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LINE-1 hypomethylation is associated with radiation-induced genomic instability in industrial radiographers

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Abstract

Global DNA hypomethylation is proposed as a potential biomarker for cancer risk associated with genomic instability, which is an important factor in radiation-induced cancer. However, the associations among radiation exposure, changes in DNA methylation, and carcinogenesis are unclear. The aims of this study were (1) to examine whether low-level occupational radiation exposure induces genomic DNA hypomethylation and (2) to determine the relationships between radiation exposure, genomic DNA hypomethylation and radiation-induced genomic instability (RIGI) in industrial radiographers. Genomic DNA methylation levels were measured in blood DNA from 40 radiographers and 28 controls using the LINE-1 pyrosequencing assay and the luminometric methylation assay. Further, the micronucleus-centromere assay was performed to measure aneuploidy of chromosomes 1 and 4 as a marker of delayed RIGI. Genomic DNA methylation levels were significantly lower in radiographers than those in controls. LINE-1 hypomethylation was not significantly correlated with recent 1-year, recent 3-year, or total cumulative radiation doses in radiographers; however, LINE-1 hypomethylation significantly

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Author Contributions

YHC designed the study, performed laboratory experiments, collected data, wrote, and revised the manuscript. YHJ and SYK performed statistical analyses of the data. HDW, YJK, and SC performed laboratory experiments. EC prepared figures and revised the final manuscript. HWC designed and conducted the study. SYC collected the samples and applied for Research Ethics Board approval. All authors approved the final manuscript.

Conflict of interests

The authors declare they have no actual and potential conflict of interests.

correlated with the cumulative radiation dose without recent 3-year exposure data (D3dose, $r = -0.39$, $p < 0.05$). In addition, LINE-1 hypomethylation was a significant contributor to aneuploidy frequency by D3dose ($F(2, 34) = 13.85$, $p < 0.001$), in which a total of 45% of the variance in aneuploidy frequency was explained. Our results provide suggesting evidence regarding the delayed effects of low-dose occupational radiation exposure in radiographers and its association with LINE-1 hypomethylation; however, additional studies using more subjects are needed to fully understand the relationship between genomic DNA hypomethylation and RIGI.

Keywords

global DNA hypomethylation; radiation; radiation-induced genomic instability; radiographers; LINE-1

Introduction

Ionizing radiation is a well-known carcinogen resulting in a large spectrum of DNA lesions, including double-strand breakages (DSB). Radiation-induced genomic instability (RIGI) is a driving force underlying radiation carcinogenesis [Ward, 1988; Little, 2000; Little, 2003]. Several experiments support a new paradigm of radiation biology in which RIGI can be transmitted from the surviving cell through multiple cell generations to be expressed in the progeny as a delayed and persistent effect [Mothersill and Seymour, 1998; Suzuki et al., 2003; Kadhim et al., 2013]. We have recently shown that delayed aneuploidy--numerical chromosome aberrations are induced by low doses of x-radiation in human fibroblasts. Fifty and 100 mGy of x-rays induce delayed aneuploidy of chromosomes 1 and 4 in the progeny of irradiated fibroblast cells [Cho et al., 2015]. Therefore, unexpected health effects likely result from delayed genomic instability after radiation exposure, raising concerns regarding the most appropriate dose limit for radiation exposure in both the public and industrial workers. Furthermore, delayed genomic instability can partially explain the mechanisms underlying radiation-induced carcinogenesis, which is poorly understood. However, despite the important implications associated with the biological effects of low-dose ionizing radiation and the impact on radiation safety and protection protocols, there are very few studies regarding delayed genomic instability after low-level radiation exposure among the general population or radiographers.

Genome-wide epigenetic alterations are known to hold substantial potential as biomarkers for environmental exposures; this, in turn, may provide insight into mechanisms of environmentally related diseases and allow for a better understanding of disease etiology. DNA methylation, a well-defined epigenetic mechanism, plays an important role in cancer development by adding or removing methyl (-CH₃) groups at CpG dinucleotides, which influences gene regulation [Jones and Baylin, 2007; Ehrlich, 2002; Feinberg and Tycko, 2004]. Genomic DNA hypomethylation resulting from demethylation in repeats or transposable elements or across the genome is associated with genomic instability, an increased number of mutational events, and subsequent development of cancer [Wilson et al., 2007; Weidman et al., 2007; Kitkumthorn and Mutirangura, 2011]. In particular, some studies report that changes in genomic DNA methylation are associated with a striking form

of genomic instability known as chromosome instability, including centromeric aberrations [Martínez et al., 2012], chromosome aberrations [Li et al., 2013], and aneuploidy [Costa et al., 2016].

Numerous studies have shown that age, environmental exposures, and life styles are potential risk factors contributing to genomic DNA hypomethylation [Zhang et al., 2011; Bollati et al., 2009; Baccarelli et al., 2009; Piyathilake et al., 2011]. Exposure to radiation, even at low doses, reportedly induced global hypomethylation in several *in vitro* and *in vivo* studies [Pogribny et al., 2004; 2005; Raiche et al., 2004]. Furthermore, several studies have suggested that radiation-induced epigenetic alterations could be involved in genomic instability, which is related to the onset of cancer [Aypar et al., 2011a; Aypar et al., 2011b; Koturbash et al., 2005]. Therefore, genomic DNA hypomethylation can serve as a biomarker for radiation exposure and an independent predictor of cancer risk and may be the missing mechanistic link for understanding RIGI and radiation carcinogenesis. However, studies on the effects of radiation exposure on DNA methylation patterns and RIGI have been primarily performed in animal models and *in vitro*; studies in the human population are very limited. Furthermore, the biological and/or carcinogenic effects of radiation exposure at low doses and the mechanisms underlying cancer development are still uncertain; however, a study examining individuals occupationally exposed to ionizing radiation at low doses may address this question.

In the present study, global DNA methylation levels in blood DNA from 40 industrial radiographers and 28 healthy male volunteers were measured. The micronucleus-centromere assay was also performed to measure micronuclei (MN) and aneuploidy of chromosomes 1 and 4 as markers of RIGI and delayed RIGI, respectively. We examined whether low-level occupational radiation exposure induces genomic DNA hypomethylation to determine the relationship between radiation exposure, genomic DNA hypomethylation, and RIGI (including delayed RIGI) in industrial radiographers.

Methods

Subjects

The study population comprised 40 male industrial radiographers and 28 healthy volunteers in Seoul, Korea. All radiographers were occupationally exposed to low linear energy transfer (LET) radiation, including X- or γ -rays at low-dose to evaluate the quality of welded joints, pipes, tanks or constructions as previously described [Cho et al., 2009]. The official personal dosimetry records based on monthly film dosimeters were collected for each radiographer for the whole working period and used to estimate occupational radiation dosages. None of the workers were ever exposed to more than the international accepted permissible dosage limit (20 mSv per year and/or 100 mSv per 5 years). The controls were office workers who had never been occupationally exposed to ionizing radiation; their blood was collected during the same periods as that of the industrial radiographers. Information regarding demographics, smoking, drinking habits, medical history, drug intake, and duration of occupational exposure to radiation (years of employment), as well as type of equipment used were obtained via personal interviews. No subjects were exposed to any chemical exposure

or medical irradiation. They also did not have a personal medical history of cancer or other chronic disease and had no drug intake in the months prior to the study.

The study protocol was approved by the local ethics committee (Research Ethics Review Board of Korea Institute of Radiological and Medical Science), and written informed consent was obtained from each participant.

Measurement of MN and aneuploidy

The cytokinesis-block micronucleus (CBMN) assay—Heparinized blood (1 ml) was collected from each subject and mixed with 9 ml RPMI 1640 (Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin (Gibco), and 1% phytohemagglutinin (Gibco). The cultures were incubated at 37 °C in an atmosphere of 95% air and 5% CO₂. Cytochalasin-B (4.0 µg/ml, Sigma Chemical Co., St. Louis, MO) was added after 44 h from the start of the culture, followed by another incubation for 28 h. The cells were collected and treated with 0.075 M KCl hypotonic solution for 3 min and fixed in a mixture of methanol:acetic acid (3:1). The cells were smeared on pre-cleaned microscope slides and air-dried. The slides were stored at –20 °C until their use for centromere labeling.

Fluorescent in situ hybridization (FISH) using centromeric probes of chromosomes 1 and 4—To detect the aneuploidy of chromosomes 1 and 4, FISH was performed using a direct-labeled centromeric probe (Vysis, Downer Grove, IL) according to the manufacturer's instructions. The direct-labeled centromeric probes conjugated with spectrum orange fluorophores (chromosome 1) and spectrum green fluorophores (chromosome 4). An anti-fade solution of p-phenylenediamine dihydrochloride/glycerol with 4',6'-diamidino-2-phenylindole (DAPI, Vysis) was used for counterstaining. Briefly, the slides were denatured using 70% (v/v) formamide in 2 × saline-sodium citrate (SSC) solution (pH 7.3) at 74 °C for 5 min and subsequently dehydrated in graded 70%, 85%, and 100% (v/v) ethanol. The DNA probes were denatured at 73 °C for 5 min and applied to each slide, which were then cover-slipped, sealed, and incubated overnight at 37 °C in a humidified chamber. The slides were washed in 0.4 × SSC (pH 7.0) at 71 °C for 2 min, followed by washing in 2 × SSC/0.1% (v/v) NP-40 (Vysis) at 37 °C for 1 min. Finally, the slides were counterstained with DAPI and stored at –20 °C.

Microscopic examination—The hybridized slides were randomized, scored blindly, and viewed using a fluorescent microscope equipped with an epifluorescent illuminator at a magnification of 100 × with the numerical aperture set at 1.4 (Nikon, Tokyo, Japan). The triple band-pass filter for DAPI/fluorescein isothiocyanate/Texas red (Chroma Technology Corp., Brattleboro, VT) was used to visualize the markers. For each slide, 1 × 10³ binucleated cells were scored. The criteria of Fenech [2000] and Sgura et al. [1997] were used to evaluate MN and aneuploidy, respectively.

Measurement of genomic DNA methylation

DNA preparation and bisulfite treatment—Genomic DNA was isolated from blood samples according to the manufacturer's protocol included with the Wizard DNA

purification kit (Promega, Madison, WI). Purified DNA was processed by bisulfite treatment using the EZ DNA methylation kit (Zymo Research, Irvine, CA) for pyrosequencing assay. The DNA was re-suspended in 20 μ l distilled water and stored at -20°C until further use.

Pyrosequencing assay—The methylation status of repetitive elements LINE-1, a surrogate marker for genomic DNA methylation, was measured by pyrosequencing assay. The primer sequences and PCR conditions have been previously described in detail [Yang et al., 2004; Wu et al., 2012]. Briefly, PCR amplification of the bisulfite converted DNA was conducted, and PCR reactions were performed using Pyromark PCR kit (Qiagen, Valencia, CA) with cycling parameters consisting of denaturation at 95°C for 15 minutes, 44 cycles of 95°C for 30 seconds followed by 56°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 10 minutes. Pyrosequencing was conducted using a PyroMark Q96 MD instrument (Qiagen), with subsequent quantification of methylation levels determined by the PyroMark-CpG software (Qiagen). We used non-CpG cytosine residues as internal controls to verify efficient sodium bisulfite DNA conversion and universal methylated and unmethylated DNAs (Zymo Research).

Luminometric methylation assay (LUMA)—Genomic DNA methylation was also determined by an alternative LUMA protocol [Karimi et al., 2006a]. Genomic DNA (400 ng) was cleaved by a methylation sensitive restriction enzyme (HpaII) and its methylation insensitive isoschizomer (MspI) in parallel reactions. Additionally, *EcoRI* was included in all reactions to normalize the amount of DNA input as previously described [Karimi et al., 2006b]. After the digestion step, the extent of cleavage was quantified via bioluminometric polymerase extension using pyrosequencing (Pyromark Q96 MD, Qiagen). The percentage of 5-methylcytosine (5-mC) was calculated using the HpaII/MspI ratio. Samples were analyzed in technical duplicates and each plate included positive, negative, and water controls.

Statistical analysis

Statistical analysis was performed using the SAS 8.1 for Windows statistical package. The differences between radiographers and controls in terms of general characteristics were tested using t - or χ^2 -tests. Differences in the methylation levels (LINE-1 and LUMA) and genotoxic endpoints (MN and aneuploidy) between the two groups were analyzed using t -tests and Mann-Whitney U tests, respectively. Pearson correlation analysis was performed to test the inter-correlation of independent variables (MN, aneuploidy, LINE-1 methylation levels, duration, and recent 1-year, recent 3-year, and total cumulative radiation doses) among radiographers. To examine causal relations between cumulative radiation dose excluding the most recent 3- or 5-year exposure (D3dose or D5dose, respectively), LINE-1, and aneuploidy frequency, a causal model was constructed and examined with a series of regression analyses. When D3dose and D5dose were tested, radiographers hired within 3 or 5 years were excluded from the analysis. The criterion for significance was determined with an alpha of 0.05.

Results

The general characteristics of the study population are listed in Table 1. All subjects in the study were healthy males, and the mean ages and patterns of smoking status and alcohol intake between radiographers and controls were not significantly different. The frequency of MN, a valuable marker for radiation-induced chromosomal aberration, and aneuploidy of chromosomes 1 and 4, a marker of delayed RIGI [Cho et al., 2015], were measured using the CBMN assay in combination with the FISH technique. Supplementary Figure 1 shows different types of aneuploidy that were detected in the present study. As shown in Figure 1, there were significant differences in both MN ($p < 0.001$, Figure 1A) and aneuploidy ($p < 0.001$, Figure 1B) frequencies between radiographers and controls. Pyrosequencing assay and LUMA were also performed to measure genomic DNA methylation levels. LINE-1 ($p < 0.01$, Figure 2A) and LUMA methylation levels ($p < 0.05$, Figure 2B) were also significantly lower in radiographers than those in controls.

Table 2 shows that the frequency of MN significantly correlated with the different radiation doses, including recent 1-year ($r = 0.31$, $p = 0.05$), recent 3-year ($r = 0.32$, $p < 0.04$), and cumulative radiation doses ($r = 0.41$, $p = 0.008$) among radiographers. Meanwhile, the frequency of aneuploidy significantly correlated with the cumulative radiation dose ($r = 0.57$, $p < 0.001$), but not with recent 1-year or recent 3-year radiation doses. These results indicate that higher aneuploidy frequency may be attributable to occupational radiation exposure in the past, but not recent exposure (in the past 1 – 3 years). In addition, there were no associations between radiation exposure and LINE-1 or LUMA methylation; however, total cumulative radiation dose correlated with LINE-1 hypomethylation, with borderline significance ($p = 0.08$).

While LUMA methylation was positively correlated with the frequency of MN and aneuploidy, there was a significant inverse correlation between LINE-1 methylation and the frequency of aneuploidy among radiographers ($r = -0.49$, $p = 0.001$), suggesting that LINE-1 hypomethylation may be involved in delayed RIGI. Therefore, we calculated the D3dose data considering lifespan of human lymphocytes and determined its association with methylation levels and aneuploidy frequency. There was no significant relationship between D3dose and genomic DNA methylation, as determined using LUMA ($r = 0.175$, $p = 0.29$). However, as illustrated in Figure 3, linear regressions between each pair of the three variables (D3dose, LINE-1 hypomethylation, and aneuploidy frequency) were significant. Specifically, D3dose significantly predicted LINE-1 hypomethylation, $\beta = -0.39$, $t(35) = -2.49$ (Figure 3A), as well as aneuploidy frequency, $\beta = 0.58$, $t(35) = 4.21$ (Figure 3B). Further, LINE-1 hypomethylation significantly predicted aneuploidy frequency, $\beta = -0.53$, $t(35) = -3.74$ (Figure 3C).

Figure 4 shows causal relations from D3dose to aneuploidy frequency (top) and from D3dose through LINE-1 hypomethylation to aneuploidy frequency (bottom). The data in the top panel ($b = 0.03$) and the causal relation data from D3dose to LINE-1 hypomethylation (bottom; $b = -0.01$) are identical to those in Figures 3B and 3A, respectively; Figure 4 presents regression coefficients, whereas Figure 3 shows Pearson's correlation coefficients. Importantly, as seen in the bottom panel, the causal relation from D3dose through LINE-1

hypomethylation to aneuploidy frequency was significant, $F(2, 34) = 13.85$, $p < 0.001$ in which a total of 45% of the variance in aneuploidy frequency was explained. Specifically, the effect of D3dose on aneuploidy frequency through LINE-1 hypomethylation was significant ($b = -0.75$), and the effect of D3dose on aneuploidy frequency controlling for LINE-1 hypomethylation was significant ($b = 0.02$).

Another causal model was constructed to investigate the relation between D5dose, LINE-1, and aneuploidy frequency; we analyzed these data in the same way as the D3dose data. The entire results are available from Supplementary Figures 2 and 3. Since there was a very high correlation between D3dose and D5dose ($r = 0.94$, $p < 0.001$), the results of D5dose and D3dose were similar to each other. In sum, the causal relation from D5dose through LINE-1 hypomethylation to aneuploidy frequency was significant, $F(2, 37) = 14.82$, $p < 0.001$ in which a total of 44% of the variance in aneuploidy frequency was explained.

Discussion

Ionizing radiation exposure, including at low-doses, can cause RIGI and delayed RIGI, with the potential for subsequent cancer development [Huang et al., 2007]. Several studies have reported that epigenetic alterations are involved in radiation exposure and RIGI [Pogribny et al., 2005; Raiche et al., 2004; Aypar et al., 2011a; Koturbash et al., 2005]; however, there are few, if any, studies regarding radiation exposure, methylation changes, and RIGI in the human population. Therefore, the present study was performed to bridge this knowledge gap and determine the relationships among low-dose radiation exposure, genomic DNA hypomethylation, and RIGI, including delayed effects, in industrial radiographers.

The frequencies of MN and aneuploidy and levels of genomic DNA methylation were significantly different between radiographers and controls, indicating that occupational exposure to radiation, including at low doses, can induce chromosomal damage (both clastogenic and aneugenic effects) as well as methylation changes in genomic DNA. These outcomes are concordant with other publications [Cho et al., 2015; Lee et al., 2015; Pogribny et al., 2004; 2005]. Therefore, we further investigated the effects of low-level occupational radiation exposure among radiographers. The frequency of MN in radiographers was significantly increased by all radiation exposure doses (recent 1-year, recent 3-year, and total cumulative during working duration); these results agree with other previous studies suggesting that MN can be used as a predictive marker for occupational radiation exposure in radiographers [Sari-Minodier et al., 2002; Cho et al., 2009]. Further, aneuploidy frequency in radiographers was significantly increased by cumulative exposure, but not by recent 1- or 3-year radiation exposure. This may suggest that radiation-exposure time points are important contributors to aneuploidy frequency in radiographers occupationally and chronically exposed to radiation.

The scoring of induced chromosomal damage (i.e. chromosome aberrations or MN) in blood T-lymphocytes after radiation exposure is classically used to estimate the absorbed radiation dose in biological dosimetry and is a reliable biomarker for predicting subsequent cancer risk [Kaddour et al., 2017; Fucic et al., 2016; Bonassi et al., 2008; IAEA, 2011]. The turnover rate of lymphocytes (both T and B lymphocytes) and transmissibility of

chromosomal damage are major challenges for the realization of retrospective biological dosimetry after radiation exposure and when assessing the associated cancer risk in healthy subjects occupationally or environmentally exposed to radiation [Voisin, 2015; Wahab et al., 2008]. The turnover or lifespan of human lymphocytes, which is estimated by the persistence of unstable chromosomal aberrations, is relatively slow (approximately 3 years) [Lucas et al., 1992], and several studies have shown that the number of lymphocytes containing radiation-induced chromosomal aberrations (or the frequency of radiation-induced chromosomal aberrations) decreased/fell gradually from about 40 days [Norman et al., 1966; Littlefield et al., 1991], 60 days [Sasaki and Norman, 1967] or 140 days to 3 years [Buckton et al., 1967; Ramalho et al., 1995; Lucas et al., 1992]. Therefore, in this study, D3dose was used to examine whether occupational low-dose radiation exposure can cause delayed effects by considering aneuploidy frequency resulting from past radiation exposure (excluding recent 3-year exposure) among radiographers. Interestingly, linear regressions showed a significant relationship between D3dose and aneuploidy frequency in radiographers (Figure 3A); these results agree with those of our previous study showing that aneuploidy of chromosomes 1 and 4 is a marker for delayed RIGI after low-dose x-irradiation. The obtained results suggest that aneuploidy of chromosomes 1 and 4 is a useful marker for determining the delayed effects of occupational low-dose radiation exposure, defined as exposure in the past, but not in the most recent 1–3 years, among radiographers.

Epigenetic alterations are associated with the early-stage biological response and/or molecular events linking environmental exposures with cancer development [Skinner, Manikkam, and Guerrero-Bosagna, 2010]. Several studies have shown that genomic DNA hypomethylation is associated with radiation exposure and RIGI [Kumar et al., 2011; Pogribny et al., 2004; Lee et al., 2015]. The obtained results show no associations between recent low-dose radiation exposure and genomic DNA methylation changes among radiographers; however, total cumulative radiation dose correlated with LINE-1 hypomethylation, with borderline significance ($p = 0.08$). Recent study by Maiehofer et al. (2017) showed similar results as ours; x-irradiation did not induce global DNA hypomethylation within the first cell cycle in human fibroblasts.

Furthermore, there was a significant correlation between LINE-1 hypomethylation and the frequency of aneuploidy among radiographers. Our previous study [Lee et al., 2015] also showed that LINE-1 hypomethylation is associated with the total cumulative radiation dose (but not recent 1.5-year exposure dose) and chromosome aberrations in nuclear power plant workers; however, LINE-1 methylation levels were higher in radiation-exposed workers than those in controls. This difference may be attributable to differences in the recruited control groups; the control group in this study includes population-based subjects who are mostly graduate students. In contrast, the controls in our previous study were office workers at nuclear power plants who were assumed to be unexposed to radiation; however, the risk of radiation exposure could be higher for the office workers at nuclear power plants than that of the population-based controls. Radiographers from these two studies were also exposed to a different source of radiation (radiation quality); the radiographers in this study were exposed to low LET radiation (X- or γ -rays), whereas the nuclear power plants workers were exposed to both high and low LET radiation. Furthermore, the method for measuring

LINE-1 methylation differed between the two studies, which may influence the measured methylation levels.

In addition, our data show that three variables, D3dose, LINE-1 hypomethylation, and aneuploidy frequency are significantly associated with each other. Therefore, we further investigated the relationship between D3dose and total aneuploidy using LINE-1 hypomethylation to determine whether LINE-1 hypomethylation is the potential mechanistic link between low-dose occupational radiation exposure and delayed RIGI. The indirect effect of D3dose on total aneuploidy via LINE-1 hypomethylation is an important outcome of this study, although the mediation effect of LINE-1 hypomethylation is small. This finding suggests that LINE-1 hypomethylation may partially explain the delayed RIGI among radiographers occupationally exposed to low dose LET radiation; however, this result should be interpreted with caution because of the small sample size used in this study.

The mechanisms underlying delayed RIGI are not fully elucidated; however, recent *in vitro* and animal studies suggest that epigenetic alterations, including LINE-1 hypomethylation, are involved in delayed RIGI after high or low LET radiation exposure and may have a causative role in cancer development [Miousse et al., 2014; Penha et al., 2017; Armstrong et al., 2012; Luzhna et al., 2015]. In particular, a study by Luzhna et al. [2015] showed that low-dose ionizing radiation induces LINE-1 hypomethylation following the up-regulation of LINE-1. Further, LINE-1 activation results in translation of LINE element in rat mammary glands. Luzhna et al. [2015] also found that radiation-induced methylation and protein level changes were persistent for 24 weeks after radiation exposure, suggesting that activation and mobilization of LINE-1 in irradiated tissue may potentially contribute to delayed RIGI. Furthermore, DNA DSB and/or DSB repair defects attributable to LINE-1 hypomethylation have been proposed as another possible mechanism of delayed RIGI [Kongruttanachok et al., 2010; Pornthanakasem et al., 2008]. LINE-1 expression attributable to radiation-induced hypomethylation, which is associated with DNA DSB formation through LINE-1-encoded endonuclease activity, may be a source of genotoxic stress and cause of delayed RIGI in cells after radiation exposure [Farkash et al., 2006; Wallace et al., 2010]. LINE-1 hypomethylation is also known to down-regulate DNA repair genes such as *PPP2R2B*, which contains intragenic LINE-1s [Aporntewan et al., 2011] and increases nuclear ataxia-telangiectasia mutated (ATM) proteins [Suyarnsestakorn et al., 2010]. ATM proteins play key roles in activating the DNA damage checkpoint, leading to cell cycle arrest, DNA repair, or apoptosis [Mavrou et al., 2008]. Thus, LINE-1 hypomethylation may indirectly lead to RIGI by interfering with ATM function and down-regulating *PPP2R2B*. A recent study by Penha et al. [2017] also reported similar results; x-irradiation induced LINE-1 hypomethylation in FRTL5 thyroid cells with slower DSB repair kinetics (DNA repair delay), which could lead to the accumulation of gene mutations.

In this study, D3dose was used to determine the delayed effects of occupational radiation exposure – aneuploidy frequency of chromosomes 1 and 4. Although D3dose was calculated based on the lifespan of human lymphocytes, total cumulative radiation doses excluding recent 7-year dose or longer should be tested to completely eliminate all genomic instability induced by recent radiation exposure [Buckton et al., 1978] and determine phenotypic changes at certain delayed times after past radiation exposures. The mean lifetime of human

lymphocyte is approximately 3 years or up to 5 years, as reported in several studies [Lucas et al., 1992; Léonard et al., 1988; Dolphin et al., 1973]. Therefore, we also analyzed the associations between D5dose, LINE-1 methylation changes, and aneuploidy frequency; the D5dose results were similar to those of D3dose. However, the small sample size precluded further statistical analysis of the possible relationship between D7dose and aneuploidy frequency via LINE-1 hypomethylation. Furthermore, the inclusion of only males in our study population limits generalizability. Therefore, additional studies with a larger number of subjects, both males and females, are needed to fully elucidate the relationship between radiation exposure, genomic DNA hypomethylation, and delayed RIGI. Despite these limitations, this is the first known report that provides suggesting evidence regarding the delayed effects of low-dose occupational radiation exposure in radiographers and its association with LINE-1 hypomethylation as a potential mechanism of delayed RIGI. The results reported here provide valuable information regarding radiation exposure in both the public and radiation-worker domains and can be used by policy makers, government regulators, and agencies to set up strategies to limit exposure. Furthermore, development of biomarkers for intrinsic radiosensitivity of healthy individuals may allow the monitoring of occupational or environmental radiation exposure and assist the stratification of patients for response to radiotherapy.

Conclusions

Our data indicate that LINE-1 hypomethylation is associated with aneuploidy of chromosomes 1 and 4, a delayed RIGI among radiographers occupationally exposed to low-dose or low-LET radiation. These data may provide evidence for the underlying mechanism associated with delayed RIGI after occupational low-dose radiation exposure in radiographers; however, additional studies with more subjects and other repetitive elements using different assays are needed to fully understand the relationship between genomic DNA hypomethylation and RIGI (including delayed RIGI) and the mechanistic pathway to radiation carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Frequency of micronuclei (MN) and aneuploidy between radiographers and controls. Mean $\pm$ SE. *** $p < 0.001$.

Figure 2. LINE-1 and luminometric methylation assay (LUMA) results between the radiographers and controls. Mean $\pm$ SE. * $p < 0.05$; ** $p < 0.01$.

Figure 3.

Scatter plots between variables. Results for (A) D3dose and LINE-1; (B) D3dose and aneuploidy frequency; and (C) LINE-1 and aneuploidy frequency are shown. D3dose = the cumulative radiation dose excluding recent 3-year exposure. r = Pearson's correlation coefficient. $*$ = $p < 0.05$; $**$ = $p < 0.01$; $***$ = $p < 0.001$.

Figure 4.

Causal relations from D3dose to aneuploidy frequency (top) and from D3dose through LINE-1 hypomethylation to aneuploidy frequency (bottom). Arrows connecting one variable to another represent unstandardized regression coefficients. $^* = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$.

Table 1.

General characteristics of study population

Variables	Number of subjects (% or range)		<i>p</i> -value
	Controls	Radiographers	
Number	28	40	
Age (mean $\pm$ SD, years)	37.8 $\pm$ 9.5 (21 – 57)	35.9 $\pm$ 5.9 (27 – 48)	0.60 ^a
Smoking status			
Never smoked	6 (21.4)	5 (12.5)	0.51 ^b
Past smoker	6 (21.4)	7 (17.5)	
Current smoker	16 (57.1)	28 (70.0)	
Current alcohol intake			
No	7 (25.0)	6 (15.0)	0.30 ^b
Yes	21 (75.0)	34 (85.0)	
Duration of employment (mean $\pm$ SD, years)	-	9.2 $\pm$ 4.6 (3 – 18)	
5	-	12 (30.0)	
6–10	-	13 (32.5)	
> 11	-	15 (37.5)	
Radiation dose (mean $\pm$ SD, mSv)			
Recent 1-year	-	5.4 $\pm$ 6.7 (0 – 28.7)	
Recent 3-year	-	20.7 $\pm$ 21.6 (3.3 – 98.3)	
Cumulative dose	-	60.5 $\pm$ 49.6 (7.8 – 281.7)	
D3dose ^c	-	39.8 $\pm$ 28.0 (1.2 – 219.0)	

SD, Standard Deviation.

^aDetermined by *t*-test.^bDetermined by χ^2 test.^cD3dose, Cumulative dose without the most recent 3-year dose.

Table 2.

Correlation matrix among variables in radiographers

	MN ^a	Aneuploidy ^b	LINE-1 ^c	LUMA ^d	1-year dose ^e	3-year dose ^e	Total dose ^g
MN							
Aneuploidy	.54***						
LINE-1	-.19	-.49**					
LUMA	.45**	.43**	-.07				
1-year dose	.31*	.18	-.01	.16			
3-year dose	.32*	.19	-.01	.11	.85**		
Total dose	.41**	.57***	-.28 [#]	.20	.43**	.50**	

Data indicate Pearson's correlation coefficient

^aMN, total frequency of micronuclei

^bAneuploidy, total frequency of aneuploidy (chromosome 1 and chromosome 4)

^cLINE-1, LINE-1 methylation levels measured by pyrosequencing assay

^dGlobal DNA methylation levels measured by luminometric methylation assay (LUMA)

^e1-year dose, Recent 1-year radiation dose

^f3-year dose, Recent 3-year radiation dose

^gTotal dose, Total cumulative radiation dose

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$,

[#]borderline significance ($p = 0.08$)