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ORIGINAL PAPER

mFISH analysis of chromosome aberrations in workers occupationally exposed to mixed radiation

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Abstract We performed a study on the presence of chromosome aberrations in a cohort of plutonium workers of the Mayak production association (PA) with a mean age of 73.3 ± 7.2 years to see whether by multi-color fluorescence in situ hybridization (mFISH) translocation analysis can discriminate individuals who underwent occupational exposure with internal and/or external exposure to ionizing radiation 40 years ago. All Mayak PA workers were occupationally exposed to chronic internal alpha-radiation due to incorporated plutonium-239 and/or to external gamma-rays. First, we obtained the translocation yield in control individuals by mFISH to chromosome spreads of age-matched individuals and obtained background values that are similar to previously published values of an international study (Sigurdson et al. in Mutat Res 652:112–121, 2008). Workers who had absorbed a total dose of >0.5 Gy external gamma-rays to the red bone marrow (RBM) displayed a significantly higher frequency of stable chromosome aberrations relative to a group of workers exposed to <0.5 Gy gamma-rays total absorbed RBM dose. Thus, the translocation frequency may be considered to be a biological marker of external radiation exposure even years after the exposure. In a group of workers who were internally exposed and had incorporated plutonium-239 at a body burden >1.48 kBq, mFISH revealed a considerable number of cells with complex chromosomal rearrangements. Linear associations were observed for translocation yield with the absorbed RBM dose from external gamma-rays as well as for complex chromosomal rearrangements with the plutonium-239 body burden.

Keywords Translocations · Complex aberrations · External gamma-rays · ^{239}Pu body burden · Mixed radiation exposure

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Introduction

The formation of chromosome aberrations has long been considered to be a sensitive biological marker of ionizing radiation (IR)-induced genome damage (Lemos et al. 2010; Iwasaki et al. 2011; IAEA 2011; Pernot et al. 2012). Unstable and stable chromosome aberrations (dicentrics and translocations, respectively) have been established as biological markers of external radiation exposure (Bauchinger et al. 2001; IAEA 2001, 2011; Edwards et al. 2005, 2007; Tucker 2008). However, in vivo studies on the effects of internal alpha-radiation to the human genome are sparse and the data often inconsistent, since the majority of data on aberration induction with alpha emitters stem from in vitro investigations (Brandom et al. 1990; Testard et al. 1997; Anderson et al. 2000, 2003, 2005, 2006; Boei et al. 2001; George et al. 2001; Moquet et al. 2001; Barquinero et al. 2004; Okladnikova et al. 2005, 2009; Tawn and Whitehouse 2005; Hada et al. 2007; Janet et al. 2008; Curwen et al. 2012). Currently, there are no data on the consequences of mixed prolonged occupational exposure with internal and external IR sources with respect to translocation yield and persistence.

Early in vivo studies which performed a routine staining did not reveal an increased chromosome aberration yield in

workers exposed to internal alpha-radiation (Dolphin 1971; Hempelmann et al. 1973; Voelz et al. 1985), while Brandom et al. (1979, 1990) reported an increase in dicentrics, rings, inversions and translocations in Rocky Flats workers after plutonium-239 aerosol inhalation. The authors concluded that the increase in chromosome aberration yield was attributed to internal exposure to plutonium-239 rather than to external gamma-rays. A study on genome instability in Sellafield workers based on a routine Giemsa analysis technique as well as on the fluorescence plus Giemsa staining method showed that the unstable aberration yield in workers exposed to internal alpha-radiation was similar to the unstable aberration yield in control individuals (Whitehouse and Tawn 2001). To the contrary, other studies of Sellafield workers' peripheral blood lymphocytes reported increased frequencies of dicentrics (Schofield 1980) and stable chromosome aberrations (Tawn et al. 1985; Whitehouse et al. 1998). Livingston et al. (2006) revealed an increased chromosome aberration yield in former workers of the Rocky Flats plutonium production facilities compared with controls; the authors of this study showed an association of the translocation yield with the exposure dose for the group of workers with high RBM doses of internal alpha-radiation due to radionuclide incorporation.

Studies of chromosome aberrations in peripheral blood lymphocytes of Mayak PA workers found a pronounced genotoxic effect of plutonium-239 and revealed a correlation between both the total number of aberrations and the number of dicentrics and an absorbed RBM dose from internal alpha-radiation due to incorporated plutonium-239 (Okladnikova et al. 2005, 2009). multi-color fluorescence in situ hybridization (mFISH) analysis of chromosome spreads of Mayak PA workers showed that the complex chromosomal rearrangements were significantly increased in plutonium production workers with high plutonium-239 body burdens compared with reactor/plutonium production workers with moderate plutonium-239 body burdens as well as to controls (Hande et al. 2005). Popova et al. (2004) demonstrated that the frequency of cells with multiple chromosome aberrations in workers of Siberian Chemical Industrial Complex with >13 nCi plutonium-239 body burden was tenfold increased relative to the background frequency. The investigation of various social groups disclosed that cells exhibiting complex aberrations showed the highest yield in workers of a radiochemical facility who had been exposed to mixed radiation (Aseeva et al. 2009).

The present study aimed to assess the spectrum and yield of chromosome aberrations in Mayak PA workers occupationally exposed to mixed radiation using the mFISH assay.

Materials and methods

The study group consisted of Mayak PA workers who had experienced prolonged occupational external gamma-ray exposure and/or internal alpha-radiation exposure due to incorporated plutonium-239 (Table 1). Chromosome spreads for 60 Mayak PA workers (42 males and 18 females) were analyzed in the present study; the mean age of the study individuals was 73.3 ± 7.2 years. 85 % of the study individuals were employed at Mayak PA during the first decade of its operation (1948–1958). 98.3 % of workers were hired prior to the age of 30 years; the mean age of workers as of the beginning of their career at Mayak PA was 23.1 ± 3.9 years (23.2 ± 4.3 years in males and 22.8 ± 2.9 years in females). Reactor workers who had experienced only external gamma-ray exposures comprised 16 % of the study group. The majority of the study individuals (84 %) were exposed to mixed radiation. Individual doses from external gamma-radiation according to an updated dosimetry system *Doses-2008* (Vasilenko et al. 2007) were used in this study.

Data on plutonium-239 (Pu-239) incorporation were provided by the Department of Internal Dosimetry of the Southern Urals Biophysics Institute. The incorporated

Table 1 Characteristics of the study group (Mayak PA workers)

	Male	Female	Both
Total dose from external gamma-rays to the whole body ± SE, Gy	1.52 ± 1.15	1.16 ± 1.01	1.41 ± 1.12
Median (min; max)	1.78 (0.0; 3.95)	0.74 (0.09; 3.12)	1.40 (0; 3.95)
Total absorbed dose from external gamma-rays to RBM ± SE, Gy	0.93 ± 0.78	0.72 ± 0.71	0.87 ± 0.76
Median (min; max)	0.93 (0.0; 2.69)	0.36 (0.04; 2.26)	0.80 (0; 2.69)
Plutonium-239 body burden ± SE, kBq	1.82 ± 3.56	4.21 ± 5.34	2.54 ± 4.27
Median (min; max)	0.18 (0; 14.91)	0.86 (0; 15.92)	0.32 (0; 4.52)
Total absorbed dose from internal alpha-particles to RBM ± SE, Gy	0.06 ± 0.11	0.18 ± 0.26	0.10 ± 0.18
Median (min; max)	0.01 (0; 0.48)	0.03 (0; 0.84)	0.01 (0; 0.84)
Period of employment			
1947–1953	27 (64.3 %)	16 (88.9 %)	43 (71.7 %)
1954–1958	6 (14.3 %)	2 (11.1 %)	8 (13.3 %)
post 1958	9 (21.4 %)	0 (0 %)	9 (15 %)
Duration of employment at Mayak PA, years	32.3 ± 13.2	21.2 ± 11.7	30.0 ± 13.7

amount of Pu-239 was estimated by measuring its urine excretion using double phosphate precipitation radiochemistry (Khokhryakov et al. 2000). Doses from the internal exposure to incorporated Pu-239 were estimated from the radionuclide urine data and accounting for its solubility properties, its biodistribution patterns in a human body and the individual's occupational history and smoking status (Khokhryakov et al. 2013). Table 1 shows dosimetric parameters of the investigated Mayak PA workers.

The control group consisted of 15 Ozyorsk residents (8 males and 7 females) who had been constantly living in the city, had not worked with ionizing radiation sources, had never been involved in any cleanup activities following nuclear accidents and had never lived in contaminated regions. The mean age of the control individuals was 62.4 ± 12.5 years.

All individuals invited to participate in the study signed a form of informed consent to voluntary participation according to the Russian Federation legislation concerning public health protection. Thereafter, blood samples were obtained from the participants.

Peripheral blood lymphocyte culturing and preparation of chromosome spreads were performed according to a standard protocol. Lymphocytes were pelleted by centrifugation and placed into a culture flask containing 10 ml PB-MAX medium (Gibco). We usually performed culturing of peripheral blood lymphocytes for 48 h according the international consensus (IAEA 2011). However, because the age of the Mayak PA workers studied here was above 70 years, we sometimes faced poor lymphocyte growth due to advanced donor age. In these cases, the culture conditions were adjusted experimentally (48–72 h) to obtain sufficient metaphase plates. Peripheral blood lymphocyte cultures were supplemented with 5′-bromodeoxyuridine in order to check cell cycle progression during a period of 68–72 h. It was found that most metaphases (87 %) were in first mitosis, so culturing conditions were adjusted to 68 h and metaphase cells were enriched by 4 h colchicine treatment (0.01 μg/ml) before fixation. To this end, cells were first treated with 0.075 M KCl hypotonic solution for 15 min at 37 °C, pelleted and fixed with ice-cold mixture of ethanol and glacial acetic acid (3 + 1 parts). The cell suspension was dropped on pre-cooled wet slides and dried in the steam of a hot water bath.

Chromosome aberrations were stained and scored by fluorescence in situ hybridization (FISH) in a multi-color format (mFISH), which allows multi-color staining of all chromosomes in a single metaphase plate. This technique is more reliable and effective than a simple FISH-assay since it allows scoring of aberrations among all chromosomes (e.g., Speicher et al. 1996).

Hybridization of chromosome was performed following the 24×Cyte lab manual protocol (Metasystems, Germany). Chromosome spreads were denatured in 0.07 N NaOH for 1 min at a room temperature, while probes were heat denaturated. After combining denatured probes and slides and coverslips, slides were incubated in a moist chamber at 37 °C for 48–72 h. Finally, slides were counterstained with DAPI in an antifade solution (MetaSystems, Germany).

Capturing of images of metaphase spreads was performed with an Axio Imager Z.2 fluorescent microscope (Carl Zeiss) equipped with filter sets for DAPI, FITC, Texas Red, Spectrum Orange, DEAC and Cy5 (MetaSystems, Germany). Karyotyping of 100–150 metaphase spreads per individual was done using the ISIS4 software (MetaSystems). An aberration yield per 100 analyzed metaphases was calculated.

Statistics: Correlation and regression analyses were performed using Statistica 6.0 standard package (Borovikov 2003). Student's t test (p value) and Fisher's F test (Draper and Smith, 1986) were employed to estimate statistical significance of the differences; $p < 0.05$ denoted a significant difference.

Results

In total, we analyzed 10,118 metaphase spreads of our study groups by mFISH. Both stable (translocations, insertions, terminal deletions and complex chromosomal rearrangements) and unstable (dicentrics) chromosome aberrations were observed in the lymphocyte chromosomes of Mayak PA workers, while controls only displayed stable chromosome aberrations (translocations). Aberrations involving three and more breaks in two or more chromosomes were considered complex chromosomal rearrangements (complexes).

The comparison of the chromosome aberration yields of the study groups revealed significantly ($p < 0.05$) increased yields of both stable and unstable chromosome aberrations in Mayak PA workers compared with the chromosome aberration yield of the control individuals (Table 2). Our findings support the observations that the chromosome aberration yield of an exposed population markedly exceeds the corresponding background level

Table 2 Chromosome aberration yield in the study groups

Group	Chromosome aberration yield per 100 cells		
	Total	Stable aberrations	Unstable aberrations
Cases ($n = 60$)	2.39 ± 0.26*	4.43 ± 0.35*	0.36 ± 0.09*
Controls ($n = 15$)	0.67 ± 0.23	1.22 ± 0.41	0.13 ± 0.08

* Statistically significant difference ($p < 0.05$) to control

Table 3 Chromosome aberration yield in relation to the absorbed RBM dose from external gamma-rays

Chromosome aberrations per 100 cells	Dose from external gamma-rays to RBM, Gy	
	0–0.5	>0.5
Stable		
All	3.6 ± 0.5	5.2 ± 0.5*
Translocations	2.2 ± 0.5	3.8 ± 0.4*
Unstable	0.4 ± 0.1	0.3 ± 0.1

* Statistically significant differences between the two dose groups ($p < 0.05$)

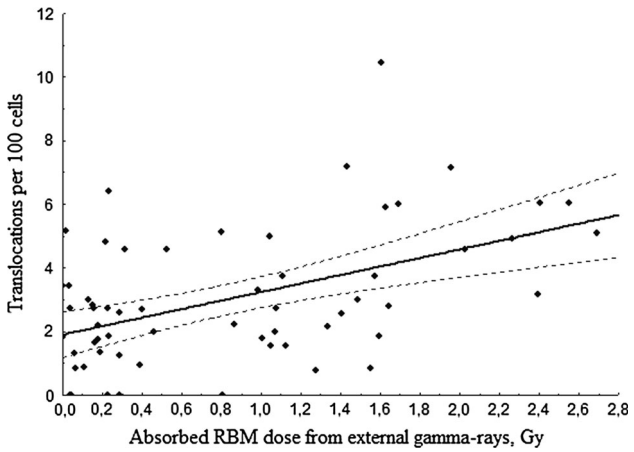

Fig. 1 Translocation yields in relation to the absorbed RBM dose from external gamma-rays. The *stippled lines* represent 95 % confidence intervals

(UNSCEAR 2000; IAEA 2001, 2011) and may hence be used as a biological marker of chronic radiation exposure, even long after the exposure event.

Table 3 presents the results of the chromosome aberration yield analyses in relation to the total absorbed RBM dose from external gamma-radiation in Mayak PA workers. The yield of stable chromosome aberrations in workers exposed to gamma-rays at total absorbed RBM dose above 0.5 Gy was significantly higher ($p < 0.05$) than that in workers exposed to gamma-rays at absorbed RBM dose below 0.5 Gy. In the two groups, the majority of stable chromosome aberrations (87.7 %) were simple translocations. A significant correlation coefficient for the translocation yield and the absorbed RBM dose from external gamma-rays was 0.5. Figure 1 demonstrates a linear relationship between the translocation yield and the absorbed RBM dose from external gamma-rays, even for exposure events that occurred more than 40 years ago.

Next, we analyzed the spectrum and the yield of chromosome aberrations in Mayak PA workers in relation to Pu-239 body burden. Chromosome aberration yield was found to be significantly increased in those workers whose Pu-239 body burden exceeded 1.48 kBq compared with workers with less than 1.48 kBq Pu-239 body burden (Table 4). Unstable chromosome aberrations in Mayak PA workers with Pu-239 body burden exceeding 1.48 kBq were found to be dicentrics. All the revealed dicentrics were reported to be constituents of complexes (Fig. 2).

Table 4 Chromosome aberration yield in relation to plutonium-239 body burden

Chromosome aberrations per 100 cells	Plutonium-239 body burden, kBq	
	below 1.48 kBq	above 1.48 kBq
Stable		
All	3.96 ± 0.37	5.57 ± 0.75*
Translocations	2.96 ± 0.31	3.31 ± 0.58
Complex	0.1 ± 0.04	0.91 ± 0.27*
Unstable	0	0.27 ± 0.12*

* Statistically significant differences between the two dose groups ($p < 0.05$)

A detailed analysis of the chromosome aberration spectrum revealed that complexes contributed a large portion of registered damages in the group of workers with >1.48 kBq plutonium-239 body burden. The complex aberration yield (0.91 ± 0.27 per 100 cells) in individuals with more than 1.48 kBq of Pu-239 body burden was significantly higher than that (0.10 ± 0.04 per 100 cells) of workers with less than 1.48 kBq of Pu-239 body burden. The significant correlation coefficient for complexes and Pu-239 body burden was 0.7, while for external gamma-irradiation there was no significant correlation for this aberration type and the absorbed RBM dose ($r = -0.06$). For the case of mixed exposures, correlation analysis failed to reveal an association of translocation yield with Pu-239 body burden ($r = -0.09$), or with the total absorbed RBM dose form internal alpha-particles ($r = 0.02$). Hence, the findings demonstrate that translocations are a biological marker responding to the external gamma-ray exposure when concerning mixed radiation exposures. This may be correlated with a higher RBE and a higher long-term toxicity by alpha emitters (Vandenbulcke et al. 2003) leading to depletion of lethally hit lymphocytes with time.

A sequential regression analysis based on linear and linear-quadratic models revealed that the linear model achieved statistical significance. Lack of significance for just one of the model parameters of the linear-quadratic model rendered the model insignificant. We attribute this fact to a high variability of the data and to a considerable number of null data estimates. Hence, the linear model was the best to fit chromosome aberration yield points in relation to Pu-239 body burden. Figure 3 demonstrates the

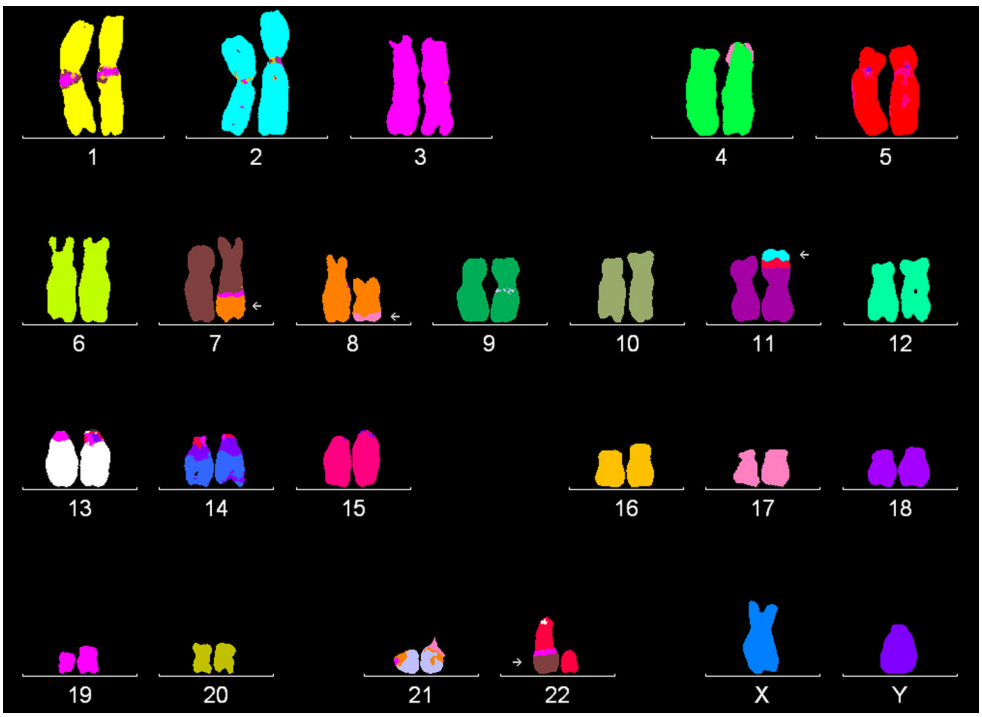

Fig. 2 Stable complex chromosomal aberration involving 2, 7, 11, 17, 22 chromosomes

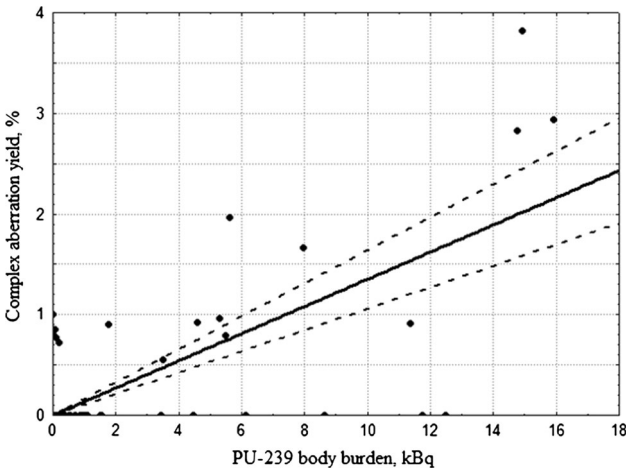

Fig. 3 Complex chromosome aberration yields in relation to plutonium-239 body burden. The *stippled lines* represent 95 % confidence limits

linear relationship (including 95 % confidence limits) between complexes and the Pu-239 body burden.

Discussion

In vitro studies revealed that high-LET radiation induces clustered DNA damages and complex chromosome aberrations (Anderson et al. 2000, 2003, 2005, 2006; Moquet et al. 2001; Barquinero et al. 2004; Hada et al. 2007; Janet et al. 2008), which were ascribed to the propensity of high-LET radiation to induce multiple DNA double-strand breaks in different chromosomes (Brenner and Sachs 1994; Anderson et al. 2002). The latter likely induce complex chromosome rearrangements or intrachromosomal aberrations. In contrast, low-LET radiation induces a relatively random spatial distribution of DNA damages in cells assuming that each damage had the same probability becoming an aberration (Edwards et al. 2005, IAEA 2011).

Profiles of aberrant cells including multiple chromosome damages induced by alpha-particle exposure were investigated in a number of studies; nevertheless, a direct comparison of the findings is inappropriate. Still there are no general criteria for identification of multiply damaged cells; no exact definition has been suggested for cells containing this type of damages. A wide range of aberrant cells with a considerable number of chromosome aberrations are defined as cells with multiple chromosome damages; these are cells containing 5 and more chromosome exchange aberrations (Awa and Neel 1986), 3 and more dicentrics or dicentric equivalents (Rozgaj et al. 2002), 5 chromosome aberrations including at least 1 exchange (Lazutka 1996), more than 1 dicentric or centric ring (Lloyd et al. 1988), 3 and more breaks in 2 or 3 chromosomes (Anderson et al. 2000, 2003, 2005). Moreover, different studies used different criteria for aberration scoring and different chromosome staining techniques causing inconsistent results. For instance, the analysis of Pu-239-induced chromosome aberrations by the simple FISH-assay

Anderson et al. (2000) found that 45–56 % of rearrangements were of a complex type, while mFISH investigation of similarly treated cells revealed an increased complex chromosomal rearrangement level of up to 83 % (Anderson et al. 2002). The analysis of peripheral blood lymphocyte irradiated with americium-241 within a 0–1 Gy dose range by three chromosome FISH revealed an aberration profile containing 34 % of complexes (Barquinero et al. 2004). Using a similar 3 color FISH setup, Moquet et al. (2001) reported that the frequency of complexes in lymphocytes was 25 % after irradiation with Pu-239 alpha-particles. Another simple FISH study showed the increased complex chromosomal rearrangement yield from 56 % (following irradiation with bismuth-213 at dose 10 mGy) to 89 % (bismuth-213 exposure at dose 500 mGy) (Tawn et al. 2007).

In all, we consider mFISH (Speicher et al. 1996) to be the most appropriate technique for identification of complexes. It enables karyotyping of all chromosomes within a single metaphase plate avoiding the need for a subsequent conversion to a whole genome equivalent aberration frequency and, thus, decreasing uncertainties of the approach. Staining of each chromosome pair with a specific color code allows to detect exchange aberrations of any type even in a case when a morphologically normal homologous chromosome is formed due to repair processes or when there is small exchange between chromosomes. Still, intrachromosomal exchanges will go unnoticed. Moreover, mFISH is eminently effective in case of chronic and mixed radiation exposure, since it reveals numerous types of chromosome aberrations in one experiment.

Stable complex chromosome aberrations may be used not only as biomarkers of high-LET radiation exposure (Hande et al. 2005), but may also act as a predictor of tumorigenesis in humans (Pejovic et al. 1992; Johansson et al. 1995; Gorunova et al. 1998; Mandahl 1996, Mitelman Database http://cgap.nci.nih.gov/Chromosomes/Mitelman). Complexes evidence high lymphocyte damage levels. While the damage of a differentiated lymphocyte may not pose a direct hazard to health, the possibility remains that similar damage to a stem or poorly differentiated cell may induce genomic instability and cancer formation, especially when the damage is not lethal. Even stable complexes may cause gene fusions leading to formation of aberrant transcripts or activation of oncoproteins which can alter cell proliferation and abrogate cell death pathways thereby driving, e.g., leukemic transformation (e.g., Aseeva et al. 2009). Epidemiological studies have previously reported increased risks of lung and liver cancer incidence in Mayak PA workers who incorporated plutonium-239 (Labutina et al. 2013), as well as a significant association of lung, liver and bone cancer mortality with the incorporated radionuclide in this cohort (Sokolnikov et al. 2008, Gilbert et al. 2013). All participants of the present study were not diagnosed with cancer at the time of blood sampling. However, the majority of cells (91 %) displaying complex chromosomal rearrangements in Mayak PA workers with more than 1.48 kBq Pu-239 body burden were capable to undergo cell divisions. It can thus not be excluded that such cells in vivo may undergo transformation leading to tumorous malignances.

Conclusion

multi-color fluorescence in situ hybridization analysis of an aged population of Mayak workers revealed significantly increased translocation and complex chromosomal rearrangement yields in Mayak PA workers exposed to mixed radiation (external gamma-rays and internal alpha-particles). The translocation yield was shown to be a biological marker for the external gamma-ray exposure, while complexes may be an indicator of internal alpha-radiation exposure even decades after Pu-239 incorporation.

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