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A CYTOGENETIC APPROACH TO THE EFFECTS OF LOW LEVELS OF IONIZING RADIATION (IR) ON THE EXPOSED TUNISIAN HOSPITAL WORKERS

SANA BOURAOUI[1], SOUMAYA MOUGOU[1], AFEF DRIRA[2], FATEN TABKA[2], NOUHA BOUALI[1], NAJIB MRIZEK[2], HATEM ELGHEZAL[1], and ALI SAAD[1,3]

[1] Farhat Hached University Teaching Hospital, Sousse, Tunisia
Department of Cytogenetic and Reproductive Biology
[2] Farhat Hached University Teaching Hospital, Sousse, Tunisia
Department of Occupational and Environmental Medicine
[3] Faculty of Medicine of Sousse, Tunisia
Common Service Units for Research in Genetics

Abstract
Objectives: The aim of this study is to assess chromosomal damage in Tunisian hospital workers occupationally exposed to low levels of ionizing radiation (IR). **Materials and Methods:** The cytokinesis-block micronucleus (CBMN) assay in the peripheral lymphocytes of 67 exposed workers compared to 43 controls matched for gender, age and smoking habits was used. The clastogenic/aneugenic effect of IR was evaluated using the CBMN assay in combination with fluorescence in situ hybridization with human pan-centromeric DNA in all the exposed subjects and controls. **Results:** The study showed a significant increase of the micronucleus (MN) frequency in the lymphocytes of the exposed workers compared to the control group (13.63±4.9‰ vs. 6.52±4.21‰, $p < 0.05$). The centromere analysis performed in our study showed that MNs in hospital staff were predominantly centromere negative (72%) and the mean negative labeled micronucleus (C–MN) frequency was significantly higher in the exposed subjects than in the controls (9.04±4.57‰ vs. 1.17±0.77‰). The multivariate regression analysis, taking into account all confounding factors, showed that only the time of exposure to IR had a significant effect on the level of MNs and C–MN. **Conclusion:** The present study shows that chromosomal damage leading to the formation of micronucleated lymphocytes is more frequent in the hospital workers exposed to IR than in the controls, despite the low levels of exposure. The results of the study confirm the well-known clastogenic properties of ionizing radiation. In regards to health monitoring, detection of early genotoxic effects may allow for the adoption of preventive biological control measures, such as hygienic improvements in the workplace or reduction of hours of occupational exposure.

Key words:
Centromere-micronucleus assay, Occupational radiation exposure, Hospital workers, Centromere negative micronuculei

INTRODUCTION

Over the years, ionizing radiation (IR) has become a universal diagnostic and therapeutic tool, making the largest man-made contribution to the population dose [1]. Thus, medical personnel represent the group most consistently exposed to low doses of IR. High doses of IR are known to produce deleterious consequences in humans, including, but not exclusively, cancer induction; however, the effect of such radiation at lower doses, as in occupational work settings, is less clear [2] and needs a comprehensive

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Corresponding author: S. Bouraoui, Department of Cytogenetic and Reproductive Biology, Farhat Hached University Teaching Hospital, Ibn El Jazzar Street, 4000 Sousse, Tunisia (e-mail: sana_bouraoui@yahoo.fr).

elucidation. The levels of exposure to ionizing radiation in hospitals have decreased in recent decades and are now far below the regulatory limit of 20 mSv/year, indeed below the detection limit of dosimeters. Some medical uses of radiation, such as nuclear medicine and interventional procedures, may expose the personnel to higher doses, and these are subjects of concern [3]. Recently, Engin et al. [4] have assessed the genomic instability in γ-radiation and X-ray-exposed hospital staff and observed that chronic exposure to low levels of IR, below the accepted limit, could induce oxidative stress and increased apoptosis frequency, compared to the non–exposed staff. Information on radiation effects can be considered in the context of cancer risk [5], since there is recent evidence indicating that low doses of radiation exert both suppression and induction of neoplastic transformation [6].

Many cytogenetic studies have been conducted among hospital workers exposed to IR. Bonassi et al. [7] observed increased rates of chromosomal aberrations in hospital workers exposed to IR. A low, but statistically significant, increase in the rate of chromosomal aberrations in circulating lymphocytes among hospital workers with thyroid nodules was also observed in workers occupationally exposed to radiation [8].

A cytological consequence of induction of chromosome aberrations is the formation of micronuclei (MNs) that are observed in interphase cells. A micronucleus is formed during cell division when the nuclear envelope is reconstituted around chromosome fragments lacking a centromere (acentric fragments) or a lagging whole chromosome that is not incorporated into the main daughter nucleus, or both. This gives rise to a separate smaller nucleus in addition to the main daughter nucleus.

The cytokinesis-blocked micronucleus (CBMN) assay is widely used, since it represents a reliable test to assess radiation-induced chromosome damage and it is a valuable biomarker in many biomonitoring studies on human populations occupationally or environmentally exposed to IR [9]. The micronucleus-centromere assay combines the CBMN assay with fluorescence in situ hybridization (FISH). It uses a pan-centromeric probe to detect micronuclei (MNs) derived from acentric chromosome fragments or whole chromosomes [10,11]. Due to its predominantly clastogenic action, IR exposure is expected to induce more negatively labeled micronuclei (C–MN) than positively labeled micronucles (C+MN). There is now some evidence that centromere identification in MNs can improve the detection of in vivo effects of clastogenic exposures in humans [12].

Studies on genotoxic effects of occupational exposure to IR at low doses are limited and they provide contradicting results. The aim of the present study is to assess chromosomal damage in occupational Tunisian hospital workers exposed to low levels of IR. For this purpose, the CBMN assay in peripheral lymphocytes of 67 radiation workers and 43 controls was used. In order to characterize the origin of MNs occurring following the exposure to IR, the MN assay with FISH and an all-chromosome centromeric probe were combined.

MATERIALS AND METHODS

Subjects

The exposed group consisted of 67 healthcare workers occupationally exposed to ionizing radiation working in 6 different units of two hospitals in Sousse. Out of the selected subjects, 7 were from the nuclear medicine department, 15 from the radiology department and 3 from the orthopedic department at the Sahloul Hospital of Sousse, 6 from the radiotherapy department, 25 from the radiology department, 5 from the physiology department and 4 from the cardiology department at the Farhat Hached Hospital of Sousse.

Occupational exposure to IR occurred for a mean duration of 18.76±9.48 years (from 5 to 47 years). The

handling time was 6.36±0.9 h/day (6–12 h/day). The mean age of the exposed group was 43.86±7.60 years (range: 30–59 years). The group consisted of 20 males and 47 females. The workers were exposed to different kinds of radiation (X-ray, γ-ray) and many radioactive isotopes (^{125}I, ^{131}I, ^{57}CO, etc.). Eighteen percent of the exposed group worked without any individual protection (gowns, lead aprons, etc.).

The control group (N = 43) (13 males and 30 females), selected from the administrative department of the Farhat Hached Hospital of Sousse, presented the mean age of 42±8.87 years (range: 24–55 years). Ten percent of the hospital personnel experienced dermic symptoms (erythema, etc.), but the difference between these disorders in the hospital staff and the controls was statistically insignificant. All participants were healthy at the moment of blood sampling and had no occupational exposure to genotoxic compounds.

The characteristics of the subjects and the controls are presented in Table 1.

During a routine occupational health examination, an occupational physician, according to a structured questionnaire, interviewed each subject. The questionaire included questions on smoking habits, medical history, drug intake, diagnostic medical irradiation, family history of cancer or genetic disease and, for the exposed subjects, the duration of occupational exposure to IR and radiation protection measures. The subjects were informed about the nature of the study and potential adverse health effects and agreed to participate in it by

Table 1. Demographic characteristics of the exposed and control populations involved in the study

Variables	Groups		p: degree of significance
	exposed (N = 67)	control (N = 43)	
Age (years) M±SD	43.86±87.60	42.20±8.87	0.205
Sex [n (%)]			
males	20 (30)	13 (30)	0.830
females	47 (70)	30 (70)	
Smoking status (n, %)			0.510
smokers	5 (7)	5 (12)	
non-smokers	62 (93)	38 (88)	
Exposure time (h/day) M±SD	6.36±0.90	–	–
Exposure to a low dose of ionizing radiation (year) M±SD	18.42±9.30	–	–
Subjects [n (%)]			
using gloves	8 (12)	–	–
using lead aprons	18 (26)	–	–
using glasses	11(16)	–	–
using screens fixed/mobile	8 (12)	–	–
using pliers	11 (16)	–	–
without any individual protection	12 (18)	–	–
with general affection	47 (70)	8 (19)	$< 10^{-3}$
with dermic affection	7 (11)	6 (14)	0.750

$p < 0.05$.

signing a written informed consent. The ethical commission of the Farhat Hached Hospital approved the protocol of this study.

Cytokinesis-blocked micronucleus assay (CBMN)

Using heparinized vacutainer tubes, 5 ml samples of whole blood were obtained and kept at room temperature for less than 6 h. The CBMN assay was performed as described by Sari-Minodier et al. [13]. The cultures were started by adding 0.7 ml of whole blood to 9.3 ml of Roswell Park Memorial Institute medium (RPMI) supplemented with 25% fetal bovine serum, heparin (50 U/ml), antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) and 1% PHA and kept for 72 h in a humidified CO_2 incubator at 37°C. Cytochalasin B (Sigma Chemicals Company, St. Louis MO) was added to the cultures (5 μg/ml) 44 h after phytohemagglutinin (PHA) stimulation. In 72 h, the cells were subjected to a mild hypotonic treatment (KCl 0.075 M), fixed twice with methanol/acetic acid solution (3/1), then smeared on pre-cleaned microscope slides and air-dried. Staining was performed with 5% Giemsa in milli-Q water for 15 min. The stained slides were then scored by light microscopy at 1000X magnification. For each slide, 2000 Giemsa-stained binucleated lymphocytes with a well-preserved cytoplasm were scored for the presence of micronuclei according to the criteria described above [14].

Fluorescence In Situ Hybridization (FISH) with a pan-centromeric DNA probe

Fluorescence hybridization was performed with a human pan-centromeric probe (Kreatech, Netherlands) directly labeled with fluorescein isothiocyanate (FITC). The remaining fixed cells were thawed and dropped onto pre-cleaned slides just before hybridization. The cells were treated twice (2X) with saline sodium citrate buffer (SSC) at 37°C for 10 min, paraformaldehyde (PFA) at room temperature for 15 min, phosphate-buffered-saline (PBS) (1X) for 5 min, Pepsine/Hcl at 37°C for 13 min and again PBS (1X) for 5 min. The cells were then dehydrated in a series of ethanol washes (70%, 80%, 100%, V/V). The DNA probe and cells were denatured at 75°C for 5 min in Hybrite™ (Vysis). After overnight hybridization at 37°C in a humidified chamber, the slides were washed in 2X SSC NP40 (Sigma) for 2 min at room temperature, 2X SSC NP40 for 5 min at 72°C and again in 2X SSC NP40 for 2 min at room temperature. The cells were counterstained with 15 μl of 4', 6-diamidoni-2-phenylindole dihydrochloride (DAPI).

The presence of centromeres in MNs was determined with an epifluorescence microscope.

Binucleated cells with well-preserved cytoplasm were preliminarily scored under DAPI for the presence of MNs. When a micronucleated cell was noted, the filter setting was changed to determine whether the micronucleus contained centromere signals. If one or more green spots (FITC-labeled centromeres) were observed inside an MN, it was classified as C+MN. A negatively labeled MN was classified as C–MN. The number of centromeric signals per MN was also recorded and C+MN were classified as C1+MN (one centromere) or Cx+MN (two or more centromeres) (Photo 1). The results were expressed as the total number of MNs, the number of C–MN, C+MN, C1+MN and Cx+MN in 2000 binucleated cells.

Statistical analysis

The effect of different variables on each studied biomarker was evaluated by Student's t-test. A multivariate regression model (with stratification and after exclusion of smokers) was applied to analyze the effect of occupational exposure to a low dose of IR and the role of confounding factors on the frequencies of MNs, C+MN, C–MN, C1+MN and Cx+MN. The level of significance was established at 0.05. All analyses were conducted using the SPSS version 10.0 (Department of Epidemiology and Medical Statistics, CHU Farhat Hached Sousse, Tunisia).

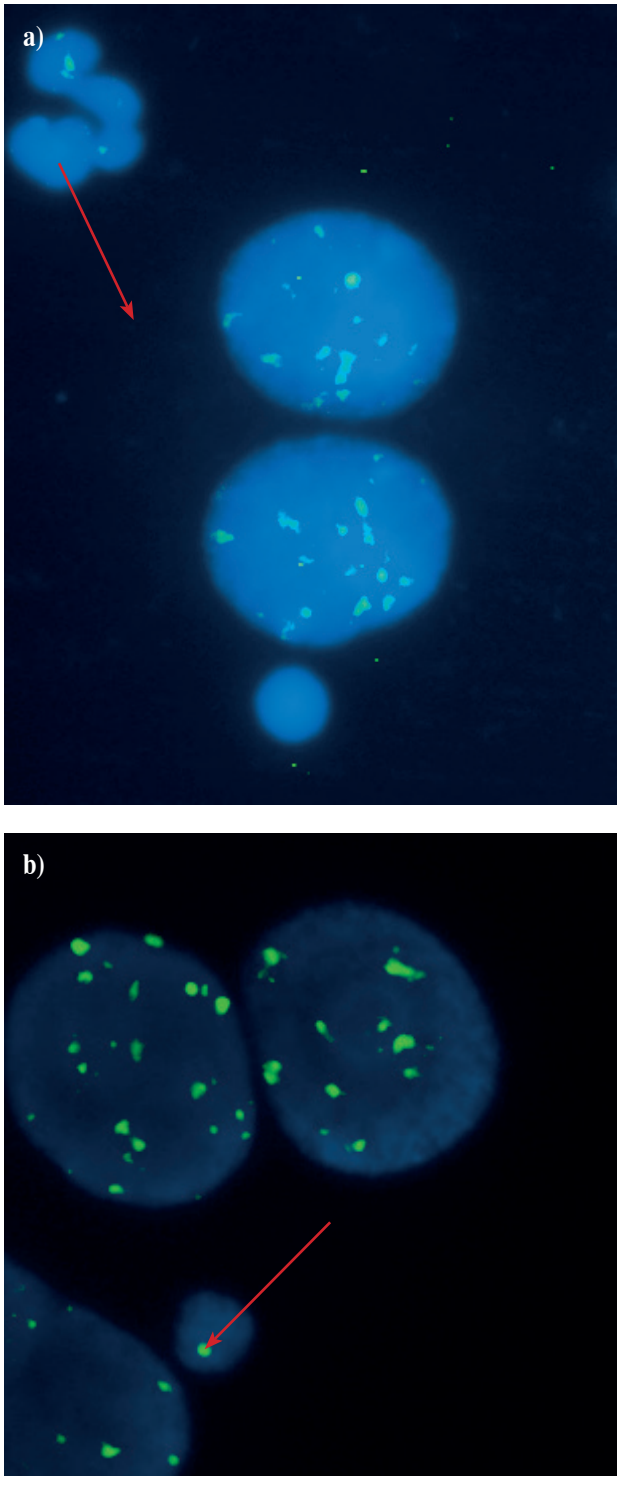


Photo 1. Example of hybridized cells by FISH analysis:
a) binucleated cell with a centromere – negative MN,
b) binucleated lymphocyte with a centromere – positive MN (indicated by arrowhead)

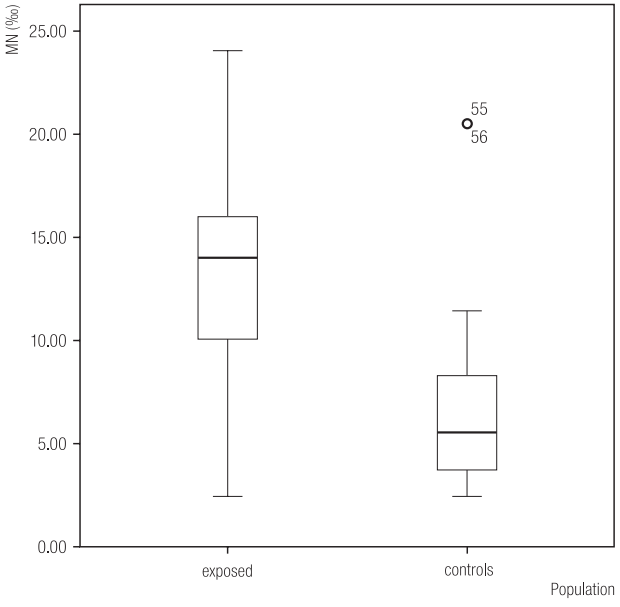
RESULTS

The MN assay was performed in 67 workers exposed to a low dose of IR and in 43 matched controls. The MN frequency was significantly higher ($p < 10^{-3}$) in the exposed group (13.63±4.9‰), compared to the controls (6.52±4.21‰), as shown in Figure 1.

The highest level of MNs was observed in the exposed subjects from the nuclear medicine (14.25± 7.93‰) and radiology departments (13.13±3.6‰), but the differences in comparison with others groups were not significant.

To assess the origin of the observed MNs, induced as a result of an aneugenic or a clastogenic effect of a low dose of IR, the FISH technique with a human pancentromeric DNA probe was used.

The frequency of MNs determined by the FISH technique was significantly higher in the exposed group (12.7±4.9‰), compared to the controls (4.27±4.1‰) ($p < 10^{-3}$). The C–MN frequency was significantly ($p < 10^{-3}$) higher in the exposed group (9.04±4.57‰),



Significant statistical difference between the exposed and the control group by the Student's t-test, $p < 0.05$.

Fig. 1. Distribution of MN per 2000 binucleated cells in the control group and the exposed group

compared to the controls (1.17±0.77‰). The C+MN frequency was higher in the exposed group (3.66±3.32‰), compared to the controls (3.1±3.6‰), but the difference was not too significant (p = 0.58). Among the centromeric micronuclei, the frequency of micronuclei containing one centromere (C1+MN) was higher in the group exposed to IR than in the controls (3.5±3.18‰ vs. 2.32±2.7‰), but the difference was not too significant, either (p = 0.81). The results of the fluorescence in situ hybridization (FISH) are summarized in Table 2.

The impact of the typical confounding factors, i.e. gender, age, time of exposure, use of individual protection equipment, general and dermic affections, on the selected endpoints was evaluated by fitting a multivariate regression model for each point (Table 3). It indicated that the time of exposure to IR influences the levels of MNs and C–MN.

The effects of the factors including the donor's age, gender, use of individual protection equipment, dermic and general affections were statistically insignificant ($p < 0.05$).

Seventy percent of the exposed group had general symptoms (nausea, vomiting, dizziness, asthenia, etc.). The differences between these symptoms in the exposed subjects and the controls were statistically significant ($p < 10^{-3}$).

Table 2. Results of the Fluorescent In Situ Hybridization (FISH) with a human pan-centromeric DNA probe and characteristics for the control and the exposed group

Biomarkers	Groups M±SD (‰)		p value
	exposed (N = 67)	controls (N = 43)	
MNs	12.70±4.89	4.27±4.10	< 10–3
C+MN	3.66±3.32	3.10±3.60	0.578
C–MN	9.04±4.57	1.17±0.77	< 10–3
C1+MN	3.50±3.18	2.32±2.69	0.815
Cx+MN	0.16±0.56	0.78±0.18	0.100

MNs – micronuclei, C+MN: centromere-positive MN; C–MN – centremere – negative MN;
C1+MN – micronuclei containing one centromere; Cx+MN – micronuclei containing two or more centromeres.
$p < 0.05$.

DISCUSSION

Ionizing radiation is known to induce mutations and cell transformations predominantly by causing single-standard double strand DNA breakage, thereby leading to chromosomal instability and carcinogenesis [15]. The levels of exposure to IR in hospitals have decreased in recent decades, and are now below the regularity limit of 20 mSv/year, and even below the detection limit of dosimeters [16].

In Tunisia, the threshold dose of permissible radiation is limited to 50 mSv/year for the exposed workers.

In the present study, the lack of data on individual physical doses of radiation results from the fact that the majority of the workers in the study sample did not use dosimeters; for this reason, radiation exposure was considered as the time per year a worker was exposed to radiation. There are other parameters that can be used to estimate exposure to radiation when the data regarding the physical dose is not available, such as data provided by biological dosimeters [17] and dicentric counting [18].

Biomonitoring of occupationally exposed people appears to be a sensitive way to evaluate the genotoxic effects of radiation exposure. This type of monitoring may be used as an indicator to detect early damage. The micronucleus assay has been employed to evaluate cytogenetic or mutagenic exposure in human populations including studies of biological dosimetry after radiation exposure [19].

The main objective of the present study was to investigate the mechanism responsible for MNs induction in Tunisian hospital workers exposed to ionizing radiation by using CMBN in combination with FISH. In the study, the frequency of MNs was significantly higher in the exposed workers compared to the controls. The previously mentioned biomonitoring studies on industrial radiographers

Table 3. Multivariate regression models linking potential confounding factors to biomarkers selected from the MN assay

Biomarkers	Independent variables	Regression coefficient Standardized Unstandardized B(S.E) Bêta	Adjusted R^2	p value
MNs	age	0.394	0.260 (0.216) 0.584	0.294
	gender	0.361	3.420 (3.079)	0.329
	time of exposure	0.699	22.093 (8.312)	0.040
	individual protection	0.155	8.170 (0)	0.648
	dermic affection	–0.143	–0.423 (0.690)	0.573
	general affection	–0.230	1.120 (0.132)	0.081
C+MN	age	–0.221	–0.081 (0.202) –0.176	0.709
	gender	0.868	4.570 (2.88)	0.188
	time of exposure	0.666	11.690 (7.78)	0.207
	individual protection	0.194	5.698 (0)	0.732
	dermic affection	0.402	0.661 (0.646)	0.364
	general affection	0.203	0.122 (0.020)	0.345
C–MN	age	0.530	0.341 (0.192) 0.654	0.150
	gender	–0.125	–1.150 (2.74)	0.690
	time of exposure	0.337	10.396 (7.4)	0.023
	individual protection	0.048	2.430 (0)	0.875
	dermic affection	–0.376	–1.084 (0.65)	0.153
	general affection	0.234	0.114 (0.04)	0.07
C1+MN	age	–0.252	–0.088 (0.165) 0.134	0.621
	gender	0.916	4.600 (2.35)	0.122
	time of exposure	0.808	13.526 (6.35)	0.100
	individual protection	0.206	5.780 (0)	0.673
	dermic affection	0.485	0.759 (0.528)	0.224
	general affection	0.543	0.304 (0.026)	0.184
Cx+MN	age	0.086	0.007 (0.058) –0.898	0.907
	gender	–0.022	–0.026 (0.830)	0.977
	time of exposure	0.591	10.867 (4.029)	0.036
	individual protection	1.178	2.770 (0)	0.011
	dermic affection	0.007	0.013 (0.353)	0.973
	general affection	–0.211	– 0.141 (0.01)	0.140

Abbreviations as in Table 2.
$p < 0.05$.

or hospital workers occupationally exposed to radiation showed an increase in the frequencies of MNs [20,21]. In other studies, there was a non-significant elevation of MNs [22], even when other genotoxic endpoints such as chromosomal aberrations or sister chromatid exchange were more elevated than in the controls [23].

The centromere analysis performed in this study showed that MNs in hospital workers were predominantly centromere negative MNs and the mean C–MN frequency was significantly higher in the exposed group than in the controls. This observation is clearly congruent with the well-known clastogenic properties of ionizing radiation. Vral et al. [10] reported a high percentage of spontaneous MNs containing centromeres, while mainly centromere negative MNs (C–MN) were induced after irradiation with low doses of 6°CO γ-ray. Sari-Minodier et al. [13] also showed that chromosomal damage leading to the formation of micronucleated lymphocytes in industrial radiographers was due to a markedly higher frequency of C–MN. In a study of miners who were chronically exposed to radionucleotides found in uranium mines, Kryscio et al. [24] reported that the percentage of centromere positive MNs (C+MN) was lower in the miners than in the controls. Thierens et al. [11] found a significant increase in C+MN (with no observed effect on C–MN) and concluded that aneugenic properties of radiation may be important after long-term chronic low-dose exposure.

The multivariate regression analysis applied to workers exposed to IR showed that C–MN and MNs frequencies were significantly associated with the time of exposure to IR after adjusting for confounding variables. Thierens et al. [25] reported similar results in which the C–MN frequency was increased due to the radiation dose that accumulated over the last 10 years in nuclear plant workers. The effect of age and gender on MNs was confirmed by the data from the Human MicroNucleus Project comprising nearly 7000 subjects [26]. The effect of gender has been described in many studies, which found higher MNs in females [27]. The increase in the MNs frequency has been primarily attributed to micronucleation of the X chromosome in females [23]. It has been also shown that a higher MN frequency observed with ageing is directly associated with decreased efficiency of DNA repair and increased genomic instability [28]. Contrary to the study of Sari-Minodier et al. [3], the influence of age and gender on the MNs frequency was not further investigated due to the relatively small size of the present study population, which could decrease the statistical power.

We were unable to find an influence of general and dermic symptoms on the levels of all the studied biomarkers, either. It has been reported that from fifty to eighty percent of patients who are treated with radiotherapy have general symptoms such as nausea and vomiting [29]. From research, no study has demonstrated these symptoms in hospital staff exposed to IR; in addition, seventy percent of the exposed group had general symptoms and the difference between these symptoms in the exposed subjects and the controls was statistically significant. Due to the lack of dosimeters, it is believed that the hospital staff was exposed to high doses of IR (more than 50 mSv/year); in addition, 18% of the hospital staff worked without any individual protection equipment, which could be responsible for the onset of disorders in the staff.

The regularity and the effectiveness of protective measures of hospital staff exposed to IR should be evaluated. Several of the analyzed exposed subjects were identified as working without any protection, although they had previously claimed to have been using it. This raised a question about the relative efficacy of the prevention measures. In this context, a correlation between the use of individual protection equipment and all the studied biomarkers (MNs, C+MN, C–MN, C1+MN and Cx+MN) was not found.

The DNA damage leading to the formation of micronucleated lymphocytes that were observed among the healthcare workers is not necessarily only due to the biological

effects of radiation. CBMN assay endpoints are related to both the environmental exposure and the genotoxic make-up of individuals. CBMN assay endpoints are not specific to the biological effects of radiation. Bonassi et al. [7] suggested that other chromosome-damaging agents might be involved in the increase in chromosome aberrations observed in the workers with uncertain exposure to IR. The working environment in hospitals is complex and includes, besides ionizing radiation, several chemical pollutants that may have genotoxic effects; thus, the possibility that the observed MNs may be partly due to chemical pollutants present in the hospital environment cannot be excluded, even if the difference between those radiation-exposed workers who reported such co-exposure and those who did not was not statistically significant.

Radiation-induced MN frequency can reflect inter-individual variability in radiosensitivity.

Various cellular repair mechanisms are responsible for the maintenance of genomic integrity in the face of environmental insults. A genetic variation in the DNA repair genes may influence individual DNA repair capacity and influence the risk of developing cancer [30].

Previous studies had shown that XRCC3 polymorphisms contribute to increased frequencies of MNs [31]. In the same context, Cho et al. [32] showed that the variant allele of XRCC3 influenced the C–MN frequency in radiographers. It may suggest an altered protein product resulting in suboptimal repair of radiation-induced damage leading to an increase in MNs.

CONCLUSION

The present study shows that chromosomal damage leading to the formation of micronucleated lymphocytes is more frequent in hospital workers exposed to a low dose of ionizing radiation than in the controls. This increase can be attributed to preferential clastogenic mechanisms.

The working environment in Tunisian hospitals is complex and includes, besides IR, several chemical pollutants that may have genotoxic effects. It cannot be determined whether the MNs frequency observed in this study is due to chronic low-dose radiation exposure, being a hospital healthcare worker, or both these factors. All these considerations highlight the constraints and difficulties involved in conducting and interpreting cytogenetic monitoring of occupationally exposed individuals. The results are particularly interesting for a developing country such as Tunisia, where biological security control measures are not too strict and extended workdays are a common phenomenon.

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