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Short communication

A case-control study of genotoxicity endpoints in patients with papillary thyroid cancer

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ABSTRACT

Thyroid cancer is one of the fastest growing cancer types worldwide. Using the cytokinesis-block micronucleus (CBMN) and comet assays, we performed a case-control study of 23 untreated papillary thyroid cancer (PTC) patients and 23 healthy volunteers. PTC patients showed higher basal DNA damage in peripheral blood lymphocytes. The CBMN assay indicated that the numbers of micronuclei, nuclear buds, and nucleoplasmic bridges among the cases were 2.67-, 2.79-, and 7.72-fold higher, respectively, than among the controls ($p < 0.05$). Comet assay tail lengths and tail intensities were 1.20- and 1.94-fold higher, respectively ($p < 0.05$). In additional, 14 thyroid tissues from PTC patients were probed for Raf-B and Ret expression; all samples were positive for at least one of these proteins.

1. Introduction

Cancer is one of the leading causes of death worldwide [1]. The World Health Organization (WHO) estimates that 30% of all cancers could be prevented by changes in lifestyle (e.g., control of smoking and obesity), lowering exposure to environmental pollution, diagnostic radiation and occupational carcinogens, or immunization against certain infectious diseases (http://www.who.int/cancer/prevention/en/).

Biomonitoring of genome integrity may be one way to improve cancer prevention. Bonassi et al. demonstrated that volunteers whose micronucleus frequencies were in the medium and highest tertiles were more prone to cancer development; thus, one of the main goals of the HUman MicroNucleus (HUMN) group was fulfilled – the micronucleus test became an established method for prediction of cancer risk [2]. The micronucleus test evolved into the cytokinesis-block micronucleus (CBMN) assay, which is now a standard biodosimetry method endorsed by the International Atomic Energy Agency (IAEA) and the WHO for measuring exposure to ionizing radiation, and a standard method for assessing the genotoxic hazard of a chemical (OECD guideline) [3]. Similarly, the comet assay can be used to biomonitor genome integrity; therefore, the ComNet project was launched in order to investigate the ability of the comet assay to be used as a cancer predictive biomarker and as a tool in defining high-risk groups and improving cancer prevention [4].

Thyroid cancer represents only 2.1% of all cancers, but it is one of the fastest growing cancer types in the world with an annual percent change (APC) of up to 24.2% [5,6]. With regard to the pathogenesis of papillary thyroid cancer (PTC), the most common type of thyroid cancer, point mutations of the BRAF oncogene and RET/PTC translocation occur in more than 80% of cases [7,8].

The aim of this pilot study was to investigate, using the CBMN and comet assays, the cytogenetic status of untreated PTC patients and compare it with that of healthy volunteers. The presence/localization of the Raf-B and Ret proteins in PTC tissues was another endpoint studied.

2. Methods

2.1. Subjects

The PTC patients took part in this study before their regular treatment at the Clinical Hospital for Tumours, Zagreb. Based on age, gender and smoking status, we also recruited volunteers for

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the control group. The characteristics of all 23 patients and 23 healthy donors are summarized in Table 1. We obtained written informed consent and asked the participants to fill out a questionnaire (approved by the Institutional and Hospital Ethics Committees) covering standard demographic questions and information on occupational, medical, and family history.

For the cytogenetic tests, a blood sample (4 mL) was drawn by antecubital venepuncture into heparinized tubes (Becton Dickinson, USA). Based on the answers given in their questionnaires, the volunteers were not exposed to potentially confounding factors (occupational chemical exposures, genetic disorders, anticancer drugs, or radiation).

2.2. Micronucleus test

The micronucleus test was performed following the guidelines of Fenech and Morley [9] with minor modifications [10]. Blood (0.5 mL) was added to RPMI 1640 medium (Gibco, USA) supplemented with foetal bovine serum (Gibco) and phytohaemagglutinin (Remel Europe Ltd., UK) and incubated at 37 °C and 5% CO_2 for 72 h. After 44 h of culture, cytochalasin-B (Sigma, USA) was added (final concentration 6 μg/mL), in order to prevent cytokinesis. Fixed human peripheral blood lymphocytes (HPBLs) were stained with 5% Giemsa solution (Merck, Germany). Thousand binucleated lymphocytes per repetition were analysed. The frequencies of micronuclei (MNi), nuclear buds (NBs), nucleoplasmic bridges (NPBs), and the cytokinesis-block proliferation index (CBPI) were scored according to Fenech [11].

2.3. Comet assay

The alkaline comet assay was performed according to Singh et al. [12] with minor modifications [13]. Three-layer-agarose samples were placed in freshly prepared lysis solution and left overnight. The slides were kept in denaturation for 20 min in freshly prepared electrophoresis buffer, followed by another 20 min of electrophoresis in a horizontal electrophoresis tank at 1 V/cm (300 mA). The slides were then washed with tris buffer, stained with ethidium bromide (10 μg mL^{-1}) and stored at 4 °C prior to analysis. Hundred randomly captured nuclei per slide were analysed using Comet assay IV (Perceptive Instruments Ltd., UK) image analysis software. For data presentation, we chose tail length and tail intensity.

2.4. Immunohistochemistry

Immunohistochemical analysis was performed according to Grah et al. [14]. Paraffinized thyroid tissue samples from 14 PTC patients were microdissected and placed onto slides for deparaffinization. After removal of paraffin and rehydration, the samples were heated for 30 min at 100 °C in pH 9 buffer (Dako, Denmark) and then immersed in washing buffer overnight (Dako). Imunnohistochemistry for Raf-B (Santa Cruz Biotechnology, USA F-7, sc-5284, mouse monoclonal antibody, dilution 1:50) and Ret (Santa Cruz Biotechnology, C-19, sc-167, rabbit polyclonal antibody, dilution 1:25) was done according to the manufacturer's instructions, and primary antibodies were incubated for 30 min followed by another 30 min of incubation with peroxidase-linked secondary antibodies (Dako REAL™ EnVision™ Detection System). Visualization of proteins were performed by adding 3,3′-diaminobenzidine (DAB), whereas the tissue was stained with Hemalaun dye (Sigma). The samples were scored based on the level of signal intensity (0: no signal; 1: weak signal; 2: medium signal; and 3: strong signal) and the percentage cells with labelled proteins (0: no cells; 1: <10%; 2: 11–50%; and 3: >50% of cells). A total score was calculated as the product of these two parameters, as follows: 0 for score 0; 1+ for score 1–3; 2+ for score 4 or 6; and 3+ for score 9. A total score of 2+ or 3+ was regarded as positively labelled tissue.

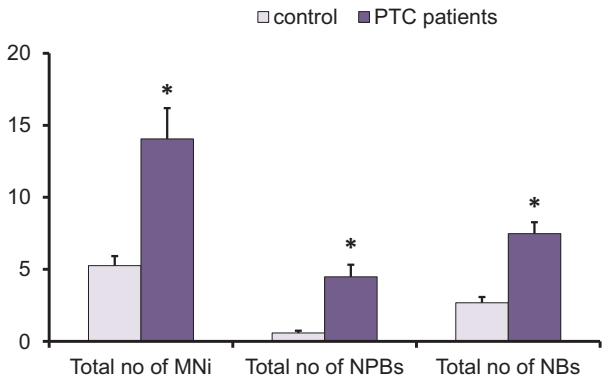

Fig. 1. Total number of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBs) from peripheral blood lymphocytes per 1000 cells in healthy control group and group of untreated papillary thyroid cancer (PTC) patients (* $p < 0.05$).

2.5. Statistical analysis

Data analysis was performed with Statistica12 (StatSoft, Tulsa, USA). Descriptive statistics are shown as mean ± standard error. The mean differences between the groups were compared by the Mann–Whitney *U*-test with continuity correction and $p < 0.05$ was considered statistically significant.

3. Results

The results of the CBMN assay are presented in Fig. 1. We observed that PTC patients had higher numbers of MNi (2.67-fold), NBs (2.79-fold) and NPBs (7.72-fold), compared to the healthy volunteers. CBPI, as expected, did not differ between the groups (2.055 ± 0.08 vs. 2.063 ± 0.08). The results of the comet assay were also higher: 1.20-fold for TL and 1.94-fold for TI, as presented in Fig. 2. All of the tested parameters reached significance at $p < 0.05$.

PTC tissue cytoplasm was positive for Raf-B in 13 of 14 cases; for Ret protein, 3 of 14 cases were positive (Table 2). There were two double-positive samples and no double-negative samples.

4. Discussion

Modern biomonitoring tools can enable surveillance and risk-assessment of human populations. According to the WHO, roughly one-third of all cancers could be prevented. Identification of high-risk populations can facilitate earlier cancer diagnosis and

Table 1
Characteristics of the study populations (healthy volunteers as control group and papillary thyroid cancer (PTC) patients): age, gender, height, weight, body mass index (BMI), smoking status and history of cancer disease in family.

	Age (years)	Gender (F:M)	Height (m)	Weight (kg)	BMI (kg m^{-2})	Active smokers	Family cancer history
Control ($N = 23$)	49.35 ± 13.95	17:6	1.68 ± 0.08	74.61 ± 15.69	26.16 ± 4.67	5	13
PTC patients ($N = 23$)	49.78 ± 13.89	17:6	1.68 ± 0.10	77.19 ± 19.99	27.32 ± 6.16	5	9

Values are presented as average ± standard deviation.