Micronuclei in Peripheral Blood Lymphocytes as a Possible Cancer Risk Biomarker: a Cohort Study of Occupationally Exposed Workers in Croatia

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Aim. To describe the cohort of Croatian workers monitored for micronuclei in peripheral blood lymphocytes and validate predictive value of micronuclei for the risk of cancer development.

Methods. Between 1985 and 1999, peripheral blood lymphocytes were analyzed with in vitro micronucleus assay in a cohort of 200 subjects occupationally exposed to genotoxic agents. The follow-up for cancer incidence and mortality was performed through the Croatian National Cancer Registry and records of occupational medicine physicians. Micronucleated cell frequency values were compared by Kruskal-Wallis test.

Results. The median micronucleated cell frequency value in the cohort was 49 (range, 30-79) per thousand cells. Micronucleated cell frequency was significantly higher in men than in women, which could be attributed to the different distribution of exposures. Micronucleated cell frequency increased with age for both sexes. Smoking habit had no influence on micronucleated cell frequency. The follow-up identified four cases of cancer. Three of them belonged to the highest micronucleated cell frequency tertile.

Conclusion. Due to a small number of cancer cases, the predictive value of micronuclei for the risk of cancer development in the cohort of Croatian workers was not estimated, but 4 identified cases were more than expected in a similar non-exposed group. The Croatian cohort will contribute to the pooled analysis of the current European study of predictive value of micronuclei for the risk of cancer development.

Key words: biological markers; epidemiology, molecular; micronucleus tests; neoplasms; occupational exposure; risk

Within epidemiological cancer research, molecular epidemiology is an area of increasingly intense activity and interest, combining molecular biology and epidemiological methods to strengthen epidemiological evidence (1,2). In the context of epidemiology, a biomarker or its products can be defined as a substance, structure, or process that can be measured in the human body and may influence the incidence or outcome of disease in human populations (3). Molecular epidemiology research focuses on three types of biomarkers: biomarkers of exposure (e.g., cytogenetic endpoints – chromosomal aberrations, micronuclei, and sister chromatid exchanges), biomarkers of susceptibility (e.g., genetic polymorphisms), and biomarkers of disease (e.g., tumor biomarkers) (2-4). Whereas tumor markers have been widely investigated (5), there have been few studies on the predictive value of cytogenetic biomarkers for cancer development (6).

Exposure to ionizing radiation and genotoxic chemicals can result in several cytogenetic endpoints, such as structural chromosome aberrations, micronuclei, and sister chromatid exchanges (7-9). It is assumed that genome damage could be associated with cancer development, but there were no studies to support this assumption due to a lack of cohorts large enough for reliable risk assessment (10).

The first international study to evaluate cytogenetic endpoints as cancer risk biomarkers was a Nordic-Italian cohort study, which showed that chromosomal aberrations predict cancer independently of exposure with relative risks of 2.35 (95% confidence interval [CI], 1.3-4.2) for Nordic countries and 2.66 (95% CI, 1.3-5.6) in Italy for the highest chromosomal aberrations frequency tertiles (6,11).

Micronuclei are fragments or whole chromosomes, which did not reach spindle poles during mitosis and remained encapsulated at telophase in a
separate nucleus. Whereas chromosome aberration assay detects only the genome damage, micronucleus assay additionally detects chromosome loss or malfunction of mitotic spindle caused by aneugenic mechanisms (12). Aneuploidy is an integral factor in the development of malignancies (13). There is a hypothesis that micronuclei and chromosomal aberrations could have a predictive value for cancer and therefore substitute chromosomal aberrations as cancer risk biomarkers or provide additional information on mechanism of action of aneugenic agents (14). In the Nordic-Italian study, the number of subjects tested for micronuclei was insufficient to yield statistically significant results (6,11). In 1997, the international Human Micronucleus Project (HUMN) was launched, aiming to standardize micronucleus assay in peripheral blood lymphocytes. The effort comprised 25 laboratories across the world, Croatia included. This part of HUMN project was completed and the results published in 2001 (15,16).

The other aim of HUMN project is to assess the predictive value of micronuclei for the risk of cancer development. This part of the project is currently under way, within the Commission of the European Communities program “Cytogenetic Biomarkers and Human Cancer Risk” (CEC QLK4-CT-2000-0062). Croatian cohort of the workers tested for micronuclei in peripheral blood lymphocytes will be a part of the international cohort for the pooled analysis.

The aim of this study was to describe the cohort of Croatian workers monitored for micronuclei in peripheral blood lymphocytes and, if possible, assess the predictive value of micronuclei for the risk of cancer development in this cohort.

Material and Methods

Study Population
The study included 143 men and 57 women from three factories and two hospitals in Croatia, who were occupationally exposed to cytostatics, ethylenoxide (ETO), formaldehyde, ionizing radiation (X-rays), strontium (gamma-rays), or vinylchloride monomer (VCM). For agents entering the body by inhalation, the maximum allowed levels are 2 ppm VCM, 1 ppm ETO, and 5ppm formaldehyde. For ionizing radiation, maximum allowed annual dose is below 50 mSv. Micronucleus assays were performed between 1985 and 1999, all by the same laboratory, and scored by the same scorer.

Inclusion criteria for the participants of the study were the following: age ≥15 years; ≥1,000 interphase cells scored; no drugs intake during a period of 6 months before sampling; and no exposure to ionizing radiation or ultrasound in diagnostic or therapeutic purposes 6 months before sampling. The interview included data on smoking habits, oral contraceptives, children, diseases, vaccination, and alcohol consumption.

Method
Blood samples were taken by venipuncture. Micronucleus testing was performed by cytokinesis-block technique in peripheral blood lymphocytes (17). Lymphocytes were stimulated by phytohemagglutinin. Micronuclei were scored after a single cell division by using binucleated lymphocytes to eliminate the confounding effect of altered cell division kinetics on the micronucleus index (Figs. 1 and 2). Cytokinesis was blocked by cytochalasin-B, which was added at a concentration of 3 μg/mL 44 h after the initiation of the culture. The cells were cultured for 72 h.

Follow-up
The follow-up for cancer incidence and mortality was performed through the Croatian National Cancer Registry database and through occupational physicians responsible for the three factories. The follow-up ended at the date of death, tumor diagnosis, or emigration, or the closing date of the study on December 31, 2000.

Statistical Analysis
Since the distribution of the results for the micronucleus frequency (percentage of micronucleated cells) was right-skewed, we used the median value as the measure of central tendency. Subjects were classified according to semi-quantitative categories of micronucleated cell frequency, ie, low (0-33 centile), medium (34-66 centile), and high (67-100 centile). Micronucleated cell frequency values were compared by Kruskal-Wallis test. All analyses were stratified by sex. Due to a small number of outcomes (four cancer cases), we could not estimate the cancer risk in the cohort.

Results
Cohort Description and Analysis
The cohort comprised 200 workers (143 men and 57 women), occupationally exposed to known carcinogenic chemical substances or ionizing radiation, yielding 1,443 person-years until the end of follow-up. The median frequency of micronucleated cells was higher for men (54‰) than for women.
at a statistically significant level (chi-square = 9.3, p = 0.002; d.f. = 1). The follow-up for cancer incidence identified 4 cancer cases. The follow-up for mortality identified one cancer death and no non-cancer deaths (Table 1).

Most of the tested workers (42%) were between 30 and 39 years old at the time of the test (Figs. 3 and 4). Median micronucleated cell frequency increased with age in both men and women. This increase was statistically significant for men (chi-square = 7.2, p = 0.027; d.f. = 2), but not for women (chi-square = 4.7, p = 0.096; d.f. = 2).

Table 2 shows the distribution of subjects by exposure to genotoxic agents. Men were exposed to VCM (78%) or ionizing radiation (X-rays), whereas women were exposed to cytostatics (70%), ethyleneoxyde, strontium, or VCM. In men, the median micronucleated cell frequency appeared higher for subjects exposed to vinylchloride monomere than to ionizing radiation, but there was no statistically significant difference (chi-square = 2.4, p = 0.120; d.f. = 1; Fig. 5). In women, the highest median micronucleated cell frequency value was in those exposed to VCM, but this finding was based on only two subjects and therefore not statistically analyzed (Fig. 6).

The median micronucleated cell frequency values for men and women smokers and non-smokers were similar (Figs. 7 and 8). The difference in micronucleus frequency between smokers and non-smokers was not statistically significant for either sex (men: chi-square = 0.5, p = 0.471, d.f. = 1; women: chi-square = 0.2, p = 0.655, d.f. = 1).

Case Description

During the follow-up of the cohort, 4 subjects developed a cancer and one of them subsequently died.

Case 1. Male worker exposed to VCM (50 ppm every three months during seven years), non-smoker, tested at the age of 35. The frequency of micronuclei per thousand was 57 at the time of testing (middle micronucleus frequency level). Three years later, he developed a small cell carcinoma of the lung.

Case 2. Male worker exposed to ionizing radiation (<50 mSv per year during 20 years), non-smoker, tested at the age of 34. The frequency of micronuclei per thousand was 76 at the time of testing (highest micronucleus frequency level). Ten years later, he developed a carcinoma of the urinary bladder.

Case 3. Male worker exposed to VCM (50 ppm every three months during 14 years), smoker, tested at the age of 42. The frequency of micronuclei per thousand was 69 at the time of testing (highest micronucleus frequency level). Seven years later he developed a gastric cancer.
Median micronucleated cell frequency was substantially higher in our study group (49.0‰) than the referent value for general population (6.5‰) (15,18), since all of the tested subjects were exposed to genotoxic agents. In the HUMN standardization study, whose results are considered referent values for general population, median micronucleated cell frequency values were higher for women than for men (7.0‰ vs 6.3 ‰). In our study, however, micronucleated cell frequency was significantly higher in men than in women, which could be attributed to different distribution of exposures. On the other hand, the increase in median micronucleated cell frequency with age for both sexes is consistent with the results of HUMN standardization study (15). There is no sign of significant cumulative effect or adaptation. Our results confirm earlier findings that smoking does not influence micronucleated cell frequency in exposed as well as in unexposed populations (19,20).

The application of cytogenetic biomarkers (chromosomal aberrations, sister chromatid exchanges, and micronuclei) is critical for health risk assessment after environmental or occupational exposure. The genetic damage measured in peripheral blood lymphocytes has been used in occupational health surveillance programs since the 1960 to assess genotoxic risks. The rationale for using this biomarker has been the hypothesis that the extent of genetic damage in peripheral blood lymphocytes reflects similar events in the precursor cells for carcinogenic processes in the target tissues (21). According to the multi-step hypothesis of cancer development, the formation of initiated cells by genotoxic compounds is causally related to cancer (22). For some agents, the distribution of chromosome breaks could be associated with specific chromosomal structure changes in specific types of cancer (23,24).

The subjects in our study were exposed to chemical agents and ionizing radiation, which have been documented to cause genome damage at low doses (25-28).

Like in other molecular epidemiology studies, possible sources of bias in studies using micronuclei frequency as a biomarker are related to biological variability and biomarker kinetics. Micronucleus frequency decreases after cessation of exposure and usually reaches control values within three months (18). Micronucleus frequency in combination with chromosomal aberration or sister chromatid exchange frequency and blood screening form the basis for decision whether the worker is transferred to another...
workplace. Such practice is common in Central European countries.

In our study, a drawback in assessing the effect of particular occupational exposures on micronucleated cell frequency was the absence of a control group. However, this will not bias the international pooled cohort analysis of predictive value of micronuclei for the risk of cancer development, since the micronucleated cell frequency is not considered the outcome variable for particular occupational exposures, but the exposure variable for the outcome of cancer incidence.

During the follow-up of the cohort, we found 4 cancer cases. The expected number of cancer cases would be less than one, taking into account the mean age at test (37 years), mean age at the end of follow-up (44 years), and age-specific cancer rates for the study period (29). Three of 4 cancer cases were in the highest micronucleated cell frequency category and the fourth case was in the middle category. Due to small numbers, the cohort study analysis was not performed. However, the Croatian cohort will contribute to the pooled analysis of the current European study of predictive value of micronuclei for the risk of cancer development. Considering the relatively young average age of the cohort and the increase of cancer risk with age, it could be expected that the further follow-up would yield enough cancer cases for the analysis of predictive value of micronuclei for the risk of cancer development in Croatia.

The availability of databases of biological data from basic research on one side, and population data from epidemiological and population registries on the other, with increasing possibilities for matching between them, offers a valuable opportunity to increase the knowledge of determinants of health and disease in human populations. The value of such databases has also been recognized by the Commission of the European Communities, which in its current 6th Framework Program encourages the establishment of biobanks. The analysis of combined data, however, requires an interdisciplinary approach and collaboration. Launching such a collaboration between cancer epidemiologists and cytogeneticists in order to analyze pooled data from available databases provides a possibility to widen the scope for cancer prevention in Croatia.

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