Chromosome analysis of nuclear power plant workers using fluorescence in situ hybridization and Giemsa assay

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The aim of this study was to evaluate the genotoxic effects of ionizing radiation in vivo in exposed Bulgarian nuclear power plant workers by using classical cytogenetic and molecular cytogenetic analyses of peripheral lymphocytes. Chromosome analysis using fluorescence in situ hybridization (FISH) and Giemsa techniques was undertaken on 63 workers and 45 administrative staff controls from the Bulgarian Nuclear Power Plant. Using the Giemsa method, the frequencies of cells studied with chromosome aberrations, dicentrics plus rings and chromosome fragments in the radiation workers were significantly higher compared with the control group ($P = 0.044$, $P = 0.014$, and $P = 0.033$, respectively). A significant association between frequencies of dicentrics plus rings and accumulated doses was registered ($P < 0.01$). In the present study, a FISH cocktail of whole chromosome paints for chromosomes 1, 4 and 11 was used. A significant association between frequency of translocations and accumulated doses was also observed ($P < 0.001$). Within the control group, a correlation was found between age and the spontaneous frequency of translocations. No correlation was found between smoking status and frequency of translocations. When compared with the control group, workers with accumulated doses up to 100 mSv showed no increase in genome translocation frequency, whereas workers with accumulated doses from 101 to 200 mSv showed a statistically significant doubling of genome translocation frequency ($P = 0.009$). Thus, in cases of chronic exposure and for purposes of retrospective dosimetry, the genome frequency of translocations is a more useful marker for evaluation of genotoxic effects than dicentric frequency.

Keywords: chromosome aberrations; occupational exposure; nuclear power plant workers; FISH; dose accumulation

INTRODUCTION

Chromosome aberrations are well-established biomarkers of the genotoxic effects of radiation exposure and their expected consequences, such as increased cancer risk [1–4].

While conventional cytogenetic analysis is the method used to determine dicentrics and rings, an unstable type of aberration, the fluorescence in situ hybridization (FISH) technique is a rapid, sensitive and suitable technique for monitoring low–dose radiation exposure [5–8], and for retrospective dosimetry [6].

The only nuclear power plant in Bulgaria, ‘Kozloduy’, is located on the North Bulgarian border on the Danube riverbank. Until 2006, there were two big departments of energy production: EP-1 (Units 1, 2, 3 and 4) and EP-2 (Units 5 and 6).

Personal occupational exposure to ionizing radiation is under regular control. Irradiation of the groups in this study is mainly external, and mainly γ-radiation, with a negligible exposure to β-radiation and neutrons. γ-radiation is measured monthly by film and thermoluminescent dosimeters.

The occupational exposure is a prolonged exposure to low doses and low dose rates. It is now known that possible adverse health effects of such radiation exposure are stochastic. Malignant diseases with different latent periods are of basic importance. Follow-up cytogenetic monitoring was
performed, during their occupational exposure, on a representative group of NPP workers subjected to regular physical dosimetry.

**MATERIALS AND METHODS**

**Study subjects**

The operation of the Bulgarian Nuclear Power Plant was started in the late 1970s. According to the Basic Norms of Radiation Protection in Bulgaria, until the year 2000 the annual individual dose limit was 50 mSv/y. Most of the employees having relatively high recorded accumulated doses were included in this study. The accumulated dose was measured during the person’s entire working time at the nuclear power plant using personal dosimeters. In this study, the doses of exposed workers for the last 5 years ranged from 2.0–3.0 mSv/year. Relatively higher doses were accumulated in the beginning of their working times. The working years varied between 12 and 32 years, except for two workers with only 2 and 5 years. A matched control group was collected from administrative staff of the NPP. Subjects with accumulated doses of up to 2 mSv were accepted as controls. Just four of the control employees had accumulated doses of between 0.98 and and 1.85 mSv.

Standard Giemsa staining and fluorescent in situ hybridization chromosome analyses were performed on 108 healthy persons: 63 male workers from NPP ‘Kozloduy’, with ages ranging from 33–53 years (mean age: 43.07 ± 0.4 years), and 45 control individuals (84.8% males and 15.2% females) with ages ranging from 18 to 57 years (mean age: 37.68 ± 1.3 years).

Workers were grouped according to their accumulated dose of radiation: up to 100 mSv (excluding those with accumulated doses up to 2 mSv), 101–200 mSv, 201–300 mSv, 301–400 mSv and >400 mSv.

NPP workers and controls were screened to ensure that study data on the relationship between cytogenetic damage and accumulated dose was not biased by differences in the two groups’ lifestyles: age at the time of NPP employment, smoking habits, alcohol and coffee intake, medication, chronic diseases, radiological medical examinations and exposure to damaging chemical agents (mainly pesticides) were obtained by the administration of personal questionnaires at the time of blood sampling. These data are summarized in Table 1.

**Lymphocyte cultures and slide preparation**

Peripheral blood lymphocytes were cultured for 50 h in RPMI 1640 (Sigma) medium supplemented with 15% fetal calf serum (Sigma), 100 µg/ml streptomycin, and 2% phytohemagglutinin. Colcemid was added for the final 2 h of culture. Bromodeoxyuridine was added to a number of cultures at the beginning of the study to determine the optimum culture time, and was added thereafter on a regular basis to ensure that the maximum numbers of cells were in their first in vitro division.

Harvesting of the cells was performed according to standard techniques with exposure to 75 mM potassium chloride followed by fixation in 3:1 methanol:acetic acid solution. Slides were prepared following storage of fixed preparations at −20°C.

**Cytogenetic methods**

Five hundred Giemsa stained metaphases from most of the test subjects were scored for dicentrics and rings by two independent observers.

Since FISH analysis of bicolored aberrations detects only a portion of the total aberrations present in a cell, the exchange frequency was converted to a whole genome equivalent using the formula derived by Lucas et al. [9]: $F_p = 2.05 f_p(1 - f_p)F_g$, where $F_p$ is the frequency of translocations detected by FISH, $f_p$ is the painted fraction of the genome, and $F_g$ is the genomic aberration frequency. Chromosomes 1, 4 and 11 comprise approximately 19% of the human genome. Human chromosome #1 comprises 8.22%, chromosome #4 6.34% and chromosome #11 4.52% of the human genome [10]. Knowing what proportion of the genome that is colored allows the calculation of correction factors for individual chromosomes that bring results for the entire genome.

Analysis was carried out manually using an Olympus microscope with an epifluorescence attachment. In addition,

| Table 1. General characteristics of the study population with respect to age, smoking and alcohol intake |
|-----------------------------------------------|
|                               | NPP workers | Controls |
| Sex                           | No. (%)     | No. (%)  |
| • men                        | 63 (100)    | 38 (85)  |
| • women                      | 0 (0)       | 7 (15)   |
| Age (years)                  | No. (%)     | No. (%)  |
| • 18–29                      | 0 (0)       | 5 (11)   |
| • 30–39                      | 23 (36)     | 20 (45)  |
| • 40–49                      | 32 (51)     | 14 (31)  |
| • >50                        | 8 (13)      | 6 (13)   |
| Smoking status               | No. (%)     | No. (%)  |
| • yes                        | 44 (70)     | 29 (64)  |
| • no                         | 19 (30)     | 16 (36)  |
| Alcohol intake               | No. (%)     | No. (%)  |
| • yes                        | 52 (83)     | 32 (71)  |
| • no                         | 11 (17)     | 13 (29)  |
a computerized image analysis system with a camera was used to capture and confirm all aberrations. Only cells containing approximately 46 chromosomes and the correct amount of painted material were included. All aberrations were described according to PAINT nomenclature [5, 6]. Simple translocations and dicentrics were recorded as reciprocal, or two-way, if both bicolored chromosomes were present in the cell and terminal, or one-way, if only one bicolored chromosome was present. Incomplete rearrangements were included in the latter category. Because recent evidence has shown that the majority of apparently terminal or one-way rearrangements are actually reciprocal [11], for the purposes of this report only total numbers of translocations (reciprocal, terminal and single interstitial translocations) and dicentrics are presented.

### Statistical analysis

Data from different groups, as well as the influence on the aberrations of age, accumulated doses and smoking status were estimated by IBM SPSS Statistics 20.

### RESULTS

#### Chromosome analysis by classical Giemsa assay

A total of 52,374 metaphases were analyzed by the classical Giemsa-staining cytogenetic method (Table 2). The frequencies of studied cells with chromosome aberrations, dicentrics plus rings and chromosome fragments in the radiation workers were significantly higher when compared to the control group \((P = 0.044, P = 0.014, \text{ and } P = 0.033, \text{ respectively; Table 2}).

When applying multiple regression analysis to estimate the influence of accumulated dose on the frequency of dicentrics plus rings, a linear relationship was found between dose and dicentric plus rings yield, with coefficient of determination \(R^2 = 0.09683\) (Figure 1).

The relationship between accumulated doses and observed chromosomal damage was corroborated by correlation analysis using the Spearman test (IBM SPSS Statistics 20). A moderate correlation was observed between accumulated doses and frequencies of cell with aberrations and chromosome fragments, with correlation coefficients ranging between \(r = 0.27\) and \(r = 0.35\), and statistical significance \((P < 0.01)\). Moderate correlation was observed with dicentrics (correlation coefficient \(r = 0.27\; P < 0.01)\).

The distribution of smokers and non-smokers was approximately equal in both groups (70% and 64% smokers in workers and controls, respectively). We observed no correlation between induced chromosomal aberrations and smoking or alcohol consumption.

Data on the chromosome analysis of peripheral lymphocytes of NPP workers in the study group with different accumulated doses are shown in Table 3. No increase is observed in the frequency of dicentrics plus rings in the group with accumulated doses up to 100 mSv. Increased frequencies were observed when the accumulated doses were between 101–200 mSv, where the frequency of dicentrics plus rings was increased 3-fold compared with the control group. For the study group with accumulated doses between 201 and 300 mSv, there was no significant difference in either the cells with aberrations nor in the frequency of dicentrics plus rings compared with the control group. In groups with accumulated doses > 300 mSv, and up to 730 mSv, the frequency of dicentrics plus rings increases more abruptly and is accompanied by a significant increase in cells with aberrations \((P = 0.03 \text{ and } P = 0.006, \text{ Mann Whitney Test}).

#### Table 2. Frequencies of chromosomal aberrations in control and NPP workers

|                  | Workers                  | Controls                  | P-value |
|------------------|--------------------------|---------------------------|---------|
|                  | No. | mean per 100 cells | S.E.M  | No. | mean per 100 cells | S.E.M  |   |
| Number of subjects | 63  | 30 521                |        | 45  | 21 853                |        |   |
| Number of cells   |      |                       |        |      |                       |        |   |
| Cells with aberrations | 599 | 1.96 | 0.14 | 314 | 1.44 | 0.11 | 0.044* |
| Dicentrics plus rings | 27  | 0.09 | 0.02 | 7   | 0.03 | 0.01 | 0.014* |
| Chromosome fragments | 399 | 1.31 | 0.08 | 202 | 0.92 | 0.08 | 0.033* |
| Chromatid fragments | 197 | 0.65 | 0.09 | 97  | 0.44 | 0.06 | 0.07  |
| Chromatid exchange | 30  | 0.11 | 0.03 | 13  | 0.1  | 0.02 | 0.2   |
| Rogue cells       | 4   | 0.0044 | 0.0044 | 1   | 0.0127 | 0.006 | 0.2   |

*\(P < 0.05\), when compared with controls. S.E.M. = standard error of the mean.
Chromosome analysis by FISH

The present study comprised a total of 139,469 FISH painted metaphases, or about 41,309 cell equivalents after conversion to the fraction of genome painted. Calculated genome frequencies in both groups—controls and workers—on exchange types of aberrations per 1000 cells are given in Table 4. As becomes evident from Table 4, more than a doubling of frequency of translocations was observed in the worker group compared with controls, the difference being significant ($P < 0.001$).

Reciprocal, terminal and few interstitial translocations (insertions) were recorded. A total of 27 interstitial translocations were found in workers, and 7 were found in the control group. This type of translocation is considered as a complex exchange. It has been experimentally established that complex-type aberrations are induced by neutron or other types of high-LET radiation [12].

Our results for controls correlate with published data [13–15], which have reported a spontaneous frequency of 3–8 translocations per 1000 cells. Our results indicate that the frequency of translocations in workers is about two times higher than the spontaneous level. The Mann Whitney test showed a significant increase in the frequency of translocations in the worker group ($P < 0.001$).

Single cells with trisomy for chromosomes 1, 4 and 11 were observed in both groups. In five workers, we found

Table 3. Cytogenetic results (classical method), pooled data for dose groups

| Dose (mSv) | No. of individuals | No. of cells | Cells with aberrations | Dicentrics + rings % ± S.E.M. | Chromosome fragments % ± S.E.M. | Chromatid fragments % ± S.E.M. |
|------------|-------------------|--------------|------------------------|-------------------------------|-------------------------------|-------------------------------|
| Controls   | 45                | 21,853       | 0.95 ± 0.1             | 0.03 ± 0.01                   | 0.95 ± 0.1                    | 0.45 ± 0.06                   |
| <100       | 6                 | 3,000        | 0.87 ± 0.2             | 0.02 ± 0.02                   | 0.43 ± 0.2                    | 0.43 ± 0.1                    |
| 101-200    | 18                | 8,561        | 1.71 ± 0.18            | 0.09 ± 0.04                   | 1.1 ± 0.1                     | 0.58 ± 0.1                    |
| 201-300    | 13                | 6,095        | 1.61 ± 0.2             | 0.07 ± 0.03                   | 0.9 ± 0.16                    | 0.55 ± 0.10                   |
| 301-400    | 14                | 6,865        | 2.09 ± 0.128*          | 0.09 ± 0.03*                  | 1.63 ± 0.4*                   | 0.64 ± 0.1                    |
| >401       | 12                | 6,000        | 2.92 ± 0.5*            | 0.13 ± 0.04**                 | 1.75 ± 0.3*                   | 0.9 ± 0.29                    |

* $P < 0.05$, ** $P < 0.01$, when compared with controls. S.E.M. = standard error of the mean

Fig. 1. Individual frequencies of dicentrics plotted against cumulative doses. The straight line represents the linear regression of the data.
Table 4. Genome frequency of translocations (FISH method), pooled data for dose groups

| Dose (mSv) | No. of individuals | No. of cells | No. of translocations | No. of genome equivalents analyzed | Genome frequency of translocations ± S.E.M. (× 10⁻³) |
|------------|-------------------|-------------|----------------------|-----------------------------------|---------------------------------------------|
| Controls   | 45                | 60,325      | 121                  | 17,939                            | 6.75 ± 0.83                                |
| Workers    | 63                | 79,144      | 325                  | 23,370                            | 13.91 ± 1.2*                               |
| <100       | 6                 | 4,948       | 11                   | 1,573                             | 6.99 ± 1.55                                |
| 101–200    | 18                | 20,081      | 62                   | 6,386                             | 9.71 ± 1.8*                                |
| 201–300    | 13                | 15,048      | 72                   | 4,639                             | 15.52 ± 3.26**                             |
| 301–400    | 14                | 19,847      | 72                   | 5,598                             | 12.86 ± 2.01**                             |
| > 401      | 12                | 19,220      | 108                  | 5,174                             | 20.87 ± 2.95*                              |

*P < 0.01, **P < 0.001, when compared with controls. S.E.M. = standard error of the mean.

multi-aberrant cells, containing ≥5 exchange-type aberrations; dicentrics, polycentrics, interstitial deletions, and chromatid/chromosome fragments. These cells were first reported in 1970 by Bloom et al. [16], and were named ‘rogue cells’ in 1986 by Awa and Neel [17], who saw them in a study of the offspring of survivors of the Hiroshima atomic bombing.

When compared with the control group, workers with accumulated doses of ≤100 mSv showed no increase in genome translocation frequency, whereas workers with accumulated doses from 101–200 mSv showed a statistically significant doubling of genome translocation frequency (P = 0.009; Table 4). Workers with accumulated doses between 201 and 300 mSv showed the same trend of statistically significant increased frequency of genomic translocations compared to control subjects (P < 0.001, Table 4).

In the group with doses of 301–400 mSv, there was a slight decrease in the frequency of translocations compared to the 201–300 mSv group, but again there was a significant difference between workers and controls (P < 0.001). In subjects with high doses (> 401 mSv), there was a statistically significant 3-fold increase in the frequency of translocations compared to control subjects (P = 0.002).

Significant association was found between accumulated doses and the observed translocation frequency by applying the Spearman correlation analysis test (IBM SPSS Statistics 20), with correlation coefficient r = 0.569 and P < 0.001. Length of employment showed a statistically significant impact on genomic frequency of translocations (correlation coefficient r = 0.524, P < 0.001).

A linear relationship was observed between accumulated radiation dose and frequency of translocations, with coefficient of determination R² = 0.2536 when applying multiple regression analysis to evaluate the influence of the dose on the frequency of translocations (Figure 2).

Age significantly correlated with genome frequency of translocations (with a correlation coefficient of r = 0.250 and P < 0.05). Within the control group, correlation was found between age and the spontaneous frequency of translocations (r = 0.416, P = 0.016).

There was also a low but statistically significant effect of alcohol consumption, increasing the frequency of translocations (correlation coefficient r = 0.233, P < 0.05), unlike smoking status, which showed no correlation.

DISCUSSION

The present study is the first to investigate nuclear industry workers in Bulgaria with low-dose chronic radiation exposure through the application of classical cytogenetic analysis and molecular cytogenetic analysis to assess the frequency of unstable and stable chromosome aberrations, respectively. In our study dicentrics and/or rings were found both in groups of workers from the restricted area of the NPP and in the control group. The frequency of dicentrics plus rings observed in the administrative staff was 0.03%, which coincides with the laboratory data released from the mid-1970s [18], and is even lower than that of the leading laboratories in the world [19–21].

Comparisons between NPP workers and administrative staff controls with the same frequency parameters were made, with individual physical dosimetry on all study participants.

The results obtained show a significant increase in the frequency of stable, as well unstable chromosomal aberrations in peripheral blood lymphocytes of exposed workers compared to controls from the administrative staff of the company. This increase is more evident for the frequency of stable translocations, with statistical significance P < 0.001, than unstable dicentrics and rings (P < 0.01). When we compared the chromosomal aberration frequencies between the control and worker groups according the accumulated doses, the most sustainable and significant increase with the dose received was observed in the incidence of translocations. The frequency of stable translocations was significantly increased in peripheral blood lymphocytes of exposed workers with accumulated doses above 101–200 mSv compared with the control group. The increase of frequency of dicentrics and rings appeared in the group of workers with doses 101–200 mSv, but reach statistical significance in
workers with accumulated doses of 301–400 mSv, compared with controls. These data suggest that FISH analysis of stable translocations is a more sensitive technique than dicentric analysis for estimation of genetic damage after prolonged radiation exposure.

Chromosomal aberration analysis by means of conventional or FISH techniques is a widely used method for monitoring genotoxic effects of low-dose environmental and professional radiation exposure [7, 19–24]. Many authors report increased genetic damage in peripheral blood lymphocytes of human populations exposed to relatively low-dose occupational radiation exposure. For example, significant increases in the incidence of dicentrics plus rings were observed in follow up studies of populations around Chernobyl [24–28], liquidators of the Chernobyl accident [27], and the population living in buildings with chronic low-dose exposure [29]. Significant association between translocation frequency and accumulated dose in nuclear workers was observed by many authors [30, 31]. In this study we found a positive correlation between the level of dicentrics and symmetrical translocations and accumulated doses.

Symmetrical aberrations, such as balanced translocations, successfully pass through cell division and accumulate over time. Increased frequency of symmetrical translocations has been noted many years after exposure to ionizing radiation [7, 32]. The advantage of FISH analysis for the evaluation of stable aberrations is the ability to obtain broader information about induced chromosomal aberrations, whatever the time of their induction.

When assessing the effects of chronic low-dose radiation on humans, it is important to take into account factors such as smoking and medical radiological studies that could affect the cytogenetic results. In this study of people professionally exposed to ionizing radiation, we found no evidence for a synergistic effect with ionizing radiation for the confounding factors investigated. We observed no correlation between induced chromosomal aberrations and smoking, coffee consumption, medication, chronic diseases, radiological medical examinations or exposure to pesticides. There was a low but statistically significant effect of alcohol consumption, increasing the frequency of translocations ($P < 0.05$), which might have been due to the low number of subjects who denied alcohol intake. Several authors [30, 33] have reported the influence of smoking on induced translocations for retired nuclear power workers, but in the current study such influence was not found to be statistically significant.

Age was significantly correlated with genome frequency of translocations in all investigated subjects in our study ($P < 0.05$). Such a correlation was found for the investigated control subjects with mean age 37.7 (18–57), but not for the group of workers, probably because their age range was more limited, from 33–53 years old. This has been confirmed by many studies on the influence of age on the frequency of stable aberrations [34–38]. It has been suggested that the increase in translocation frequency with age is caused by age-dependent biological processes [39].

Logically, length of employment showed a statistically significant impact on genomic frequency of translocations ($P < 0.001$).
In this study we found ‘rogue cells’, multi-aberrant cells with dicentrics, polycentrics, translocations and multiple fragments. Such single cells with multiple aberrations of a chromosome type found in very low frequencies were observed in peripheral blood lymphocytes of clinically healthy individuals with no proven effect from a mutagen. There are many assumptions about the origin of ‘rogue cells’, but so far their origin is unclear. According to some authors, their presence is associated with viral infection by the human polyoma virus JC and its replication [40]. The possibility of radiation effects, such as low-dose alpha radiation, cannot be ruled out [41], as this has been observed in newborns in the Indian state of Kerala, famous for its high natural radiation background, in uranium miners in Namibia, and in liquidators of the Chernobyl accident, and others [41–43].

We observed a linear relationship between the frequency of stable and unstable aberrations and accumulated dose when applying regression analysis with coefficients of determination for the frequency of dicentrics and rings: \( R^2 = 0.097 \), and for the frequency of translocations: \( R^2 = 0.254 \). As can be seen, the linear relationship is more pronounced for the frequency of stable aberrations. A linear relationship between accumulated dose and low frequency of translocations was reported by Tawn, 2004 for retired workers from the Sellafield nuclear plant [31].

In conclusion, our study demonstrates that accumulated radiation dose is the dominant influence on chromosome aberration frequency in the peripheral blood lymphocytes of NPP workers. We can say that in cases of chronic low-dose radiation exposure, such as for the radiation exposure of NPP workers, the genome frequency of stable translocations is an extremely useful marker for evaluation of genotoxic effects.

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