological differentiation and neck metastasis are general prognostic factors: neck metastasis is accepted as a particularly poor prognostic sign [5]. However, different tumour behaviours can be observed even in tumours with similar histopathological diagnosis, grade and stage, leading investigators to study additional prognostic factors and conduct new molecular studies.

The accumulation of genetic damage leading to genomic instability is a recognised cause of cancer [6,7]. Therefore, it is important to measure the genetic damage [8]. In human peripheral lymphocytes (PBL), the CBMN assay is one of the standard cytogenetic tests for evaluation of genetic damage because of its sensitivity, reliablity and low cost. CBMN is a comprehensive method that allows determination of multiple markers such as MN, NPB and NBUD arising from different genetic mechanisms [9,10]. The mechanisms of MN are chromosome and/or chromatid breaks and mal-segregated chromosomes. Dicentric chromosomes and gene amplifications are the mechanisms of NPB and NBUD, respectively. MN and NBUD may sometimes occur via breakage of NPBs [9,11]. These nuclear anomalies, which are markers of genomic instability, are frequently seen in cancer [6]. Numerous studies have validated that there is a positive correlation between increased MN, NPB and NBUD frequencies in PBL and cancer risk [6,8,12-15].

The CBMN assay is a useful method to monitor genomic instability, and many recent events reveal that the increased genomic instability in PBL might be an early event in carcinogenesis [6,16]. To the best of the authors' knowledge, there is no data about MN, NPB and NBUD frequencies in patients with LC.

In the present study, we aimed to determine the frequencies of MN, NPB and NBUD in PBL collected from individuals with LC, who were diagnosed by the ear, nose and throat (ENT) department, and healthy individuals who lived in Çanakkale, Turkey. For this purpose, we carried out the CBMN assay.

Methods

Population

A total of 102 volunteers, 51 with LC and 51 healthy control subjects, participated in this study. LC patient files were reviewed. Patients' age, gender, occupation, pesticide exposure, clinical history, anatomical location of disease, smoking history, radiotherapy history, physical examination, and TNM classifications were recorded (Tab. I). Cancers were staged according to the 2002 AJCC guidelines [17]. All patients had undergone video laryngoscope examination. Lymph node positivity was evaluated with both physical examination and ultrasonography. The type of the treatment, recurrence and metastasis frequency were noted. Lymph node invasion and tumour grade were recorded as well. Pathology reports were evaluated. LC patients were divided based on anatomical location of disease (supraglottic, glottic, subglottic), surgery type (partial, total laryngectomy, microlaryngoscopic resection), radiotherapy history and lymph node positivity of the neck. The control group was selected from healthy individuals who had no cancer cases in their family and who were over 40 years of age. This research was approved by the Ethics Committee of the Faculty of Medicine. Informed consent was obtained from each patient and control.

Table I. Size and characteristics of the studied population.

	Case (%)	Control (%)
N	51	51
Age		
Mean ± sd	64 ± 8.3	51 ± 7
Range	38-81	41-67
Gender		
Male	51 (100)	24 (47.1)
Female	0	27 (52.9)
Smoking		
Yes	51 (100)	39 (76.0)
No	0	12 (24.0)
Tumour stage		-
T1	18 (35.3)	
T2	22 (43.1)	
T3	9 (17.6)	
T4	2 (3.9)	
Tumour Location		-
Glottic	46 (90.2)	
Supraglottic	5 (9.8)	
Lymph node metastasis		-
Yes	11 (21.6)	
No	40 (78.4)	
Radiotherapy		-
Yes	24 (47.1)	
No	27 (52.9)	
Occupational exposure to pesticide		-
Yes	24 (47.1)	
No	27 (52.9)	

Chemicals

Chemicals used in the study were purchased from the following suppliers; cytochalasin B, culture medium RPMI 1640, foetal calf serum from Sigma (Germany), phytohaemagglutinin from Biological Industries (Israel), Giemsa, methanol, glacial acetic acid, potassium chloride from Merck (Germany).

Cytokinesis-block micronucleus assay

Fenech's method was used with minor modifications [10]. A 5 mL peripheral blood sample obtained from each donor taken in sterile heparinised tubes. A culture medium mixture, including 4 mL medium (RPMI 1640), 1 mL serum (foetal calf serum), 0.2 mL phytohemagglutinin (as a mitogen), was prepared for each donor. 0.5 mL whole blood, taken from each donor, was added to the medium. All cultures were incubated at 37°C for 72 h. At hour 44, cytochalasin B (6 μg/L) was added to each culture to stop cytokinesis. After 72 h, the cultures were harvested. At the harvest stage, the cells were firstly treated with cold potassium chloride (0.075 mol/L) as a hypotonic solution and culture tubes were centrifuged at 1000 rpm for 5 min; the supernatant was discarded. Secondly, the pellet was resuspended and fixed in methanol-acetic acid (7:1, v/v) three times. 5% Giemsa was used to stain slides. We evaluated 1000 binucleated cells under a light microscope for each individual. The microscopic evaluation of the slides was performed at x1000 magnification according to Fenech's criteria [18].

Statistical evaluation

We used frequency distribution, one sample t test, one-way analysis of variance (ANOVA) and correlation analysis. We used the SPSS (Statistical Package for the Social Sciences) 10.0 statistical program to evaluate the data. The Kolmogorow-Smirnov goodness-of-fit test was used to check the normal distribution of data. The MN, NPB and NBUDS rates were compared between LC patients and the control group. Results with $p < 0.05$ were considered statistically significant.

Results

A total of 102 volunteers, 51 with LC diagnosis by the ENT department and 51 control subjects, participated in the study. In the cancer group, all patients (n = 51) with LC were male, smokers, and the mean age was 64 (38-81). The tumour locations were 90.2% glottic and 9.8% supraglottic. In the control group, 47.1% were male, 52.9% were female and 76% were smokers.

Table II shows the MN, NPB and NBUD values of the control group and the cancer group. In the cancer group, the MN, NPB and NBUD values were 28.1, 1.31 and 3.33, respectively. In the control group, the MN, NPB and NBUD values were 13.9, 0.29 and 0.02, respectively. The analyses showed that there were significant differences between the control and the cancer groups in terms of MN, NPB and NBUDS values ($p = 0.01$, $p = 0.004$, $p = 0.01$, respectively).

Table II. Descriptive statistics of MN, NPB. and NBUDS values in cancer and control groups.

		N	Mean ± SD (‰)	p
MN	Cancer group	51	28.1 ± 15.4	0.01
	Control group	51	13.9 ± 4.07	
NPB	Cancer group	51	1.31 ± 2.28	0.004
	Control group	51	0.29 ± 0.75	
NBUD	Cancer group	51	3.33 ± 3.60	0.01
	Control group	51	0.02 ± 0.14	

MN: micronucleus; NPB: nucleoplasmic bridge; NBUD: nuclear bud; SD: standard deviation.

The MN, NPB and NBUDS values were compared in the patients with and without radiotherapy treatment (Tab. III). The average MN, NBP and NBUD frequencies in the group treated with radiotherapy were 32.8, 1.88 and 4.13, respectively. In the group treated without radiotherapy, the average MN, NBP and NBUD frequencies were 24.0, 0.81 and 2.63, respectively. There were no significant differences between the two groups in terms of MN, NPB and NBUD frequencies ($p > 0.05$).

The MN, NPB and NBUD frequencies were compared in patients with and without pesticide exposure (Tab. IV). The results showed that the mean of MN, NBP and NBUD frequencies in the patients with pesticide exposure were higher than in the patients without pesticide exposure. However, the differences in the median values of the MN and NBUD frequencies between the two groups were statistically significant ($p = 0.01$, $p = 0.02$ respectively).

Discussion

LC comprises 25-30% of all head and neck malignancies, and is the most common cancer of the upper respiratory

Table III. Descriptive statistics of MN, NPB, NBUD in LC with and without radiotherapy treatment.

	RT	N	Mean ± SD (‰)	p
MN	With RT	24	32.7 ± 20.6	0.06
	Without RT	27	23.9 ± 6.5	
NPB	With RT	24	1.8 ± 2.8	0.11
	Without RT	27	0.8 ± 1.4	
NBUDS	With RT	24	4.13 ± 4.52	0.14
	Without RT	27	2.63 ± 2.41	

MN: micronucleus; NPB: nucleoplasmic bridge; NBUD: nuclear bud; SD: standard deviation; RT: radiotherapy.

Table IV. Descriptive statistics of MN, NPB and NBUD values according to pesticide use status of patients in the cancer group.

	Pesticide exposure	N	Mean ± SD	p
MN	Yes	24	36.0 ± 17.7	0.01
	No	27	21.1 ± 8.4	
NPB	Yes	24	1.92 ± 2.82	0.09
	No	27	0.78 ± 1.52	
NBUD	Yes	24	4.63 ± 4.61	0.02
	No	27	2.19 ± 1.81	

MN: micronucleus; NPB: nucleoplasmic bridge; NBUD: nuclear bud; SD: standard deviation.

tract [19,20]. In the current study, we evaluated genomic damage in patients with LC. For this purpose, the CBMN assay was used in PBL. CBMN is sensitive, reliable and has low cost. Moreover, it is a comprehensive and preferred method allowing assessment of chromosomal damage [9,10,18]. In genetic toxicology, genetic damage at chromosome level is important because the mutation of a chromosome is a notable event for carcinogenesis [18].

The MN, NPB and NBUD frequencies were determined in the patient group, and compared with those of the control group. The analyses demonstrated that there were significant differences between the control and cancer groups in terms of MN, NPB and NBUD frequencies ($p = 0.01$, $p = 0.004$, $p = 0.01$, respectively). To our knowledge, there is no data related to genomic damage assessed in PBL in patients having LC. However, there are many studies on genomic damage in different cancer types. The result on the MN frequency obtained in the present study is consistent with the results of other studies [8,12-15]. There are only a few studies evaluating the frequency of NPB, NBUD and MN [21]. Pardini [21] reported that MN and NBUD frequencies were significantly elevated in bladder cancer patients compared to controls. Similarly, a significant relationship was found in the present study. However, contrary to Pardini [21], NPB, MN and NBUD were all significantly increased in the present study. NPB is an indicator of dicentric chromosomes in the anaphase stage of mitosis. It occurrs because of misrepair of DNA breaks and/or telomere end fusions. NBUDs caused by gene amplification and MN arise from chromosome fragments and/or whole chromosome, but MN and NBUD may sometimes occur via breakage of NPBs. Our findings showed that MN, NPB, and NBUDs frequencies representing the above-mentioned genetic mechanisms were increased in the cancer group, which was statistically significant.

Many epidemiological and experimental studies [2,22,23] and the 2004 and 2010 monographs of the International Agency for Research on Cancer (IARC) concluded that smoking habit is an important risk factor in cancer [24] and LC was more common in men [2]. In the present study, all the patients with LC were men and smokers. The result is consistent with previous findings investigating the relationship between smoking/gender and LC.

Since the two groups did not match in terms of gender and smoking habit, we compared only males and smokers in both groups. The mean of the MN, NPB, and NBUD frequencies for smokers were compared between the patient group ($n = 51$) and the control group ($n = 39$). The analyses demonstrated that the MN, NPB, and NBUD frequencies were significantly higher in the cancer group than the control group ($p < 0.001$, $p < 0.001$, $p < 0.001$ respectively). Similarly, the male controls ($n = 24$) and the male patients ($n = 51$) were compared in terms of MN, NPB, and NBUD values and a significant difference was found between the two groups ($p < 0.001$, $p < 0.01$, $p < 0.001$ respectively). As a result, gender and smoking habit were not found to be the confounding factors for the increase in MN, NPB, and NBUD frequencies.

There are many pesticides in the 2A (probably carcinogenic to humans) and 2B (possibly carcinogenic to humans) lists of the IARC [25]. Moreover, there have been relationships between many types of cancer and pesticide exposure such as brain, leukaemia, kidney, lung and prostate cancers [25]. There are some studies on cancer risk in farmers exposed to pesticides, which indicated a link between pesticide exposure and increased risk of cancer [26,27]. However, only a few studies examined the relationship between pesticide exposure and head and neck cancers. These studies reported that pesticide exposure might be a risk factor for head and neck cancers [28]. In our study, approximately half of the patients with LC had previous pesticide exposure. Therefore, we compared MN, NPB and NBUD frequencies in patients with and without pesticide exposure. The results showed that the mean of the NBP frequencies was higher and that the mean of the MN and NBUD frequencies were significantly higher in patients with pesticide exposure ($p = 0.001$, $p = 0.02$ respectively). Therefore, our findings suggest that pesticide exposure might be a risk factor that increases genomic instability and risk of LC.

In the cancer group, the frequencies of MN, NPB, and NBUD were compared between patients with and without radiotherapy treatment. All biomarkers were found to be higher in patients with radiotherapy treatment, but none of the increases was statistically significant ($p > 0.05$). To our knowledge, there is no data comparing genomic damage in patients with and without radiotherapy treatment. However, in the literature, there are some studies evaluating genomic damage in cancer patients undergoing radiotherapy. These studies showed that genomic damage increased significantly in cancer patients undergoing radiotherapy [29,30].

The main limitation of the present study was that it had a small number of patients. For this reason, future studies should be designed with larger cohorts. Another limitation of the study was lack of information on pesticide exposure of patients with LC, such as what types of pesticides were used.

Conclusions

The MN, NPBs and NBUDs frequencies of patients with LC were significantly higher than those in the control group. When we compared the genetic damage in the pesticide-exposed and nonexposed patients with LC, genetic damage was significantly higher in the patients with LC who were exposed to pesticides. Our findings suggest that pesticide exposure might be a risk factor that increases genomic instability and risk of LC. However, these data must be supported by future studies with a larger number of cases.

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