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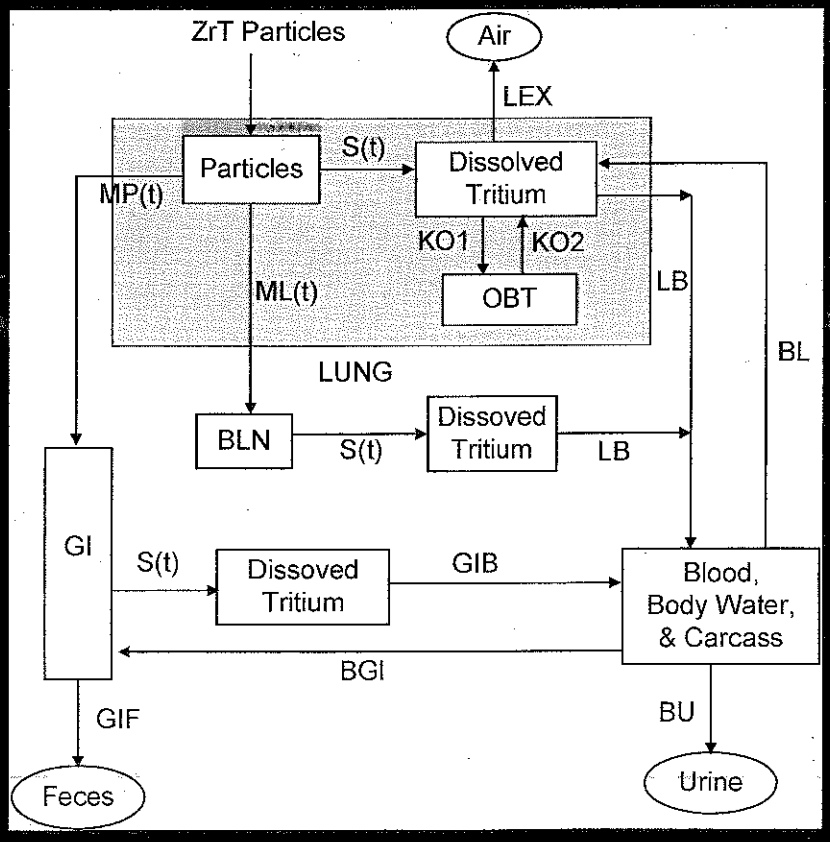

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Paper

DETECTION OF PREMATURE SEGREGATION OF CENTROMERES IN PERSONS EXPOSED TO IONIZING RADIATION

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Abstract—**We have analyzed the frequency of premature centromeric division (PCD) in medical personnel professionally exposed to low doses of radiation. They had chromosome aberrations (CAs) involving dicentric chromosomes, ring chromosomes, acentric fragments, chromosome breaks, and chromatid breaks. The study included 30 exposed subjects and 23 controls who were each analyzed by a conventional cytogenetics procedure and subsequently by fluorescent in situ hybridization (FISH). The latter was applied particularly in order to verify PCD in a specific chromosome (chromosome 18) in both metaphases and interphase nuclei. The results revealed a significant difference ($p < 0.001$) in frequencies between the two groups (exposed and controls) for all the observed variables (CAs), metaphases with PCD (MPCD), total number of chromosomes with PCD (TPCD), number of PCD metaphases in acrocentric chromosomes (MAPCD), and the total number of acrocentric chromosomes with PCD (TAPCD). The doses of ionizing radiation absorbed by the subjects' bodies were measured with thermoluminescent dosimeters once a month during the duration of occupational exposure. They were expressed in mSv, as mean annual effective doses for the period of exposure. The Spearman rank test showed a high positive correlation between total life effective dose and frequency of CAs and PCD. Based on the results obtained in this study, we suggest that PCD, as a phenomenon manifesting chromosomal instability (CIN), should be considered as a suitable cytogenetic biomarker for individuals occupationally exposed to ionizing radiation.**

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Key words: analysis, statistical; chromosome aberration; cytogenetics; exposure, occupational

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INTRODUCTION

It is well known that changes of genetic material in human lymphocytes can be induced by ionizing radiation (IR) and the development of unstable and stable structural chromosome aberrations (CAs) may be observed as the consequence of a major clastogenic effect (Rozgaj et al. 1999, 2002; Kosuba et al. 1995; Lamberti et al. 1989; Milkovic-Kraus et al. 1992; Schmid et al. 1989; Lloyd et al. 1988; Pincheira et al. 1999; Bonassi et al. 1997; Bauchinger 1995; Bigatti et al. 1988; Maffei et al. 2002; Garaj-Vrhovac et al. 2006; Cardoso et al. 2001; Touil et al. 2000). Besides its clastogenic effect, IR can also induce aneuploidy (aneugenic activity) as demonstrated by using fluorescence in situ hybridization (FISH) in both mouse (Boei and Natarajan 1995) and human populations accidentally exposed to IR (Natarajan et al. 1991, 1994, 1996).

Similar observations by Lengauer et al. (1997) and Musacchio and Hardwick (2002) revealed the relation between aneuploidy and premature centromere division (PCD). The latter is observed as early separation of one or more chromosomes in their centromere regions during prometaphase/metaphase, while the remaining chromosomes have distinctive X- or V-like morphology (Rushkovsky et al. 2003). As already described (Corona-Rivera et al. 2005), the phenomenon of PCD can be divided into three main categories: 1) Low frequency of PCD (up to 3% of the mitoses) observed in colchicine-treated lymphocyte cultures from normal individuals (Domínguez and Rivera 1992; Chamla and Saura 1993); 2) High frequency of PCD (5% or more) with mosaic aneuploidies (mosaic variegated aneuploidy) involving a variety of chromosomes observed in individuals with microcephaly, growth deficiency, severe mental retardation and risk of malignancy (Kawame et al. 1999; Plaja et al. 2001; Kajii et al. 2001; Jacquemont et al. 2002); 3) High frequency of PCD (5% or more) as the sole chromosome abnormality, either in association with miscarriages and

infertility (Rudd et al. 1983; Gabarrón et al. 1986; Bajnoczky and Gardo 1993; Keser et al. 1996) or as a trait considered to be harmless (Madan et al. 1987; Chamla 1988; Domínguez and Rivera 1992).

Furthermore, PCD is described as an almost constant finding in disorders such as Robert's syndrome (Judge 1973; Freeman et al. 1974; German 1979; Parry et al. 1986; Resta et al. 2006), Fanconi's anemia, and ataxia teleangiectasia (Buhler et al. 1987). The PCD phenomenon in the X chromosome in older women described by Fitzgerald et al. (1975) is also an age dependent aneuploidy (Izakovic and Vahancik 1984; Bajnoczky 1985; Bukvic et al. 2001). Dysfunction of centromeres, such as premature centromeric splitting and centromeric puff, in different types of neoplasia is reported as a manifestation of impaired mechanisms of space and time regulation of mitosis leading to further chromosomal instability (CIN) (Litmanovic et al. 1998).

It is not known whether PCD itself causes aneuploidy in tumor cells, but currently there are indications supporting the hypothesis about a correlation between PCD and aneuploidy (Lengauer et al. 1997). It is possible that PCD reflects some problems at the spindle checkpoint leading to chromosome loss/gain during mitosis (Vig and Wodnicki 1974; Musacchio and Hardwick 2002).

Treatment of cells with arsenite can disturb the mitotic events and subsequently induce chromosome loss, suggesting an association between the mitotic disorders, aneuploid/aneugenic potential and chromosome/gene instability during the process of carcinogenesis (Yih et al. 1997). Sakurai et al. (1999) reported that patients with type 1 multiple endocrine neoplasia, carrying a heterozygote MEN1 mutation, revealed higher PCD expression in peripheral blood lymphocytes when exposed to an alkylating diepoxybutane (DEB) agent, compared with healthy controls and a group affected by neoplasia without the mutation. Some other chemicals (i.e., pesticides) could be considered as potential inducers of PCD as noted in peripheral blood lymphocytes after in vitro treatment (Dolara et al. 1994).

The literature data indicate that PCD occurs as a consequence of an inappropriate time of segregation and separation of centromeres (Vig et al. 1989). It has to be emphasized that centromere division in the human chromosome complement is not an accidental chaotic process. Namely, Vig et al. (Vig and Sterner 1991; Vig et al. 1993) reported that the normal sequence of centromere separation starts from chromosome 18, followed by chromosomes 2, 10 and 8, while the acrocentric chromosomes divide last. On the other hand, Garcia-Orad et al. (2000) observed that the order of centromere splitting is chromosome 2, 8, 17, 18, followed by chromosomes 13–15 and 21–22.

In this study, in comparison with controls, we analyzed the PCD frequency in persons occupationally exposed to low doses of radiation. They had CAs including dicentric chromosomes, ring chromosomes, acentric fragments and chromosome breaks. A conventional cytogenetics procedure and subsequently FISH were employed for this purpose, the latter particularly in order to verify PCD in a specific chromosome (chromosome 18) in both metaphases and interphase nuclei. The aim of this study was to assess if PCD could be considered as a biomarker in workers exposed to IR.

MATERIAL AND METHODS

Subjects

Table 1 shows the characteristics of the studied groups regarding sex (male or female), age (in years), working experience (WE in years), duration of occupational exposure to radiation (DOE in years), smoking habit (smoker or non-smoker), and total life effective dose in mSv. Personal dosimeters were used for the estimation of exposure. The doses of ionizing radiation absorbed by subjects' bodies were measured with thermoluminescent dosimeters (TLD) once a month during the DOE and expressed in mSv as the mean of annual effective doses for the period of exposure. Routine personal dosimetry was performed using an automatic TLD reader (Harshaw Model 6600 with LiF:Mg Ti cards; Thermo Scientific, 81 Wyman Street, Waltham, MA 02454). The glow curve was used for sophisticated data processing with Harshaw CGCD (Computerized Glow Curve Deconvolution) software (German et al. 2000). The groups were matched for age and working experience. The sex ratio and smoker/non-smoker ratio were similar for both the control and exposed groups.

Our study included 30 subjects employed at the Clinical Center of Serbia, who were professionally exposed (daily continual exposure) to low doses of x

Table 1. General characteristics of the studied population.[a]

	Control	Exposed
Sex		
Men	15	14
Women	8	16
Age (y) (mean ± SD)	37.17 ± 7.77	36.00 ± 8.94
WE (y) (mean ± SD)	12.91 ± 6.76	12.70 ± 7.37
DOE (y) (mean ± SD)	0	12.70 ± 7.37
Smoking		
Smokers	14	15
Non-smokers	9	15
Mean total life effective dose (mSv) (range)	0	13.32 (4.81–24.76)

[a] WE—working experience; DOE—duration of occupational exposure to radiation.

radiation and 23 controls (C) not occupationally exposed to IR or chemical mutagens at their work places.

Detailed information on the occupational and medical history for all examined subjects was obtained by completing a targeted questionnaire including demographic data, smoking, alcohol intake, use of medication, and duration of exposure to radiation or chemicals. Only subjects who had not been exposed to any mutagen except for IR (exposed group) or any mutagen at all (control group) were included in the analyses.

Chromosomal aberrations (CAs) and premature centromeric division (PCD)

CAs and PCD in lymphocytes were analyzed according to a standard protocol (IAEA 1986). Whole blood cultures were prepared using RPMI 1640 medium supplemented with 10% of fetal calf serum (Life Technologies, http://www.lifetechnology.com/). The lymphocytes were stimulated with phytohaemagglutinin (PHA) (INEP-Zemun, Yugoslavia) at a concentration of 5 μg mL^{-1} for 48 h at 37°C. During the last 2 h of incubation Colchicine (0.05 μg mL^{-1}; Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103) was added to the medium. The cells were exposed to hypotonic solution (20 min) by stepwise addition of 0.075 M KCl followed by fixation (3 × 20 min) with cold methanol/acetic acid (3:1). Fixed cells were spread on slides and dried over a flame. The slides were aged for the next 5–7 d. Giemsa stained slides were coded and scored blind under a light microscope. Two hundred well-spread metaphases per subject were screened for PCD and chromosome damage. PCD was diagnosed when a separation between sister chromatids was equal to or more than the thickness of the chromatid (Madan et al. 1987; Rushkovsky et al. 2003).

Application of FISH for the analysis of PCD of chromosome 18 in metaphase and interphase nuclei

FISH was applied for the analysis of PCD in interphase nuclei and metaphases. We used the alpha satellite DNA probe L1.84 (Devilee et al. 1986) specific for the centromeric region of chromosome 18 to detect this chromosome in persons exposed to low radiation doses. The probe L1.84 was labeled with biotin-14-dATP in a nick translation reaction using a bio-nick labeling system (Gibco-BRL, Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008).

Hybridization and detection of the biotin-labeled probe were performed as described (Wilkinson 1995) with some modifications. Briefly, for each slide approximately 100 ng of the probe was precipitated and dissolved in 16 μL hybridization buffer consisting of 50% formamide, 10% dextran-sulphate, 1% SDS (Sodium Dodecyl Sulfates), 1 × Denhardt's, 2 × SSC (Saline Sodium Citrate) and 0.04M sodium phosphate pH 7.0. The probe was denatured for 10 minutes at 65°C and held on ice. The target DNA was denatured in 70% formamide/2 × SSC at 65°C for 3 min, quenched immediately in cold 70% ethanol, dehydrated through an ethanol series (70, 90, 90, 95%) and air-dried. The probe was placed on a slide, sealed under a coverslip with rubber cement, and incubated in a humidified box at 37°C overnight. After removal of coverslips in 2 × SSC at room temperature, the slides were washed twice, first in 50% formamide/2 × SSC, then in 2 × SSC for 10 min at 42°C. The slides were incubated in TNFM (4 × SSC, 0.05% *T*ween 20, 5% *N*on-*F*at *M*ilk) for 20 min at 37°C. After incubation with Fluorescein Avidin DCS (Vector Labs, 30 Ingold Road, Burlingame, CA 94010), the slides were washed in 4 × SSC/0.05% Tween 20 for 5 min at 42°C, and then with 10 × PBS (phosphate buffered saline) for 5 min at room temperature and air-dried. The slides were mounted in 1 μg mL^{-1} DAPI (Diamidino phenylindole) counterstain in Vectashield Antifade Buffer (Vector Labs) and viewed under an Olympus BX51 fluorescent microscope (Olympus America Inc., 3500 Corporate Parkway, P.O. Box 610, Center Valley, PA 18034-0610) with appropriate filters for detection of fluorescein (Spectrum Green) and DAPI and analyzed using Cytovision 3.1 software (Applied Imaging Corp., 5282 East Paris S.E., Grand Rapids, MI 49512). Chromosome 18 was scored as PCD+ in interphase nuclei and metaphases if a bipartite centromere signal was detected and as PCD− when one dot-like signal was detected.

Statistical analysis

Frequencies of CAs: chromatid and chromosome breaks, acentrics, dicentrics and ring chromosomes, as well as four parameters of PCDs, were evaluated for at least 200 metaphase cells. The frequencies were estimated for several predictor variables including ionizing radiation exposure (control group vs. exposed group), sex (men vs. women), smoking habit (smokers vs. non-smokers), age and working experience (WE). Four PCD parameters were included in the analysis: frequency of metaphase cells with PCD on any chromosome (MPCD), total number of chromosomes with PCD (TPCD), frequency of metaphase cells with PCD on acrocentric chromosomes (MAPCD), and total number of acrocentric chromosomes with PCD (TAPCD).

The control and exposed groups, sex qualification and smoking habits were coded as binary (0 or 1). Age, WE, and total life effective dose were used as continuous predictor variables.

The data for CAs and PCDs were tested for normal dispersion. MPCD and TPCD data fit a normal distribution curve ($p > 0.2$ for both variables). The frequency of chromatid breaks, chromosome breaks, acentrics, dicentrics, rings, and MAPCD and TAPCD showed statistically significant deviation from normal distribution ($p < 0.01$).

The effects of discrete predictor variables (ionizing radiation exposure, sex qualification and smoking habit) on differences in frequency of CAs and PCDs were tested by one-way ANOVA (analysis of variance) for parameters with normal distribution and the Mann-Whitney U test for parameters with discrete distribution. The presence of significant linear correlation between CAs and PCDs was determined with the Spearman rank test (nonparametric correlation test) on the whole sample (control + exposed) and the exposed group only. Correlations between continuous predictor variables (age, WE, and total life effective dose) and frequencies of CAs and PCDs were calculated as Pearson's correlations for variables with normal distribution and the Spearman rank correlations for discrete variables. The Poisson regression model with the canonical logarithmic link function was used for evaluating the effects of both discrete and continuous predictor variables on CAs and PCDs with discrete distribution only. The fit of the Poisson regression model was assessed using Pearson's X^2 and the deviance. Pearson's X^2 divided by the degrees of freedom was used as a scale factor to account for over- or under-dispersion. Since the analyzed model is log-linear, $\exp(\beta_i)$ can be interpreted as relative risk (RR). The analyses were performed using Statistica 5 (StatSoft, Inc., 2300 East 14th Street, Tulsa, OK 74104) and SAS 6.12 software on a PC.

Since FISH is more precise in estimating PCD than classical cytogenetic analysis, this molecular cytogenetic technique was performed simultaneously with conventional CA analysis. For that purpose, four subjects from the exposed group and four subjects from the control group were randomly selected and a FISH procedure was applied as described previously. The results were compared using the nonparametric Mann-Whitney U test.

RESULTS

To analyze CAs and PCDs by conventional cytogenetic methods a total of 10,600 metaphase cells were scored, 4,600 in the control group and 6,000 in the exposed group (200 per individual). The ratios of aberrations per number of analyzed cells are presented in Table 2. In comparison with frequencies for the control group, frequencies of cells with aberrations in the exposed group were increased by 2-fold for chromatid breaks, MPCD and TPCD, by 3-fold for MAPCD and TAPCD, by 5-fold for chromosome breaks, and by 8-fold for acentrics. No cells with dicentrics or rings were found in the control group, while for the exposed group the ratio was 1 cell with a dicentric per 187 cells and 1 cell with a ring per 1,500 cells. Fig. 1 illustrates the frequencies of all CAs and PCDs in the exposed and control groups.

Table 2. Ratios of aberrations per number of analyzed cells for the control and exposed groups.[a]

	Control	Exposed
MPCD	1/15.28	1/7.19
TPCD	1/12.17	1/6.48
MAPCD	1/148.39	1/39.74
TAPCD	1/112.20	1/30.93
Chromatid b.	1/176.92	1/68.97
Chromosome b.	1/575.00	1/109.09
Acentric	1/766.67	1/96.77
Dicentric	—	1/187.50
Ring	—	1/1,500

[a] MPCD—frequency of metaphase cells with PCD on any chromosome; TPCD—total number of chromosomes with PCD; MAPCD—frequency of metaphase cells with PCD on acrocentric chromosomes; TAPCD—total number of acrocentric chromosomes with PCD.

One-way ANOVA and the Mann-Whitney U test revealed that the frequencies of CAs and PCDs were significantly higher in the exposed group ($p < 0.001$) except for rings ($p > 0.05$). Furthermore, sex was without influence on the frequencies for most variables except for MAPCD (sex mean ± SD: males 2.17 ± 1.87; females 2.96 ± 2.49; $p < 0.01$) and TAPCD (sex mean ± SD: males 3.00 ± 2.62; females 4.00 ± 3.20; $p < 0.01$) and there was no statistically significant differences in frequencies for all CAs and PCDs between smokers and non-smokers. The Mann-Whitney U tests for checking differences between the sexes in the whole sample (control group and exposed group together) gave statistically significant results for MAPCD and TAPCD

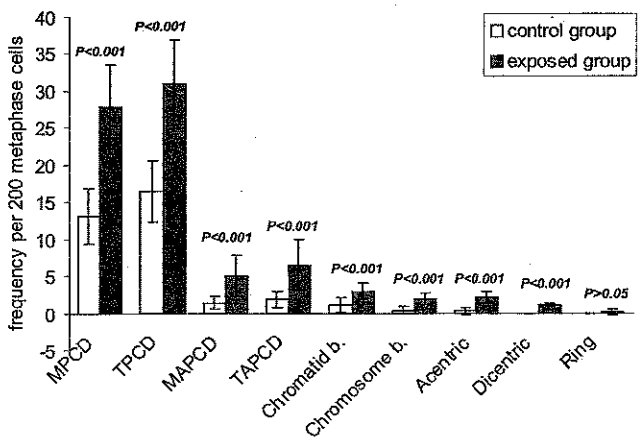

Fig. 1. The mean values (±SD) of frequencies of nine aberration parameters (for abbreviations see Material and Methods).

only, which indicated that another sex related U test should be performed for these variables within the control group and exposed group separately. These tests showed no differences between the sexes in the control group but pronounced differences appeared between them in the exposed group for both parameters ($p < 0.001$), with higher mean values for females (variable: sex mean ± SD; MAPCD: males 3.07 ± 2.20, females 6.75 ± 1.91; TAPCD: males 4.21 ± 3.14, females 8.44 ± 2.31).

The Spearman rank test showed the existence of a positive linear correlation between CAs and PCDs for most variable pairs for the whole sample (control + exposed), which indicated that an increase of frequency of one variable was coupled with an increase of frequency in the other variable (Table 3). Exceptions were the somewhat lower correlations of chromatid and chromosome breaks with MAPCD and TAPCD and the absence of correlation of rings with other variables. In the exposed group there were no correlations between CAs and PCDs for most variable pairs (Table 3).

Correlations of the WE with the age, the total life time dose, PCDs and CAs were presented in Table 4. Positive correlations of the MPCD and TPCD with WE (an increase in WE led to an increase in the MPCD and TPCD frequencies) were established, but the correlation coefficients were higher in the exposed group compared with the control group (Table 4). The analysis of residuals revealed one extreme case of an exposed woman aged 40 y (WE—12 y) with a very low frequency of MPCD and TPCD. After exclusion of this outlier from the exposed group, refitted correlation coefficients were calculated and they were higher (Table 4). However, according to data presented in Table 4, the correlations of WE with discrete dependent variables (MAPCD, TAPCD, chromatid breaks, chromosome breaks, acentrics, dicentrics, rings) were inconsistent. Thus, total life effective dose showed a high positive correlation with

Table 4. Correlation coefficients (r) between the variable pairs. ^{ref}—Refitted correlation coefficients (see results).[a]

	Control		Exposed	
	r	p	r	p
WE—age	0.86	***	0.91	***
WE—total life time dose	—	—	0.85	***
WE—MPCD	0.57	**	0.76 (0.80^{ref})	**
WE—TPCD	0.52	**	0.78 (0.79^{ref})	**
WE—MAPCD	0.60	**	0.33	ns
WE—TAPCD	0.66	***	0.41	*
WE—Chromatid b.	0.18	ns	0.46	*
WE—Chromosome b.	0.07	ns	0.2	ns
WE—Acentric	0.16	ns	0.36	*
WE—Dicentric	—	—	0.36	ns
WE—Ring	—	—	0.08	ns

[a] ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. WE—working experience; MPCD—frequency of metaphase cells with PCD on any chromosome; TPCD—total number of chromosomes with PCD; MAPCD—frequency of metaphase cells with PCD on acrocentric chromosomes; TAPCD—total number of acrocentric chromosomes with PCD.

WE (DOE), as was expected (Table 4). High positive correlations were established between total life effective dose and frequency for most CAs and PCDs when the whole sample was analyzed (Table 5). The exception was the low but significant correlation between total life effective dose and frequency of rings (Table 5). In the exposed group only, analyses showed positive correlation between total life effective dose and frequency for most CAs and PCDs, except for MAPCD, TAPCD, and chromosome breaks (Table 5). The relationships between total effective dose and frequencies of MPCD and TPCD for the exposed group are presented in Fig. 2. In the Poisson regression analysis the magnitude of the association between predictor variables and the frequency of CAs and PCDs was expressed as a relative risk factor (RR) with 95% confidence intervals and the corresponding p values. Table 6 shows the RRs for five variables (MAPCD, TAPCD, chromatid breaks, chromosome

Table 3. Spearman rank correlations between variable pairs for the whole sample (control + exposed) below diagonal and the exposed group above diagonal.[a]

	MPCD	TPCD	MAPCD	TAPCD	C.b.	Ch.b.	Acentric	Dicentric	Ring
MPCD	1	0.97^{***}	0.07^{ns}	0.16^{ns}	0.2^{ns}	0.11^{ns}	0.08^{ns}	0.24^{ns}	0.1^{ns}
TPCD	0.98^{***}	1	0^{ns}	0.1^{ns}	0.25^{ns}	0.15^{ns}	0.1^{ns}	0.26^{ns}	0.14^{ns}
MAPCD	0.63^{***}	0.63^{***}	1	0.93^{***}	-0.17^{ns}	-0.24^{ns}	0.36^{ns}	0.1^{ns}	-0.09^{ns}
TAPCD	0.67^{***}	0.68^{***}	0.96^{***}	1	-0.09^{ns}	-0.27^{ns}	0.28^{ns}	-0.01^{ns}	-0.07^{ns}
Chromatid b. (C.b.)	0.62^{***}	0.63^{***}	0.39^{**}	0.41^{**}	1	0.18^{ns}	0.25^{ns}	0.32^{ns}	0.02^{ns}
Chromosome b. (Ch.b.)	0.68^{***}	0.68^{***}	0.37^{**}	0.39^{**}	0.54^{***}	1	0.07^{ns}	0.07^{ns}	-0.20^{ns}
Acentric	0.70^{***}	0.72^{***}	0.65^{***}	0.67^{***}	0.57^{***}	0.67^{***}	1	0.16^{ns}	-0.02^{ns}
Dicentric	0.85^{***}	0.86^{***}	0.65^{***}	0.67^{***}	0.68^{***}	0.77^{***}	0.83^{***}	1	0.29^{ns}
Ring	0.25^{ns}	0.27^{*}	0.18^{ns}	0.19^{ns}	0.19^{ns}	0.11^{ns}	0.20^{ns}	0.30^{*}	1

[a] ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. MPCD—frequency of metaphase cells with PCD on any chromosome; TPCD—total number of chromosomes with PCD; MAPCD—frequency of metaphase cells with PCD on acrocentric chromosomes; TAPCD—total number of acrocentric chromosomes with PCD.

Table 5. Correlation coefficients (r) between total life effective dose and frequency of PCD and CA for the whole sample (control + exposed) and the exposed group.[a]

	Control + exposed		Exposed	
	r	p	r	p
Total life effective dose—MPCD	0.88	***	0.68	***
Total life effective dose—TPCD	0.88	***	0.71	***
Total life effective dose—MAPCD	0.65	***	0.24	ns
Total life effective dose—TAPCD	0.71	***	0.30	ns
Total life effective dose—Chromatid b.	0.69	***	0.40	**
Total life effective dose—Chromosome b.	0.73	***	0.12	ns
Total life effective dose—Acentric	0.82	***	0.40	**
Total life effective dose—Dicentric	0.92	***	0.42	**
Total life effective dose—Ring	0.38	**	0.37	**

[a] ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. MPCD—frequency of metaphase cells with PCD on any chromosome; TPCD—total number of chromosomes with PCD; MAPCD—frequency of metaphase cells with PCD on acrocentric chromosomes; TAPCD—total number of acrocentric chromosomes with PCD.

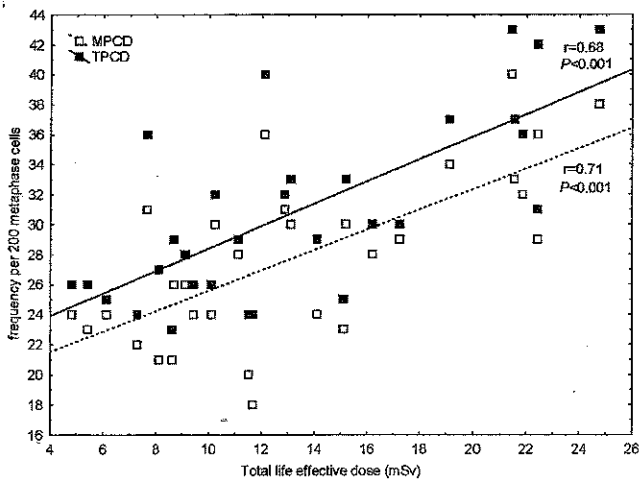

Fig. 2. Relationship between total effective dose and frequencies of metaphase cells with PCD on any chromosome (MPCD) and the total number of chromosomes with PCD (TPCD) for the exposed group.

breaks, and acentrics) regarding radiation exposure (exposed vs. control group). For four variables (MAPCD, TAPCD, chromosome breaks and acentrics) RRs were statistically significant ($p < 0.05$). The other predictor variables (WE, age, total life effective dose, sex and smoking habits) did not show statistically significant RR values because the predictor variable group (exposed vs. control group) had a very high influence on the results. Exceptions were MAPCD (RR = 2.076, $p < 0.001$) and TAPCD (RR = 1.947, $p < 0.001$) regarding sex, with higher frequencies in exposed women. These results agree with the results of Mann-Whitney U tests (see results above). Poisson regression analysis could not be performed for frequencies of dicentrics and rings because there were no cells with these aberrations in the control group (Poisson regression could not be fitted, but see the results of Mann-Whitney U tests above).

Table 6. The results of Poisson regression, predictor-group (exposed versus control).[a]

		95% confidence		
Aberration	RR	Lower	Upper	p
MAPCD	6.018	3.282	11.032	***
TAPCD	5.975	3.225	11.069	***
Chromatid break	2.078	1.099	3.931	ns
Chromosome break	5.943	2.477	14.260	**
Acentric	7.099	2.995	16.829	**

[a] ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. MAPCD—frequency of metaphase cells with PCD on acrocentric chromosomes; TAPCD—total number of acrocentric chromosomes with PCD.

According to the data presented here, the PCD frequency of metaphase cells on any chromosome, induced by ionizing radiation exposure, was considered as the only predictor factor in the samples that were subsequently analyzed by FISH. FISH was used to monitor the centromeric region of chromosome 18 in mitoses and interphase nuclei.

Figs. 3 and 4 show examples of metaphases and interphase nuclei that were scored as PCD− or PCD+. FISH analysis that did not reveal PCD are shown in Figs. 3a and 4a, where only interphase nuclei and metaphases with two separate dot-like signals specific for each chromosome 18 (scored as PCD−) were detected. Figs. 3b and 4b show interphase nuclei and metaphases with one dot-like signal (scored as PCD−) on one chromosome 18 and one bipartite centromeric signal on the other chromosome 18 (scored as PCD+) suggesting that PCD occurred in one chromosome 18 only. However, in this study, two bipartite centromeric signals that would suggest the complete and premature segregation of centromeric regions of both chromosomes 18 were not detected.

Fig. 5 illustrates the frequency of chromosome 18 PCD, expressed as a percentage in the exposed and control groups. FISH revealed that the level of chromosome 18 PCD in the exposed persons was 10.64% in chromosome metaphases and 10.76% in interphase nuclei. However, in the control group the percentage of chromosome 18 PCD was 4.65% and 5.23% in chromosome metaphases and interphase nuclei, respectively. The relative numbers of metaphases and interphase nuclei with PCDs were significantly higher in the exposed group (Mann-Whitney U test, $p < 0.05$; Fig. 5). However, our studies showed no statistically significant difference in chromosome 18 PCD presentation between interphase nuclei and metaphases within the same group (exposed or control group).

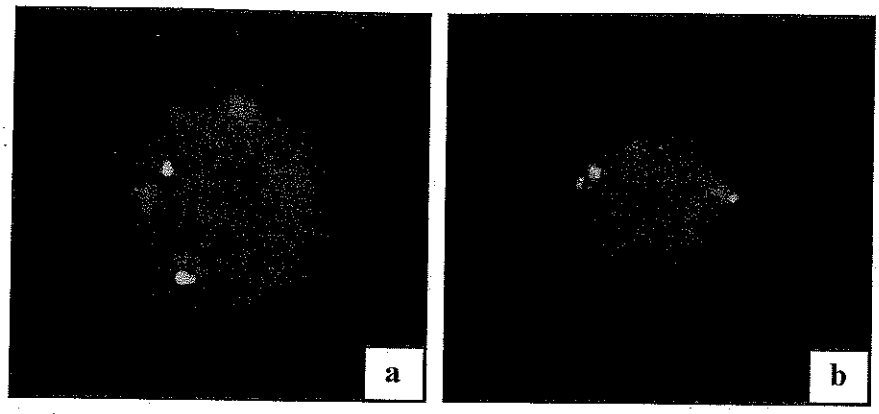

Fig. 3. Fluorescent in situ hybridization on interphase nuclei using chromosome 18 alpha satellite DNA centromeric probe L1.84: (a) Interphase nuclei of the control subjects with two separate signals, one signal for each centromeric region of chromosome 18 (scored as PCD−); (b) Interphase nucleus with one bipartite signal on one chromosome 18 representing premature centromeric division (scored as PCD+) and one signal on the other chromosome 18 (scored as PCD−).

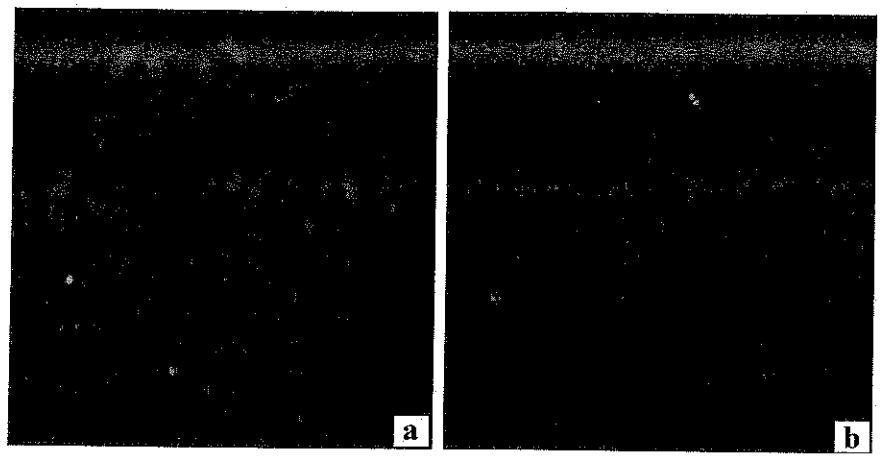

Fig. 4. Fluorescent in situ hybridization on metaphases using chromosome 18 alpha satellite DNA centromeric probe L1.84: (a) Normal metaphase with two separate signals, one signal for each centromeric region of chromosome 18 (scored as PCD−); (b) Metaphase with one bipartite signal on one chromosome 18 representing premature centromeric division (scored as PCD+) and one signal on the other chromosome 18 (scored as PCD−).

DISCUSSION

In this paper we have presented results demonstrating the significantly higher frequency ($p < 0.001$) of CAs in individuals exposed to low radiation doses compared to control non-exposed persons. Total life doses are given for all examinees from the exposed group. Our results indicate that IR induced CAs and PCDs in correlation with the size of the dose.

Controversial findings of the influence of exposure duration on the incidence of CAs have been reported by different authors. Many positive results were presented (Evans et al. 1979; Milacic 2005; Bonassi 2002), while Jha and Sharma (1991) did not find any correlation between the CA frequency and the duration of employment in a controlled area.

Using a conventional cytogenetics procedure we found errors of centromere separation in peripheral blood lymphocytes of all examined subjects, but the PCD frequency was significantly increased in the exposed group compared to the controls. This was confirmed by subsequent application of the FISH procedure.

Similarly to our results, Major et al. (1999) reported PCDs in individual chromosomes in subjects that already had CAs and they suggested that the two phenomena are not mutually exclusive or dependent on each other. However, other authors observed PCD as the only chromosome abnormality occurring in healthy individuals (Domínguez and Rivera 1992; Chamla and Saura 1993; Corona-Rivera et al. 2005). Accordingly, the question could be raised whether PCD may be considered as a normal finding, or should be regarded as the biological expression of the cellular response to IR. Buhler et al. (1987) suggested that the PCD phenomenon was not an accidental finding. If this is the case, the frequency of PCD should be different in the control and the exposed groups. The results presented here indicate

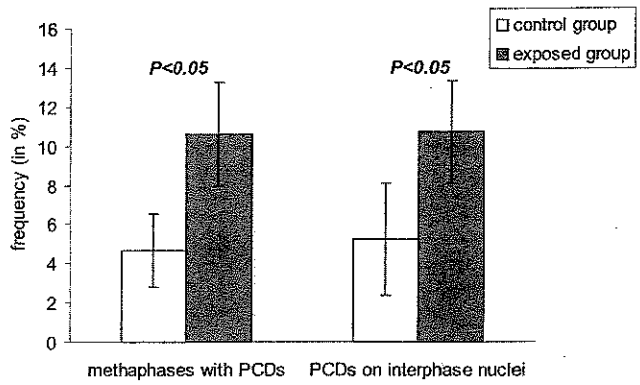

Fig. 5. The mean values (±SD) of frequencies of two aberration parameters, metaphases with PCDs and PCDs on interphase nuclei (in %) obtained by FISH.

that the PCD frequency was significantly lower in the control than in the exposed group. FISH results also revealed chromosome 18 PCDs in the control group with similar frequencies in metaphases and interphase nuclei (5%). This could be explained partially by the observations of Ikeutchi et al. (2004), who showed that PCD could be induced by an increasing hypotonic treatment (technical procedure). Other authors suggested that PCD might depend on the action of yet unknown environmental factors to which subjects from the control group were exposed (Major et al. 1999). Furthermore, it is well known that the phenomenon of PCD is associated with several human conditions/disorders (microcephaly, growth deficiency, severe mental retardation and risk of malignancy) (Kawame et al. 1999; Plaja et al. 2001; Kajii et al. 2001; Jacquemont et al. 2002) and that PCD may be increased in some illnesses such as Alzheimer's disease (White et al. 1981; Moorhead and Heyman 1983; Migliore et al. 1997; Spremo-Potparevic et al. 2004). Numerous studies in vitro and in vivo have indicated that PCD can be induced by some genotoxic agents (Gimmier-Luz et al. 1990; Major et al. 1999; Dolara et al. 1994). None of these conditions occurred in subjects from the control group and, to the best of our knowledge, the examinees were not exposed to any genotoxic agents.

Although there were no differences between the sexes for most of the tested variables, our study demonstrated a higher PCD frequency for both MAPCD and TAPCD in women than in men. No statistically significant differences between smokers and non-smokers were observed for all nine tested variables (CA and PCD). This confirms the observations of Tawn and Cartmell (1989), Chung et al. (1996), and Lovreglio et al. (2006) who reported that smoking habit is without influence on CAs. However, regular regression analysis revealed an increased correlation coefficient for two variables (MPCD and TPCD) in the exposed group regarding age and work experience in comparison with the control group.

Furthermore, Poisson regression analysis established significantly high RR for four variables (chromosome breaks, acentric fragments, MAPCD and TAPCD) connected with radiation exposure. It is interesting to point out that aneuploidy could be considered as a consequence of centromeric dysfunction, such as PCD, and that PCD is a phenomenon representing CIN, which plays an important role in malignant transformation (Lengauer et al. 1997). CIN can occur via chromosomal loss or breakage. Therefore, PCDs and CAs are useful biomarkers that could also provide a tool for measurement of both chromosomal loss and breakage in subjects professionally exposed to IR. Since PCD may be observed as a phenomenon representing the manifestation of CIN in exposed persons, our studies led us to consider PCD as a possible parameter of genotoxic risk for persons occupationally exposed to low doses of IR.

It has been suggested that IR may damage the structural elements of the chromosome necessary for normal disjunction (Bond and Chandley 1983). Such damage might lead to DNA adducts or DNA-protein cross-linking, which can increase chromosome loss (Mormiter et al. 1981). PCD of sister chromatids may also conceivably cause aneuploidy (Vig 1984). Touil et al. (2000) showed that the aneugenic effect of radiation is less clearly dose dependent at lower doses, suggesting an apparent threshold below which no change could be demonstrated. At high radiation doses the major mechanism for γ-ray-induced aneuploidy is related to chromosome loss through non-disjunction, as has been demonstrated using x rays (Touil et al. 2000).

In conclusion, the results obtained in this study using cytogenetic techniques and FISH to monitor PCD have shown that an increased frequency of bipartite signals (PCD+) can be recorded in both individual metaphases and interphase nuclei in subjects exposed to IR. As a phenomenon representing the manifestation of chromosomal instability, we suggest that PCD should be considered as a possible cytogenetic biomarker for individuals occupationally exposed to IR.

The results presented here were obtained from preliminary studies on a relatively small sample, so further studies including a larger number of subjects combined with in vitro investigations should be performed in order for the observed phenomena to be understood better. However, we believe that our data may still contribute to a clearer understanding of the PCD phenomenon, which might allow closer insight into molecular events underlying CIN.

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