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DNA methylation as a potential biomarker for radiation exposure

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이 영 현

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지도교수 정 해 원

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이 영 현

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위 원 장	성 주 헌	(인)
부 위 원 장	조 성 일	(인)
위 원	최 경 호	(인)
위 원	김 수 영	(인)
위 원	정 해 원	(인)

DNA methylation as a potential biomarker for radiation exposure

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Younghyun Lee

Date approved:

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Joohon Sung

Sung-il Cho

Kyungho Choi

Su Young Kim

Hai Won Chung

Abstract

DNA methylation as a potential biomarker for radiation exposure

Younghyun Lee

Major in Molecular Epidemiology

Department of Public Health

Graduate School of Public Health

Seoul National University

Epigenetic changes, including DNA methylation, microRNA expression and histone modification, are heritable and stable alterations in regulation of gene transcription. Although the changes are required for normal health and development, they can also contribute to diseases including cancers. The epigenetic changes could be a missing link among radiation exposure, radiation-induced genomic instability, and radiation-induced carcinogenesis. While recent studies have shown that ionizing radiation (IR) can affect epigenetic changes in animal and cancer cell lines, there is a lack of information on epigenetic alterations following irradiation and involved

mechanisms in human.

This study aimed to evaluate DNA methylation changes, one of the epigenetic alterations, in normal human blood irradiated with gamma rays *in vitro* and investigate the effects of low-dose radiation exposure on DNA methylation in peripheral blood DNA from nuclear power plant workers. I also evaluated whether radiation-induced DNA methylation states were associated with genomic instability.

I evaluated global DNA methylation levels using DNA methylation quantification kits and examined the methylation levels of specific locus and repeat element such as long interspersed element-1 (LINE-1) and Satellite 2 (Sat2) by MethyLight assay in human blood. I also evaluated the association between DNA methylation and the frequency of micronuclei or chromosomal aberrations, indicators of genomic instability in radiation exposed workers. The expression of DNA methyltransferases (DNMTs) was measured by using quantitative real-time PCR to investigate the mechanisms of radiation-induced DNA methylation alterations.

In vitro study revealed that γ-irradiation led to global DNA hypomethylation in normal human leukocytes and the expression of DNMTs was decreased with γ-irradiation. These results indicated that global hypomethylation detected in this study may be the consequence of decreased DNMTs expression by γ-irradiation. LINE-1 hypermethylation after 0.5, 1, and 2 Gy of γ irradiation was observed in γ-ray exposed blood. It was also observed to be significantly methylated in Mut L homologue-1 (*MLH1*) promoter region in cells irradiated with 2Gy γ radiation, and the expression of

MLH1 mRNA was also decreased.

This study found that global DNA methylation levels were lower in radiation-exposed workers than in controls. The methylation levels were negatively associated with recent 1.5 year radiation dose, while the levels were increased with total cumulative dose in radiation-exposed workers. Global DNA methylation levels were increased with *DNMT1* expression, and the expression levels were correlated with recent 1.5 year radiation dose. These findings suggest that recent 1.5 year radiation exposure caused a decrease of *DNMT1* expression, which resulted in global DNA hypomethylation. LINE-1 methylation levels were higher in radiation-exposed workers than in controls, while Sat2 methylation levels had no significant differences between workers and controls. Total cumulative radiation dose was significantly associated with LINE-1 methylation levels after adjusting for age, smoking status and alcohol intake in radiation-exposed workers.

Global DNA methylation levels were also correlated with chromosomal aberrations in workers, while the association was not found in controls. In contrary to this result, the frequency of micronuclei was not correlated with global DNA methylation and repeat element methylation levels. This finding suggests that global DNA hypomethylation is associated with radiation-induced double strand breaks.

These results support the hypothesis that exposure to ionizing radiation may influence DNA methylation in human blood. These findings of this study suggest the possible use of DNA methylation levels as a potential biomarker to monitor IR exposure for radiation-related workers. In addition,

the findings of this study support a link between global DNA methylation and genomic instability in radiation-exposed individuals.

Keywords: ionizing radiation, DNA methylation, DNA methyltransferases, genomic instability, chromosome aberrations.

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Chapter I. Introduction

1. Background

Ionizing radiation (IR) is widely accepted as a severe DNA damaging agent, which can lead to serious health problems (Little 1999). Even though IR is a well-known genotoxic agent and human carcinogen, a number of people have exposed to IR via occupational, diagnostic, or therapeutic uses (De Potter et al. 2006; Erven and Van Limbergen 2007). Thus, it is still becoming important to understand IR's biological effects.

While radiation-induced genomic instability and carcinogenesis have been studied well, the mechanisms underlying these processes are poorly understood (Ullrich and Ponnaiya 1998; Little 2000; Aypar et al. 2011a). Recent studies have shown that radiation induced epigenetic alterations in animal and cancer cell lines, and have suggested that the epigenetic changes may be a missing link among radiation exposure, radiation-induced genomic instability, and radiation-induced carcinogenesis (Pogribny et al. 2004; Kovalchuk and Baulch 2008; Aypar et al. 2011b).

Epigenetic changes, including DNA methylation, histone modification and microRNA expression, are heritable and stable alterations in gene expression. Particularly, DNA methylation can be changed by various environmental toxicants and has a potential to diagnose environmental diseases or monitor exposure to environmental toxicants (Kim et al. 2012).

IR exposure has been reported to affect DNA methylation patterns. Gamma ray exposure of hamster and human cancer cell lines led to global

hypomethylation (Kalinich et al. 1989). X-ray irradiation of mice also affected methylation of the promoter region of p16 tumor suppressor gene (Kovalchuk et al. 2004). Together with the evidence for altered promoter methylation in a variety of cancers (Gronbaek et al. 2007), these data can provide a link among radiation exposure, epigenetics and carcinogenesis.

It was also suggested that changes in genomic DNA methylation following irradiation are potentially correlated with initiation of genomic instability (Aypar et al. 2011b). Kaup et al. (2006a) found that radiation-induced genomic instability (RIGI) in HPV-G cells is correlated with epigenetic changes following exposure to ^{60}Co gamma radiation. In addition, high levels of DNA methylation are associated with a closed conformation of chromatin (Weber and Schubeler 2007; Cedar and Bergman 2009), whereas unmethylated DNA tends to get repackaged in a more open configuration that can lead to DNA damage, impaired DNA repair and eventually genomic instability (Pogribny et al. 2005; Aypar et al. 2011b).

Recently, some studies have reported on altered DNA methylation patterns following exposure to radiation in plants, rodents, and cell lines (Kovalchuk et al. 2003; Pogribny et al. 2004; Kaup et al. 2006a), but the results are inconsistent. Exposure of 0.5–10 Gy ^{60}Co γ rays to four different cell lines (Chinese hamster ovary clone K-1, Chinese hamster lung fibroblast clone A03, HeLa close S-3, and Mouse neuroblastoma C-1300 clone N1E-115) resulted in hypomethylation 1-3 days post-irradiation (Kalinich et al. 1989). Another group evaluated Murine cell line m5S/1M and Chinese hamster CHO/K-1 cells using 10 Gy X rays and observed no change in DNA

methylation over 3-day post-irradiation period (Tawa et al. 1998).

It was also reported that radiation exposure led to sex- and tissue-specific global DNA hypomethylation in mice and rats (Pogribny et al. 2004; Raiche et al. 2004a). In addition, radiation-induced DNA methylation changes can be different by the use of different radiation dose or radiation quality (Goetz et al. 2011).

The variability in these findings suggests that epigenetic responses post-irradiation can be different among different types of cells, different types of tissues and different radiation dose. Therefore, it is important to evaluate the methylation changes in normal human cells or tissues to use epigenetic alterations as a biomarker for radiation exposure in human populations. It also needs to investigate the effects of chronic low-dose radiation on DNA methylation in normal human population.

1.1. Epigenetic alterations by environmental toxicants.

Epigenetic changes, including DNA methylation, histone modification and microRNA expression, are heritable and stable alterations in gene expression. Particularly, DNA methylation can be altered by various environmental toxicants and has a potential to diagnose environmental diseases or monitor exposure to environmental toxicants (Kim et al. 2012).

In mammals, DNA methylation occurs almost exclusively at CpG dinucleotides. It plays an important role in developmental processes, imprinting, cell proliferation and maintenance of genome stability. Alterations in DNA methylation have emerged as one of the most consistent molecular alterations in carcinogenesis (Jones and Baylin 2007).

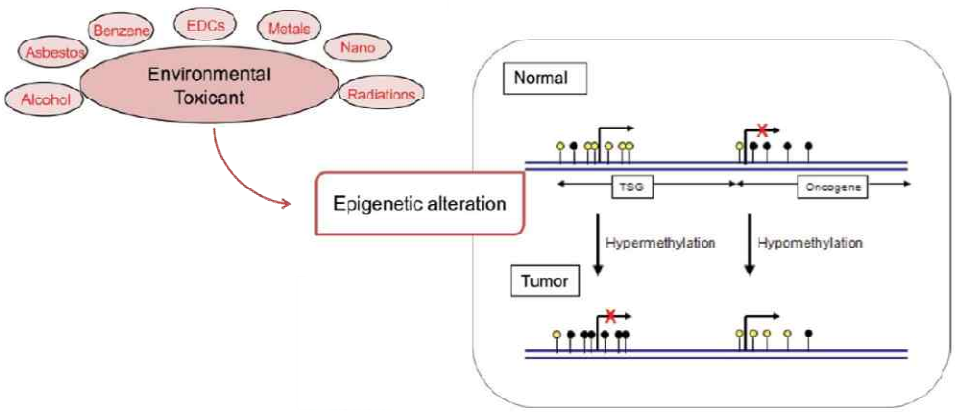

Figure I-1. Environmental toxicants to induce epigenetic alterations; ○, unmethylated; ●, methylated (Kim et al. 2012; Yang and Park 2012).

A global loss of DNA methylation is thought to contribute to

carcinogenesis by reactivating retrotransposable elements, inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007). DNA hypermethylation of gene promoter regions has been linked to carcinogenesis, transcriptionally silencing genes involved in tumor suppression, DNA repair and cell cycle regulation (Baylin 2005). Environmental carcinogens can alter DNA methylation and in turn control the expression of genes (Figure I-1) (Tremolizzo et al. 2010; Kim et al. 2012).

Epidemiologic studies have investigated the association between environmental risk factors and DNA methylation (Table I-1). Exposure of low-dose benzene was associated with altered DNA methylation of *p15*, *MAGE-1*, LINE-1, and Alu sequences (Bollati et al. 2007). Prenatal maternal lead exposure was also inversely associated with Alu and LINE-1 methylation (Pilsner et al. 2009). LINE-1 and *MGMT* hypomethylation was observed in subjects exposed to high-dose polycyclic aromatic hydrocarbon (PAH), and the alterations could be used as markers for PAHs exposure (Duan et al. 2013). In addition, the studies have reported associations between DNA methylation and several health outcomes including cancers and other diseases (Shimabukuro et al. 2007; Lim et al. 2008; Chouliaras et al. 2010; Wilhelm et al. 2010). These epidemiologic studies investigating associations among environmental risk factors, DNA methylation and different disease endpoints suggest the potential for measuring DNA methylation as a biomarker of risk for the environmental pollutants.

Table I-1. Effects of environmental exposures on DNA methylation [a]

Exposure	Gene	↑/↓[b]	Tissue	References
Benzene	Repeat elements (Alu, LINE-1)	↓	Human blood	Bollati et al. (2007)
	p15	↑		
	MAGE-1	↓		
Lead	Repeat elements (Alu, LINE-1)	↓	Human blood	Pilsner et al. (2009)
Arsenic	Global	↑	Human blood	Pilsner et al. (2007)
	Global	↓	Rat liver	Zhao et al. (1997)
	p53	↑	A549 cells	Mass and Wang (1997)
DES	Global	↓	Mouse Uterus	Li et al. (1997); Li et al. (2003)
PAHs	Repeat elements (Alu, LINE-1)	↑	Human blood	Pavanello et al. (2009)
	p53, HIC1	↓		
	Repeat elements (LINE-1)	↓	Human blood	Duan et al. (2013)
	MGMT	↓		
Cadmium	Global	↓	TRL1215 rat liver cells	Takiguchi et al. (2003)

[a], modified from Baccarelli and Bollati (2009)

[b], Increase(↑) or decrease(↓) in DNA methylation

DES, Diethylstilbestrol; PAHs, Polycyclic aromatic hydrocarbons

1.2. Ionizing radiation biomarkers

Ionizing radiation is a well-known human carcinogen that can induce a variety of biological effects depending on the physical nature, duration and doses of exposure (Pernot et al. 2012). However, the magnitude of health risks at low doses remains controversial due to a lack of direct human evidence (Ma et al. 2010). Improved evidence on the magnitude of health risk of low dose radiation is anticipated to emerge from the integration of epidemiological and biological research through molecular epidemiology incorporating biomarkers and mechanistic studies (Pernot et al. 2012).

There are a number of endpoints that can be assessed in an epidemiological setting and show potential in one or more of the four categories of biomarkers: biomarkers of exposure, susceptibility, persistent effects and/or late effects (Table I-2) (Pernot et al. 2012). Identification of these biomarkers can improve the evidence on the relationship between radiation exposure and effects.

Several biomarkers have been used to investigate exposure, effects and susceptibility to ionizing radiation, but they are established and validated at moderate and high doses (Ma et al. 2010). There is a need, therefore, to validate potential biomarkers at low doses and improve the evidence available on low dose radiation risk assessment. Epigenetic modification is one of the potential biomarkers.

In addition, epigenetic changes may be heritable and persist at

delayed times post-irradiation in the progeny of the irradiated cells (Aypar et al. 2011b). Accumulative evidences have suggested that epigenetic alterations are key factors underlying the molecular mechanisms of transgenerational effects such as genomic instability (Kovalchuk and Baulch 2008; Merrifield and Kovalchuk 2013). Kaup et al. (2006a) demonstrated DNA methylation alterations in cells exposed to the medium from the irradiated human keratinocytes persists for 20 passages. The global loss of DNA methylation was also observed in the progeny of irradiated C57Bl/6 mice (Koturbash et al. 2006). Consequently, the study on radiation-induced epigenetic alterations may be critically important to understand the IR's biological effects including non-targeted radiation effects as well as effects of direct radiation exposure.

Table I-2. Temporal classification of IR biomarkers (Pernot et al. 2012)

Biological classification of IR biomarkers		Temporal classification of IR biomarkers			
		Exposure	Susceptibility	Late effects	Persistent effects
Cytogenetics	Dicentrics	√	P	P	P
	Translocations	√	P	P	√
	Micronuclei	√	P	P	
Nucleotide pool damage and DNA damage	γ-H2AX	√	P	P	P
	Extracellular 8-oxo-dG	(oxidative stress)	P		
Germline inherited mutations/ variants and induced mutations	SNP, CNV and inherited gene mutations		√	P	P
	CNA	P			P
	HPRT	√			√
Transcriptional and translational changes	Changes in the mRNA levels of the ATM/CDK2/p53 pathway	√	P		
	Changes in RNAs identified by transcriptomics	√	P	P	P
Epigenetic modifications	Histone modifications	P	P	P	P
	DNA methylation	P	P	P	P
	miRNA	√	P	P	P
	Phosphoproteomics	P	P		
Other biomarkers	ROS	√	P	P	P
	Metabolites and metabolomic		√P	P	P

√, direct evidence that this biomarker could be used as such; P, potential or theoretical use; CNA, copy number alteration; CNV, copy number variant; ROS, reactive oxygen species

1.3. The studies on radiation-induced DNA methylation and their limitations.

1.3.1. Radiation-induced DNA methylation

Exposure to radiation has been known to result in epigenetic modifications which will affect gene regulation (Aypar et al. 2011b; Pernot et al. 2012). First, the remodeling of the chromatin structure via histone modifications can affect the initiation of the transcription of genomic DNA (Bannister and Kouzarides 2011). Second, DNA methylation can inhibit the transcription by preventing the binding of transcription factors to the promoter region of the gene (Gronbaek et al. 2007). Third, miRNA can bind to transcribed mRNAs preventing their translation into proteins (Cowland et al. 2007).

DNA methylation, one of the epigenetic modifications, is well known to be involved in carcinogenesis and the control of gene expression in specific tissues (Gronbaek et al. 2007). A global loss of DNA methylation is thought to contribute to carcinogenesis by reactivating retrotransposable elements, inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007). DNA hypermethylation may be also linked with structural changes in chromatin, decreased transposon movement and decreased gene expression (Robertson 2005; Slotkin and Martienssen 2007; Yauk et al. 2008). Several studies have shown that exposure to IR can

lead to both hypo- and hypermethylation (Table I-3). Gamma ray exposure led to global hypomethylation in hamster and human cancer cell lines (Kalinich et al. 1989). Spleen tissue of X-irradiated mice also showed global hypomethylation (Pogribny et al. 2004). In the same study, X-ray irradiation induced hypermethylation of the p16 tumor suppressor gene in tissue.

As shown in Table I-3, results induced by ionizing radiation are inconsistent. Exposure of 0.5–10 Gy ^{60}Co γ rays to four different cell lines (Chinese hamster ovary clone K-1, Chinese hamster lung fibroblast clone A03, HeLa close S-3, and Mouse neuroblastoma C-1300 clone N1E-115) resulted in hypomethylation 1-3 days post-irradiation (Kalinich et al. 1989). Another study group observed no change in DNA methylation in Murine cell line m5S/1M and Chinese hamster CHO/K-1 cells exposed to 10 Gy X rays over 3-day post-irradiation period (Tawa et al. 1998). More recently, global hypermethylation was observed 20 population doublings after irradiation of human fibroblast cells using 0.1–1 Gy ^{60}Co γ rays (Kaup et al. 2006a).

It was also shown that radiation exposure led to sex- and tissue-specific DNA methylation changes in mice and rats (Table I-3) (Pogribny et al. 2004; Raiche et al. 2004a). Significant global hypomethylation was observed in the female liver post-irradiation, but no effect on male liver was shown (Pogribny et al. 2004). Tawa et al. (1998) showed global hypomethylation in mouse liver tissue after X-ray exposure, but did not found any changes in brain or spleen tissue. The methylation of p16 promoter was more pronounced in male mice liver tissue exposed to radiation than in female tissue (Kovalchuk et al. 2004). In the same study, no significant changes in p16

methylation were found in the muscle tissue of exposed males and females.

In addition, radiation-induced genome methylation changes can be different by the use of different radiation dose or radiation quality (Table I-4) (Aypar et al. 2011a; Goetz et al. 2011). Low LET X-ray exposure resulted in hypomethylation of LINE-1 following 2 Gy irradiation and hypermethylation after 0.5 Gy irradiation in human hamster hybrid cell line (Aypar et al. 2011a). LINE-1 methylation change induced by 1 Gy X-ray was significantly different from the proton and iron-ion response in AG01522 cells (Goetz et al. 2011).

Due to these variability of the results of studies on the methylation status after irradiation, it is difficult to predict the effects of ionizing radiation on DNA methylation in human populations. Though radiation-induced DNA methylation changes were often investigated in human populations, the studies were mainly performed in radiation-exposed cancer patients (Table I-5). Thus, further studies are needed to confirm the changes induced by different doses and the effects of confounding factors such as age and other exposures in normal human population.

Table I-3. Studies on Global DNA methylation changes induced by ionizing radiation

Irradiated subjects	Cell or tissue type	Effects measured	Exposure conditions	Reference
SiHa cell line	Human cervical carcinoma	Global hypomethylation	0~8 Gy γ-ray	Kumar et al. (2011)
SaOS2 cell line	Human osteosarcoma			
GM10115 cell line	Human hamster hybrid	Global hypermethylation	0.5, 2Gy X-ray	Aypar et al. (2011a)
C57BL mice	Male & Female spleen	Global hypomethylation	0~5Gy X ray	Pogribny et al. (2004)
	Male & Female lung	No changes		
	Female liver	Global hypomethylation		
	Male liver	No changes		
Pinus silvestris	Tree crowns	Global hypermethylation	20-40Gy γ-ray	Kovalchuk et al. (2003)
C57BL/6NJcl mice	Liver	Global hypomethylation	10Gy X ray	Tawa et al. (1998)
	Brain & spleen	No changes		
CHO K-1 cell line	Chinese hamster ovary	No changes	4~10Gy X ray	
CHO K-1 cell line	Chinese hamster ovary	Global hypomethylation	0.5~10 Gy γ-ray	Kalinich et al. (1989)
C-1300 N1E-115 cell line	Mouse neuroblastoma			
V79A03 cell line	Chinese hamster lung fibroblast			
Hela S-3 cell lines	Human cervical carcinoma			
SaOS2 cell line	Human osteosarcoma			

Table I-4. Radiation-induced DNA methylation changes by radiation dose or radiation quality

Irradiated subjects	Global	LINE-1	Sat2	Radiation quality	Radiation dose	Reference
Human-hamster hybrid cell line[a]	↑	↑	-	Low LET	0.5 Gy	Aypar et al. (2011a)
	(↑)	↓	-	X ray	2 Gy	
	(↑)	-	-	High LET	0.1 Gy	
	(↓)	↓	-	Fe ions	1 Gy	
Human colorectal carcinoma cell line[b]	↓	-	-	Low LET	0.1 Gy	Goetz et al. (2011)
	↓	↓	-	X ray	1 Gy	
	(↑)	↓	-	High LET	0.1 Gy	
	↑	↓	-	Fe ions	1 Gy	
Human skin fibroblasts[c]	(↓)	(↑)	-	Low LET	0.1 Gy	Goetz et al. (2011)
	(↓)	↑	-	X ray	1 Gy	
	(↑)	↓	-	High LET	0.1 Gy	
	(↑)	↓	-	Fe ions	1 Gy	
Human keratinocytes[d]	-	-	(↑)	Low LET	0.5 Gy	Kaup et al. (2006a)
	-	-	↑	γ ray	5 Gy	

↑, significant increase compared to control

↓, significant decrease compared to control

(↑), increase but no significant changes compared to control

(↓), decrease but no significant changes compared to control

[a], GM10115 cell line; [b], RKO cell line;

[c], AG01522D cell line; [d], HPV-G cell line

Table I-5. Studies on the association between radiation exposure and DNA methylation changes in human population

Subjects	Tissues	Effects measured	Reference
plutonium-exposed workers at MAYAK, the Russian nuclear facility	Lung Adenocarcinoma	Higher prevalence for methylation of the p16 gene	Belinsky et al. (2004)
plutonium-exposed workers at MAYAK	Lung Adenocarcinoma	Increase in the prevalence of methylation of GATA5	Lyon et al. (2007)
occupationally exposed workers at hospitals	Spermatozoa	Global hypermethylation	Kumar et al. (2013)

1.3.2. Radiation-induced DNA methylation and genomic instability

Recent evidences have suggested that epigenetics such as DNA methylation and histone modification may be the mechanistic link among irradiation, genomic instability and carcinogenesis (Aypar et al. 2011b).

Genomic instability is defined as the increase in acquisition of multiple changes in the genome (Morgan et al. 1996). Radiation-induced genomic instability (RIGI) can be observed in cells that survive at delayed times after irradiation and sometimes manifest in their progeny over many generations (Aypar et al. 2011b). RIGI can manifest as delayed mutation, decreased plating efficiency, micronuclei formation, change in ploidy, gene amplification, microsatellite instability and chromosomal instability (Chang and Little 1992; Morgan 2003a; Morgan 2003b). The notable types of chromsomal instability of genome involve aneuploidy, gene deletion and chromosome aberrations (Kadhim et al. 1992; Morgan et al. 1996; Little 2003). RIGI may be one of the pathways by which radiation exposure can lead to cancer, therefore, understanding the mechanisms underlying genomic instability may help elucidate the processes involved in the development of radiation-induced carcinogenesis (Aypar et al. 2011b).

The mechanisms of RIGI have been extensively studied, but alterations such as radiation-induced mutations or double-strand breaks alone could not account for the initiation or perpetuation of RIGI (Limoli et al. 1997;

Morgan et al. 1998; Aypar et al. 2011b). As non-targeted effects, including RIGI, affect changes in cellular phenotype without direct radiation exposure or without altering DNA sequence (Aypar et al. 2011b), the mechanistic role of epigenetic changes in RIGI has attracted considerable attention.

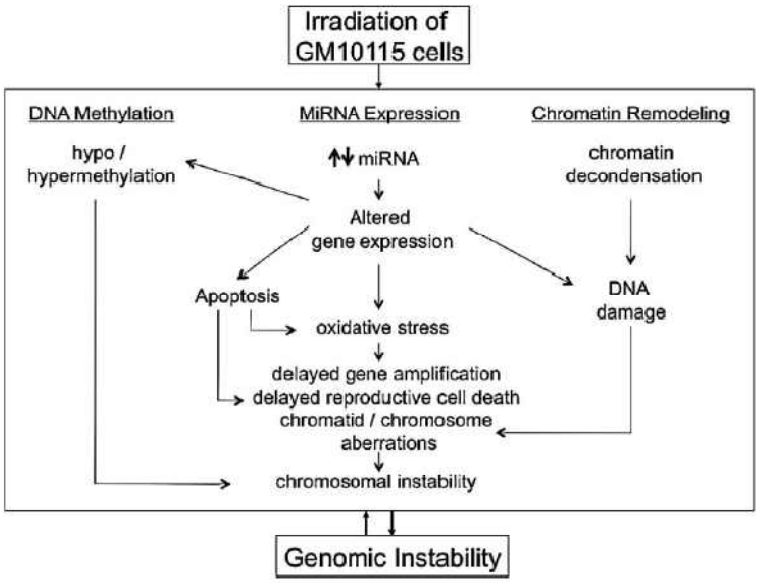

Figure I-2. Hypothesized model for the contribution of epigenetic alterations to radiation-induced genomic instability in the unstable clone (Aypar et al. 2011b).

Recent evidences have supported the hypothesis that epigenetic mechanisms may be involved in RIGI (Figure I-2). Pogribny et al. (2005) found that radiation exposure led to decreased trimethylation of histone H4 lysine, which result in relaxed heterochromatin organization that would impair genome stability. Several studies have also shown that euchromatic regions were more susceptible to radiation-induced DNA damage and gamma H2AX

accumulation (Falk et al. 2008; Vasireddy et al. 2010). In addition, Cha et al. (2009) and Shin et al. (2009) suggested that radiation exposure alters miRNA profiles that can affect apoptosis, regulation of cell cycle, and DNA damage and repair.

Recent studies also suggested that DNA methylation changes, one of epigenetic modifications, may be linked to RIGI. High levels of DNA methylation are associated with a closed conformation of chromatin, whereas unmethylated DNA tends to get repackaged in a more open configuration (Weber and Schubeler 2007; Cedar and Bergman 2009). The relaxed heterochromatin organization that could lead to DNA damage, impaired DNA repair and eventually genomic instability (Pogribny et al. 2005; Aypar et al. 2011b). In addition, ROS production post-irradiation can lead to radiation-induced DNA methylation changes which may result in RIGI (Roy et al. 2000).

While these evidences have supported the hypothesis that DNA methylation is associated with RIGI, the studies on the correlation between RIGI and DNA methylation changes are rare. Only one study using human cells has directly correlated RIGI in HPV-G cells with DNA methylation changes following exposure to ^{60}Co gamma-radiation (Kaup et al. 2006a). The RIGI was shown to be associated with DNA hypermethylation of satellite 2 (Sat2) sequences, and non-specific DNA hypomethylation was also observed. Therefore, it remains to understand related mechanisms and clarify whether DNA methylation change is associated with low dose radiation-induced genomic instability in human populations.

2. Purposes

The epigenetic regulation of radiation responses can be involved in carcinogenesis and epigenetics may be the mechanistic link between irradiation and genomic instability (Aypar et al. 2011b). Epigenetic parameters seem to be the mediators of the indirect radiation effects including radiation-induced genome instability, bystander, and transgenerational effects (Kovalchuk and Baulch 2008). Thus, this study was attempted to evaluate the effects of ionizing radiation on DNA methylation, one of the epigenetic alterations, in normal human blood.

The details of purposes in this study are following;

1. In order to evaluate the changes of DNA methylation in γ-ray exposed normal human blood.

2. In order to verify DNA methylation changes as a biomarker for monitoring the workers occupationally exposed to low dose of radiation

3. In order to examine the association between DNA methylation and genomic instability markers

Chapter II. DNA methylation alterations in response to radiation exposure *in vitro*

1. Introduction

Ionizing radiation (IR) is a well-known genotoxic agent and human carcinogen. Even though IR is a potentially lethal threat to all organisms, it is also widely used to effectively diagnose and treat cancer (De Potter et al. 2006; Erven and Van Limbergen 2007). With an increasing number of individuals being exposed to IR via occupational, diagnostic, or treatment-related routes, it is becoming important to understand IR's biological effects in order to address the adverse impacts in human (Merrifield and Kovalchuk 2013). While radiation-induced carcinogenesis is well studied (Little 2000; Huang et al. 2003; Williams 2008), its impact on epigenetic mechanisms remains to be elucidated.

The epigenetic changes could be the missing link between radiation exposure, radiation-induced genomic instability, and radiation-induced carcinogenesis (Aypar et al. 2011b). Therefore, it is important to understand the changes in epigenetic profiles following irradiation (Aypar et al. 2011a).

One important epigenetic mechanism is DNA methylation that plays an important role in developmental processes, imprinting, cell proliferation and maintenance of genome stability (Jones and Baylin 2007). Aberrant cytosine methylation is common in carcinogenesis. Methylation changes in cancer include global hypomethylation and de novo methylation of promoter regions and transcriptional repression of genes (Jones and Gonzalgo 1997). Since global hypomethylation and tumor suppressor hypermethylation are

common precursors of malignant transformation, their detection may be a useful biomarker test for carcinogens such as ionizing radiation (Pogribny et al. 1999).

Recently, some studies have shown that IR can affect DNA methylation (Koturbash et al. 2005b; Loree et al. 2006). However, there is a lack of consensus among these studies regarding the direction and magnitude of change in methylation after irradiation. Kalinich et al. (1989) demonstrated that exposure of ^{60}Co γ-rays (0.5–10 Gy) to four different cell lines (Chinese hamster ovary clone K-1, Chinese hamster lung fibroblast clone A03, HeLa close S-3, and Mouse neuroblastoma C-1300 clone N1E-115) resulted in hypomethylation 1–3 days following irradiation. Another group evaluated Murine cell line m5S/1M and Chinese hamster CHO/K-1 cells using 10 Gy X rays and observed no change in DNA methylation over 3 days post-irradiation period (Tawa et al. 1998). More recently, global hypermethylation was observed 20 population doublings after irradiation of human fibroblast cells using 0.1–1 Gy ^{60}Co γ rays (Kaup et al. 2006a).

It was also reported that radiation exposure led to sex- and tissue-specific global DNA hypomethylation in mice and rats (Pogribny et al. 2004; Raiche et al. 2004a). In addition, radiation-induced genome methylation changes can be different by the use of different radiation dose or dose rates, cell lines or post-irradiation times (Goetz et al. 2011). Therefore, it is important to evaluate the epigenetic changes in normal human cells to use epigenetic alterations as a biomarker for radiation exposure in human populations. In this study, I evaluated the post-irradiation status of specific

locus, repeat element, and genome-wide DNA methylation to determine the effect of ionizing radiation on epigenetic changes in normal human blood leukocytes.

2. Materials and Methods

2.1. Blood culture and Irradiation

Heparinized (50 U/mol sodium heparin) whole blood was obtained from a healthy donor with consent. Blood was irradiated with ^{137}Cs-generated γ-rays using Gamma-cell Elan 3000 (Nordion International, Canada) at a dose rate of 5.0 Gy/min with doses of 0.5, 1 and 2 Gy. Following irradiation, blood was cultured in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) that contained 10% fetal bovine serum (FBS) and 100 U/ml each of penicillin and streptomycin, and stimulated with 1% phytochemagglutinin (Gibco, CA). The cultures were maintained at 37 °C under an atmosphere of 5% CO_2.

2.2. DNA extraction and Bisulfite modification

Genomic DNA from the blood was isolated using the Wizard DNA extraction kit (Promega, Madison, WI, USA), according to the protocol provided by the supplier. The quantity and quality of the isolated DNA was measured on the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Cambridge, UK). The genomic DNA was modified with sodium bisulfite treatment using the EZ Methylation Kit (Zymo Research, Irvine, CA, USA) for use in repeat element and specific locus methylation assays.

2.3. RNA extraction and cDNA synthesis

Total RNA isolation was carried out using the AxyPrep™ Blood Total RNA Miniprep Kit (Axygen, Union City, CA, USA), according to the manufacturer's instructions. The quantity and quality of the isolated RNA was measured on the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Cambridge, UK). cDNA was synthesized using 1 μg of RNA as a template with the reverse transcription system (Bioneer, Daejeon, Korea). The produced cDNA was stored at -20 ℃ for later analysis of gene expression.

2.4. Assessment of global DNA methylation

Global DNA methylation levels were quantified with a commercially available Methylflash™ Methylated DNA Quantification Kit (Epigentek Group Inc., New York, USA), according to the manufacturer's instructions with 100ng of genomic DNA. The methodology for estimation of global methylation levels used in this study takes into account methylation of all CpG, including promoter and non-promoter CpG, that is irrespective of their position in the genome. Many studies have used this method to evaluate the global DNA methylation levels in mouse, rats and human (Kim et al. 2013; Lou et al. 2013; Patel et al. 2013). In this assay, methylated DNA was recognized using 5-methylcytosine (5-mC) antibody

and quantified by reading the absorbance at 450 nm in a microplate reader. The amount of methylated DNA is proportional to the OD intensity measured, and the level of DNA methylation was expressed in terms of percentage of methylation.

2.5. Repeat Element DNA Methylation Assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as described previously (Weisenberger et al. 2005). In brief, DNA was PCR-amplified in a ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) under the following conditions; 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 s and 55˚C for 1 min. The primers and probes for repeat elements including LINE1, Sat2 and AluC4 were previously described (Weisenberger et al. 2005). AluC4 was used to normalize for the amount of input DNA, respectively. Percentage of methylated reference (PMR) values were calculated by dividing the LINE1(or Sat2):AluC4 ratio of a sample by the LINE1 (or Sat2): AluC4 ratio from CpGenome^{TM} Universal methylated DNA and multiplying by 100. All samples were assayed in triplicate.

2.6. Specific Locus DNA Methylation Assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as described previously (Eads et al. 2001; Widschwendter et al. 2004). In brief, DNA was PCR-amplified in a ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems) under the following conditions; 95˚C for 10 min, followed by 50 cycles at 95˚C for 15 s and 55˚C for 1 min. The primers and probes for CDKN2A, GSTP1, MGMT, TERT, MLH1, RASSF1A and β-actin (ACTB) were previously described (Widschwendter et al. 2004). ACTB was used to normalize for the amount of input DNA, respectively. The percentage of methylation at a specific locus was calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT = (CT_{,Target} - CT_{,Reference})_{sample} - (CT_{,Target} - CT_{,Reference})_{fully\ methylated\ DNA}$ and multiplied by 100 (Livak and Schmittgen 2001). For the $2^{-\Delta\Delta CT}$ method to be valid, the amplification efficiencies of the test genes and reference gene must be approximately equal. This was examined using real-time PCR for the detection of serial dilutions of DNA with a 10-fold range and gene-specific primers of each genes and ACTB. The ΔCT ($CT_{,Target\ gene} - CT_{,Reference}$) was calculated for each DNA dilution and a plot of the log DNA dilution vs. ΔCT was made. All amplification efficiencies were similar. All samples were assayed in triplicate.

2.7. Quantitative real-time PCR for gene expression

Gene expression levels were quantitatively measured with a real-

time quantitative PCR instrument (ABI 7300, Applied Biosystems, Foster City, CA, USA), using and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers. The primers for DNMT1, DNMT3a, DNMT3b, GAPDH, MLH1, ACTB were previously described (Saito et al. 2001; Furukawa et al. 2002). Amplification conditions consisted of an initial 10 min denaturation step at 95˚C , followed by 45 cycles of denaturation at 95˚C for 15 s and annealing and extension for 1 min at 60˚C. The expression levels for DNMTs and MLH1 were calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT= (CT_{,Target}–CT_{,Reference})_{test} - (CT_{,Target}–CT_{,Reference})_{calibrator}$ and multiplied by 100 (Livak and Schmittgen 2001). GAPDH and ACTB were used to normalize gene expression levels. The amplification efficiencies of the test genes and reference gene were similar. All samples were assayed in triplicate.

2.8. Statistical analysis

Means and standard deviations were calculated for all data from three replicates. Comparisons between treatment groups and corresponding controls were analyzed using One-way analysis of Variance (ANOVA) followed by Dunnett's test as a post hoc test. Statistical analysis was performed using SPSS statistics v. 20 (SPSS Inc., Chicago, IL, USA), and p-values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of γ-radiation on global methylation level in normal human leukocytes

To explore the effects of γ-radiation on DNA methylation, I first examined the effects of γ-radiation on the global methylation status of normal human leukocytes. The cells were exposed to 0, 0.5, 1, or 2 Gy of γ-radiation. At 72h post-irradiation, genomic DNA was extracted and methylation levels were measured by using an ELISA-based assay. The effect of of γ -radiation on the percentage of 5-methylcytosine (5-mc) in cellular DNA is shown in Figure II-1. Exposure of γ-radiation decreased global DNA methylation level in a dose-dependent manner (control, 100%; 0.5 Gy, 95.97%; 1Gy, 82.91%; 2Gy, 74.21%).

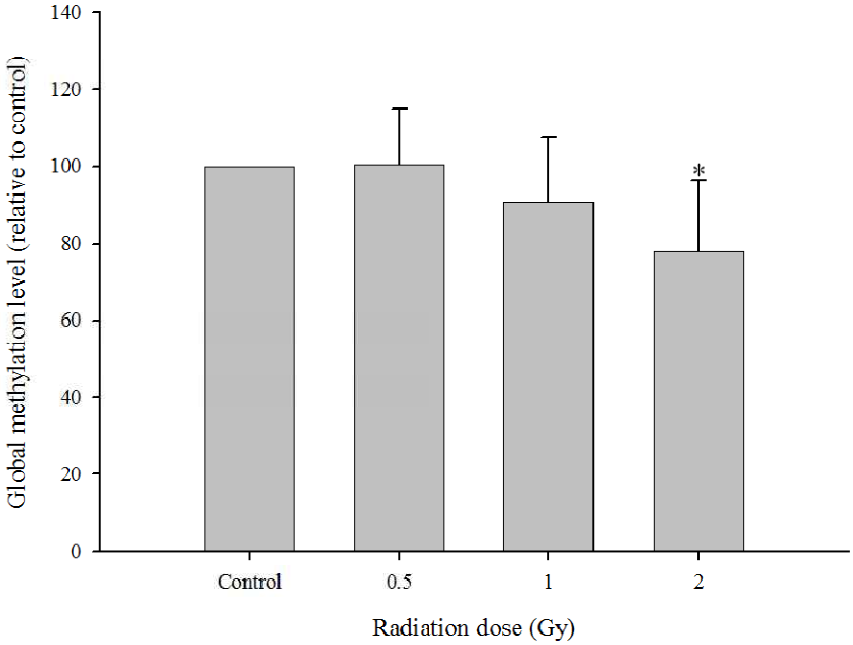

Figure II-1.Effect of γ-radiation on global DNA methylation in normal human leukocytes. Normal human leukocytes were exposed to various doses of γ-radiation. At 72h post-irradiation, genomic DNA was extracted, and global methylation status was quantified in a ELISA-based assay. Values were expressed as percentage of control in triplicate determinants and represent mean ± SD. Results were statistically analyzed with Dunnett's post hoc test after one-way ANOVA test (*, $p < 0.05$ compared to control).

3.2. Effect of γ-radiation on repeat element methylation level in normal human leukocytes

I also examined the effects of γ-radiation on the repeat element methylation level of normal human leukocytes, because analyzing the methylation of repeat elements can serve as a surrogate marker for global DNA methylation. DNA methylation level of Sat2 and LINE-1 repeat elements was evaluated by MethyLight assay. As shown in Figure II-2., the levels of Sat2 and LINE-1 were higher in irradiated cells than in controls. Particularly, there was significant differences in LINE-1 methylation at 0.5, 1, or 2 Gy of γ-radiation relative to controls (0.5Gy, 113.20%; 1Gy, 113.84%, 2Gy, 110.46%). No significant differences on Sat2 methylation level were found in either group ($p>0.05$).

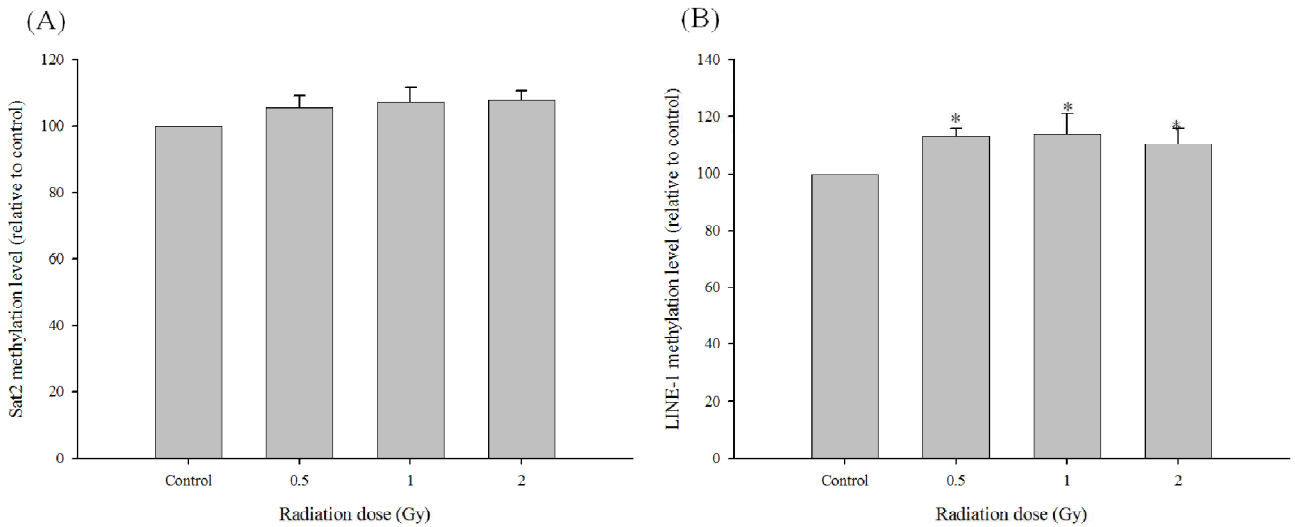

Figure II-2. Effect of γ-radiation on repeat element methylation level in normal human leukocytes. Normal human leukocytes were exposed to various doses of γ-radiation. At 72h post-irradiation, genomic DNA was extracted and modified with sodium bisulfite treatment. DNA methylation levels of Sat2 (A) and LINE-1 (B) elements were analyzed by using MethyLight assay. Values were expressed as percentage of control in triplicate determinants and represent mean ± SD. Results were statistically analyzed with Dunnett's post hoc test one-way ANOVA test (*, $p < 0.05$ compared to control).

3.3. Specific locus DNA methylation

I also investigated the promoter methylation status of six human genes: *p16*, *MGMT*, *GSTP1*, *RASSFF1A*, *hTERT*, and *MLH1* in normal human leukocyte DNA. The promoter regions of *p16*, *MGMT*, *GSPT1*, *RASSFF1A*, and *TERT* showed no significant differences in their methylation statuses after irradiation. Only *MLH1* was found to be significantly hypermethylated in its promoter region in the cells exposed to 2Gy γ radiation ($p<0.05$; Table II-1 and Figure II-3). The expression of *MLH1* mRNA was decreased in blood irradiated with 2 Gy compared to control ($p<0.05$, Table II-2).

Table II-1. Summary of methylation levels of specific genes in normal human leukocytes exposed to γ-radiation

	Radiation dose (Gy)			
	Control	0.5	1	2
p16	0.0 %	0.0 %	0.0 %	0.0 %
RASSF1A	0.0 %	0.0 %	0.0 %	0.0 %
MGMT	0.0 %	0.0 %	0.0 %	0.0 %
MLH1	0.062 ± 0.02 %	0.065 ± 0.06 %	0.10 ± 0.09%	0.16 ± 0.03 % *
GSTP1	0.0 %	0.0 %	0.0 %	0.0 %
hTERT	0.0 %	0.0 %	0.0 %	0.0 %

Results were statistically analyzed with Dunnett's post hoc test one-way ANOVA test (*, $p < 0.05$ compared to control).

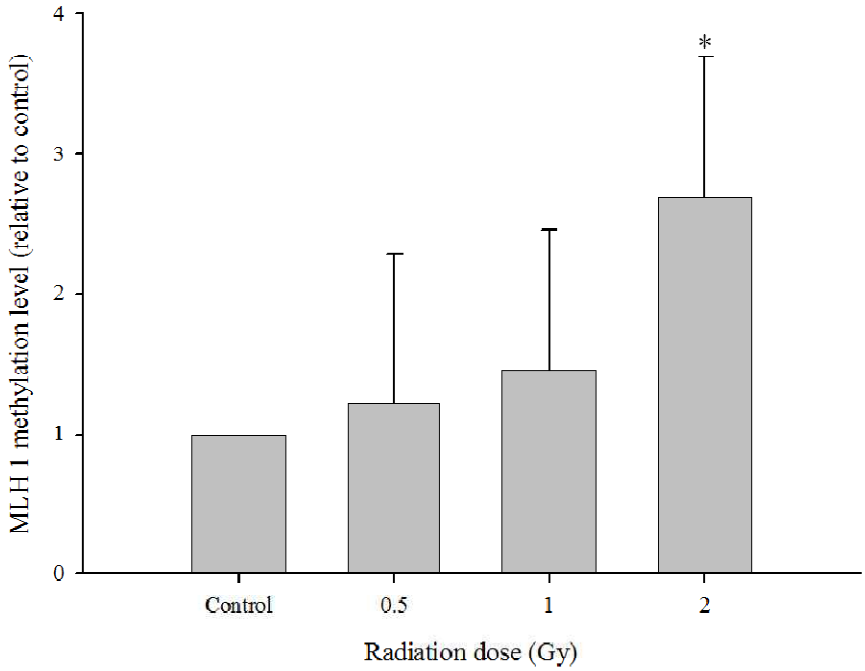

Figure II-3. Effect of γ-radiation on *MLH-1* methylation level in normal human leukocytes. Normal human leukocytes were exposed to various doses of γ-radiation. At 72h post-irradiation, genomic DNA was extracted and modified with sodium bisulfite treatment. DNA methylation levels of *MLH-1* were analyzed by using MethyLight assay. Values were derived as fold change in irradiated cells compared to the controls in triplicate determinants and represent mean ± SD. Results were statistically analyzed with Dunnett's post hoc test one-way ANOVA test (*, $p < 0.05$ compared to control).

Table II-2. Effect of γ-radiation on *MLH-1* expression level in normal human leukocytes

	MLH-1
	Mean ± SD
Control	1.00
0.5 Gy	1.56 ± 0.54
1.0 Gy	1.27 ± 0.28
2.0 Gy	0.80 ± 0.05*

Results were statistically analyzed with Dunnett's post hoc test one-way ANOVA test (*, $p < 0.05$ compared to control).

3.4. mRNA expression levels of DNMTs

As DNMTs play a crucial role in DNA methylation, I determined the expression profiles of *DNMT1*, *DNMT3a* and *DNMT3b*. As shown in Table II-3, the expression of *DNMT1*, *DNMT3a* and *DNMT3b* was significantly lower in 2Gy γ-irradiated cells than the non exposed control (*DNMT1*, 0.21 ± 0.18; *DNMT3a*, 0.41 ± 0.27; *DNMT3b*, 0.50 ± 0.13, Table II-3).

Table II-3. mRNA expression levels of DNMTs after radiation exposure in normal human leukocytes

	DNMT 1	*DNMT 3a*	*DNMT 3b*
	Mean ± SD	Mean ± SD	Mean ± SD
Control	1	1	1
0.5 Gy	1.37 ± 1.01	1.67 ± 1.58	1.09 ± 0.76
1.0 Gy	0.67 ± 0.037 *	1.02 ± 0.79	0.69 ± 0.25
2.0 Gy	0.21 ± 0.18 *	0.41 ± 0.27 *	0.50 ± 0.13 *

Results were statistically analyzed with Dunnett's post hoc test one-way ANOVA test (*, $p < 0.05$ compared to control).

4. Discussion

Exposure to ionizing radiation may result in a variety of epigenetic alterations as well as genetic effects. Although the effects of radiation on DNA methylation patterns have been studied, radiation-induced DNA methylation changes can be different by the use of different radiation dose, cell lines or post-irradiation times (Goetz et al. 2011). Therefore, it is important to evaluate the epigenetic changes in normal human cells to use the alterations as a biomarker for radiation exposure in human populations. In this study, I evaluated the post-irradiation status of specific locus, repeat element, and global DNA methylation to determine the effect of ionizing radiation on epigenetic changes in normal human blood.

4.1. Global methylation changes induced by ionizing radiation

Global methylation levels of irradiated and control cells were measured by DNA methylation quantification kits after irradiation. A few previous reports have investigated the effects of radiation exposure on global DNA methylation in animals and cancer cell lines. Kalinich et al. (1989) reported that ^{60}Co γ radiation led to the reduction of DNA methylation levels in Chinese hamster ovary (CHO) clone K-1, Chinese hamster lung fibroblast (V79) clone A03, HeLa clone S-3 and Mouse neuroblastoma C-1300 clone

NIE-115 cell lines. Tawa et al. (1998) also observed the induction of genome hypomethylation in the X-irradiated mice. In consistent with these results, γ-irradiation led to global DNA hypomethylation in normal human leukocytes in the present study. Global loss of DNA methylation tends to get repackaged in a more open configuration (Pogribny et al. 2005) and it may cause carcinogenesis by reactivating retrotransposable elements inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007).

The mechanisms underlying the DNA methylation changes induced by radiation have been proposed to involve DNMTs expression. DNMTs catalyze the transfer of a methyl group from S-adenosylmethionine to the cytosine at CpG dinucleotide to produce 5-methylcytosine. *DNMT1* is considered a maintenance DNA methyltrasferase, whereas *DNMT3a* and *DNMT3b* are responsible for *de novo* DNA methylation (Jones and Baylin 2002; Laird 2003). Altered DNA methylation was associated with reduced expression of *DNMT1* and *DNMT3a* in radiation-exposed mice (Pogribny et al. 2005). In chronically exposed female mice liver, decreased expression of *DNMT1*, *DNMT3a* and *DNMT3b* led to hypomethylation of the genome (Raiche et al. 2004b). In the present study, the expression of DNMTs was decreased with γ radiation. The global hypomethylation I detected in this study may be the consequence of decreased DNMTs expression resulting from γ irradiation.

4.2. Repeat element methylation changes induced by ionizing radiation

Methylation status of repeat elements such as LINE-1 and Sat2 is important given that together these elements, which consist of interspersed repeats and tandem repeats comprise approximately 45% of the human genome and that they are normally highly methylated (Lander et al. 2001; Jordan et al. 2003). Aberrant repeat element methylation is another common epigenetic change that evolves during the carcinogenic process (Chalitchagorn et al. 2004; Estecio et al. 2007). It has been suggested that hypomethylation of these sequences promoted genomic instability and tumor progression (Kazazian and Goodier 2002). Koturbash et al. (2007) showed that LINE-1 elements were hypomethylated in the bystander rat spleen 7 months after cranial exposure of 20 Gy X-rays. Hypomethylation of LINE-1 and Alu elements was also observed in RKO and AG01522D cell lines after 0.1 and 1 Gy of high LET Fe ion irradiation (Goetz et al. 2011).

Evaluation of repeat element DNA methylation using MethyLight assays did not show hypomethylation of LINE-1 after exposure to γ-ray in this study. Unexpectedly, I observed LINE-1 hypermethylation after 0.5, 1, and 2 Gy of γ irradiation. However, Goetz et al. (2011) demonstrated a trend for hypermethylation in AG01522D human diploid skin fibroblast cell lines post 1Gy of X-rays. Aypar et al. (2011a) also showed significant hypermethylation of LINE-1 elements in GM10115 human-hamster hybrid cell line following

exposure to 0.5 Gy X-rays. These results indicate that there is not a clear link between radiation exposure and hypomethylation. While DNA hypomethylation is thought to contribute to carcinogenesis by inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007), DNA hypermethylation may be linked to structural changes in chromatin, decreased transposon movement and decreased gene expression (Robertson 2005; Slotkin and Martienssen 2007; Yauk et al. 2008). Specifically, DNA packaging in heterochromatin, which results from LINE-1 hypermethylation in part, appears to delay DNA strand break processing, leading to slow down DNA repair (Rube et al. 2011).

I also investigated the methylation of Sat2 element after exposure to γ-ray. LINE-1 methylation levels were higher in exposed cells than in non-exposed cells, while any change of Sat2 methylation was not found in this study. Thus, Sat2 methylation appears to be inappropriate as a biomarker to investigate the radiation-induced epigenetic changes.

The changes in repeat element methylation observed in this study were not large. It could be argued that the differences between irradiated cells and control are modest and it is not sufficient to suggest a significant biological response. However, it must be considered that the LINE-1 assay evaluates just 10 CpG within 81 bp of DNA in this study. As a result, the changes in this study may represent a large change in methylation.

Koturbash et al. (2007) suggested that decreased levels of DNMTs and methyl-binding protein were linked to the significant global and LINE-1 hypomethylation. Although decreased DNMTs expression in irradiated cells.

was observed in my study, LINE-1 hypomethylation was not found in this study. This discrepancy may be because the process of DNA methylation is affected by other epigenetic factors besides DNMTs (Esteller 2007). Evidence suggests that chromatin remodeling enzymes and histone methylation are also essential for DNA methylation patterns (Geiman and Robertson 2002). Therefore, further studies are needed to investigate the expression of other epigenetic factors and address the mechanisms of LINE-1 methylation.

Analyzing the methylation of repeat elements can serve as a surrogate marker of global DNA methylation due to its high frequency in the genome (Yang et al. 2004). Some studies evaluated the methylation of repeat elements as a surrogate marker of global DNA methylation to investigate the epigenetic changes induced by benzene, lead and persistent organic pollutants (Bollati et al. 2007; Rusiecki et al. 2008; Wright et al. 2010). However, radiation-induced methylation changes of repeat elements were not similar to global DNA methylation changes in this study. In addition, the different outcomes in radiation-induced changes between global DNA methylation and repeat elements methylation were observed in other studies (Aypar et al. 2011a; Goetz et al. 2011). Although the differences could be due to cell types, experimental conditions, or assays used to evaluate the methylation levels, the levels of repeat elements methylation may be inappropriate as a surrogate of global DNA methylation to investigate the radiation-induced epigenetic changes. Furthermore, it also needs to study the changes and roles of repeat elements methylation changes itself following irradiation.

4.3. Change in *MLH1* methylation levels induced by γ radiation

Promoter methylation status of the six human genes [*p16*, *MGMT*, *GSTP1*, *RASSFF1A*, *hTERT*, and *MLH1*] was assessed by using MethyLight assay. The genes were chosen because it is known that their expression is linked to cancer and it is also involved in cellular responses to irradiation. Workers in plutonium plants had a 3.5 times greater risk for p16 methylation in their lung cancers than controls (Belinsky et al. 2004). The expression of *GSTP1* and *hTERT* was reduced in radiation-exposed workers and cell lines (Schuck et al. 2002; Fachin et al. 2009). Methylation of *MGMT* promoter correlated with response to radiotherapy in glioblastoma patients (Rivera et al. 2010). Promoter regions of these genes are hypermethylated in cancers and the hypermethylation of these genes correlates with their loss of transcription in human tumors (Belinsky et al. 1998; Esteller et al. 1998a; Herman et al. 1998; Esteller et al. 1999; Burbee et al. 2001). However, no changes in DNA methylation at the CpG sites analyzed for the promoter regions of *p16*, *MGMT*, *GSTP1*, *RASSFF1A*, and *hTERT* were observed in this study. It may be difficult to measure the methylation levels of the genes in normal blood, because the methylation levels of these genes were relatively low. Further studies, thus, are needed to determine whether radiation influences promoter methylation of these genes by using other assays.

Only *MLH1* methylation level was significantly increased in the cells

irradiated with 2Gy γ-radiation compared to control. For evaluation of radiation-induced changes, the cells showing at least a weak positive PCR were estimated as positive (PMR > 0.1%) because of the relatively low methylation levels observed for this gene (Friedrich et al. 2005). In the cells irradiated with 2Gy γ-radiation, it was found to be significantly methylated in *MLH1* promoter region in this study, and the expression of *MLH1* mRNA was also decreased.

MLH1, a key DNA mismatch repair protein, play a central role in correcting DNA mismatches during the DNA replication. Alterations in the expression of DNA repair-associated enzymes, such as *MLH1*, may affect mutation rates and genomic stability (Bucci et al. 2005; Martin et al. 2010). Transcriptional silencing of *MLH1* by promoter hypermethylation was associated with microsatellite instability phenotype in endometrial cancer (Esteller et al. 1998b). Contrary to its role in correcting replication errors, the role of *MLH1* in drug-induced DNA damages is detrimental to cells. Consequently, silencing of *MLH1*, due to hypermethylation, has been implicated acquired resistance to chemotherapeutic drugs *in vitro* (Strathdee et al. 1999). Loss of *MLH1* in mouse embryo fibroblast cell lines was associated with a modest increase in clonogenic survival following ionizing radiation (Fritzell et al. 1997). Yan et al. (2009) found that loss of *MLH1* in colorectal cells was associated with resistance to prolonged low-dose rate ionizing radiation. Although the changes of methylation and expression of *MLH1* by radiation exposure was small in this study, these alterations in chronically exposed radiation workers or cancer patients undergoing the radiotherapy

with high dose rate can be serious. Thus, further studies are needed to demonstrate the impact of radiation-induced *MLH1* hypermethylation.

In conclusion, ionizing radiation induced epigenetic changes including global DNA hypomethylation, repeat elements hypermethylation and *MLH1* hypermethylation. However, further studies are needed to clarify the functional impact of the radiation-induced epigenetic changes in population studies because normal cells cannot be propagated long enough to observe radiation-induced changes in theses end points.

Chapter III. DNA methylation alterations in the workers occupationally exposed to low dose radiation

1. Introduction

The risk of low dose radiation have been increasingly highlighted for special attention with environmental, diagnostic, and therapeutic exposure (Ma et al. 2010). The biological effects of chronic low dose radiation exposure and its relationship to carcinogenesis have been studied in recent years. It is reported that even low doses of IR used in diagnostic procedures can lead to the development of radiation-induced cancers (Liu et al. 2002; Brenner and Hall 2004). Low dose radiation can also result in genomic instability that manifests as chromosomal aberrations and gene mutations (Morgan 2003a; Nagar et al. 2003). Genomic instability markers increased significantly in the progeny of cells irradiated with 50 or 100cGy (Maxwell et al. 2008). Current evidences have shown that low dose radiation responses may be associated with epigenetic regulation (Ma et al. 2010; Aypar et al. 2011b). Recent studies have suggested that the epigenetic changes could be a missing link among radiation exposure, radiation-induced genomic instability, and radiation-induced carcinogenesis (Aypar et al. 2011b).

One important epigenetic mechanisms is DNA methylation that plays an important role in developmental processes, imprinting, cell proliferation and maintenance of genome stability (Jones and Baylin 2007). Alterations in DNA methylation have emerged as one of the molecular alterations in carcinogenesis (Jones and Baylin 2007). A global loss of DNA methylation can contribute to carcinogenesis by reactivating retrotransposable elements,

inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007). In addition, DNA hypermethylation may be linked to structural changes in chromatin, decreased transposon movement and decreased gene expression (Robertson 2005; Slotkin and Martienssen 2007; Yauk et al. 2008). DNA hypermethylation of gene promoter regions has also been linked to carcinogenesis, transcriptionally silencing genes involved in tumor suppression, DNA repair and cell cycle regulation (Baylin 2005). Since these global hypomethylation and tumor suppressor hypermethylation are common precursors of malignant transformation, their detection may be a useful biomarker test for carcinogens (Pogribny et al. 1999).

Recent studies also suggested that changes in genomic DNA methylation following irradiation is potentially correlated with initiation of genomic instability. Kaup et al. (2006b) found that radiation-induced genomic instability (RIGI) in HPV-G cells is correlated with epigenetic changes following exposure to ^{60}Co gamma-radiation. The RIGI was shown to be associated with DNA hypermethylation of satellite 2 (Sat2) sequences, and non-specific DNA hypomethylation was also observed in the study. These studies on radiation-induced methylation changes have shed light on the mechanisms of radiation-induced genome instability (Kovalchuk et al. 2004).

The existing data of radiation effects on DNA methylation patterns are limited. Studies on radiation-induced changes in DNA methylation have focused on animal and cancer cell lines. Even so, there is a lack of consensus among researches regarding the direction and magnitude of change in methylation after irradiation (Kalinich et al. 1989; Tawa et al. 1998; Kaup et

al. 2006b). It was also reported that radiation-induced genomic methylation changes can be different by the use of different radiation dose, cell lines or post-irradiation period (Goetz et al. 2011).

Therefore, it is important to evaluate the methylation changes in normal human cells to use epigenetic alterations as a biomarker for radiation exposure in human populations. In the present study, I investigated the effects of low-dose radiation exposure on DNA methylation using peripheral blood DNA from nuclear power plant workers. I also evaluated whether radiation-induced DNA methylation states were in turn associated with the frequency of micronuclei or chromosomal aberrations, indicators of the degree of genomic instability.

2. Materials and Methods

2.1. Study population and blood collection

The study population was composed of 176 male workers occupationally exposed to radiation from nuclear power plants and 30 controls who had never been occupationally exposed to ionizing radiation. The study protocol was approved by the Research Ethics Review Board of Seoul National University, and written informed consent was obtained from each individual before their inclusion in the study. Information regarding age, smoking status, drinking habits, and duration of work were obtained via personal interviews. To obtain the occupational radiation dose, the official personal dosimetry records were collected for the whole working periods. All subjects consented to participate in the study and volunteered to offer their blood samples. Peripheral whole blood samples from each subject were drawn into heparin-containing tubes and processed for analysis immediately upon arrival in the laboratory.

2.2. DNA extraction and Bisulfite modification

Genomic DNA from the blood of subjects was isolated using the Wizard DNA extraction kit (Promega, Madison, WI, USA), according to the

protocol provided by the supplier. The quantity and quality of the isolated DNA was measured on the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Cambridge, UK). The genomic DNA was modified with sodium bisulfite treatment using the EZ Methylation Kit (Zymo Research, Irvine, CA, USA) for use in repeat element and specific locus methylation assays.

2.3. RNA extraction and cDNA synthesis

Total RNA isolation was carried out using the AxyPrep™ Blood Total RNA Miniprep Kit (Axygen, Union City, CA, USA), according to the manufacturer's instructions. The quantity and quality of the isolated RNA was measured on the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technology, Cambridge, UK). cDNA was synthesized using 1 μg of RNA as a template with the reverse transcription system (Bioneer, Daejeon, Korea). The produed cDNA was stored at -20 ℃ for later analysis of gene expression.

2.4. Assessment of global DNA methylation

Global DNA methylation levels were quantified with a commercially available Methylflash™ Methylated DNA Quantification Kit (Epigentek Group Inc., New York, U.S.A.), according to the manufacturer's instructions with 100ng of genomic DNA. The methodology for estimation of global

methylation levels used in this study takes into account methylation of all CpG, including promoter and non-promoter CpG, that is irrespective of their position in the genome. Many study have used this method to evaluate the global DNA methylation levels in mouse, rats and human (Kim et al. 2013; Lou et al. 2013; Patel et al. 2013). In this assay, methylated DNA was recognized using 5-methylcytosine (5-mC) antibody and quantified by reading the absorbance at 450 nm in a microplate reader. The amount of methylated DNA is proportional to the OD intensity measured, and the level was expressed in terms of percentage of methylation. Intra-assay coefficients of variation (CVs) ranged from 0.0009 to 0.35 (mean, 0.12) and inter-assay variability ranged from 0.006 to 0.32 (mean, 0.18).

2.5. Repeat Element DNA Methylation Assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight assay as described previously (Weisenberger et al. 2005). In brief, DNA was amplified in a ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) under the following conditions; 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 s and 55˚C for 1 min. The primers and probes for repeat elements including LINE1, Sat2 and AluC4 were previously described (Weisenberger et al. 2005). AluC4 was used to normalize for the amount of input DNA, respectively. Percentage of

methylated reference (PMR) values was calculated by dividing the LINE-1(or Sat2):AluC4 ratio of a sample by the LINE-1 (or Sat2): AluC4 ratio from CpGenome^{TM} Universal methylated DNA and multiplying by 100. All samples were assayed in duplicate. Intra-assay coefficients of variation (CVs) ranged from 0.0083 to 0.18 (mean, 0.06) and inter-assay variability ranged from 0 to 0.32 (mean, 0.09).

2.6. Specific Locus DNA Methylation Assay

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight assay as described previously (Eads et al. 2001; Widschwendter et al. 2004). In brief, DNA was amplified in a ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) under the following conditions; 95˚C for 10 min, followed by 50 cycles at 95˚C for 15 s and 55˚C for 1 min. The primers and probes for *CDKN2A*, *GSTP1*, *MGMT*, *TERT, MLH1*, *RASSF1A* and *β-actin* (ACTB) were previously described (Widschwendter et al. 2004). ACTB was used to normalize for the amount of input DNA, respectively. The percentage of methylation at a specific locus was calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT= (CT_{,Target}–CT_{,Reference})_{sample} –(CT_{,Target}–CT_{,Reference})_{fully\ methylated\ DNA}$ and multiplied by 100 (Livak and Schmittgen 2001). For the $2^{-\Delta\Delta CT}$ method to be

valid, the amplification efficiencies of the test genes and reference gene must be approximately equal. This was examined using real-time PCR for detection of serial dilutions of DNA with a 10-fold range and gene-specific primers of each gene and ACTB. The ΔCT ($CT_{,Targetgene}$–$CT_{,Reference}$) was calculated for each DNA dilution and a plot of the log DNA dilution vs. ΔCT was made. All amplification efficiencies were similar. All samples were assayed in duplicate.

2.7. Quantitative RT-PCR to detect DNA methyltransferase mRNAs

Total RNA was isolated from each blood specimen, and cDNA was synthesized as described above. Primers used to amplify *DNMT1* RNA, 5'-CCCCTGAGCCCTACCGAAT-3' (sense) and 5'- CTCGCTGGAGTGGACTTGTG-3' (anti-sense), resulted in a 142 bp product (Saito et al. 2001). Primers used to amplify *DNMT3a* mRNA, 5'-TTCTACCGCCTCCTGCATGAT-3' (sense) and 5'-GCGAGATGTCCCTCTTGTCACTA-3' (anti-sense), resulted in a 113 bp product (Saito et al. 2001). Primers used to amplify *DNMT3b* mRNA, 5'-GAATTACTCACGCCCCAAGGA-3' (sense) and 5'-ACCGTGAGATGTCCCTCTTGTC-3' (anti-sense), resulted in a 101 bp product (Saito et al. 2001). To standardize the amount of RNA applied, glyceraldehydephosphate dehydrogenase (*GAPDH*) mRNA was amplified using 5'-GAAGGTGAAGGTCGGAGTC-3' (sense) and 5'-GAAGATGGTGATGGGATTTC-3' (anti-sense). PCRs were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City,

CA, USA) on a real-time PCR instrument (ABI 7300, Applied Biosystems). Amplification conditions consisted of an initial 10 min denaturation step at 95˚C , followed by 45 cycles of denaturation at 95˚C for 15 s and annealing and extension for 1 min at 60˚C. cDNA from healthy control's blood was used as a calibration sample. For relative quantification, the mRNA levels of the DNA methyltransferases (DNMTs) were calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT = (CT_{,DNMT} - CT_{,GAPDH})_{sample} - (CT_{,DNMT} - CT_{,GAPDH})_{calibration\ sample}$ and multiplied by 100 (Livak and Schmittgen 2001). Quantitative PCRs were performed in duplicate for each sample–primer set, and the mean of the 2 experiments was used as the relative quantification value.

2.8. Blood culture for chromosome aberration analysis and cytokinesis-block micronucleus assay

Heparinized blood samples (1 ml) were seeded into 9 ml of culture medium, RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), 100 units/ml penicillin (Gibco) and 1% phytohemagglutinin (PHA, Sigma). The cultures were incubated at 37 ℃ in an atmosphere of 95% air and 5% CO_2.

2.9. Chromosome aberration analysis

The cultures were incubated for 48 hours from the start of the culture with PHA and colcemid (0.1 μl/ml) was added to the cultures 3 hours prior to harvesting. Chromosome preparations were performed according to the standard procedures (IAEA, 2001). The cells were treated with 0.075 M KCl hypotonic solution and fixed in the mixture of methanol : acetic acid (3:1). The slides were coded and scored blindly. For each slide, 500 cells were scored. This data was provided from Lee (2013).

2.10. Cytokinesis-block micronucleus assay

Cytochalasin-B (4.0 μg/ml, Sigma) was added after 44 hours from the start of the culture, followed by another incubation for 28 hours. The cells were treated with 0.075 M KCl hypotonic solution and fixed in the mixture of methanol : acetic acid (3:1). For each slide, 1000 binucleated cells were scored and the frequencies of micronuclei were scored according to the criteria set by Fenech M. (2000). This data was provided from Lee (2013).

2.11. Statistical analysis

Statistical analysis was performed using the SAS version 9.3 statistical package. Global DNA methylation levels, Sat2 methylation levels, and *DNMT3a* and *DNMT3b* expression levels was log-transformed, and *DNMT1* gene expression level was square-root transformed to meet

assumptions of normality. The differences between radiation exposed workers and controls in terms of general characteristics were tested using Mann-Whitney U test and $\chi 2$ test. Mann-Whitney U test was used to compare the methylation levels in radiation-exposed workers with controls. The relationship between radiation exposure and DNA methylation levels was tested by kendall rank correlation. Multiple linear regression model was used to evaluate the independent association between the methylation levels and various variables including age, smoking status, alcohol intake, and radiation exposure. The correlation between DNA methylation levels and genomic instability markers was tested by kendall rank correlation. p-values < 0.05 were considered statistically significant.

3. Results

3.1. General characteristics of nuclear power plant workers and controls

The population characteristics are shown in Table III-1. The study subjects were composed of healthy males. The age of radiation workers ranged from 29 to 59 years with a mean of 47 years and that of the controls ranges from 28 to 65 years with a mean of 46 years. The proportion of drinkers was 81.82 % in workers and 63.33 % in controls. There was no significant difference in age or smoking status between the two groups indicating that radiation exposed workers and control subjects were closely matched. The dosimetry records over the duration of work showed that the range of the last 1.5 year, the last 5.5 years and the cumulative-radiation doses of workers is 0-25.22, 0-81.86, 12-400.25 mSv. Although the level of exposure was below the accepted annual limit of 20 mSv, radiation exposed workers exhibited significantly higher levels of chromosomal aberrations ($p<0.0001$) and micronuclei ($p<0.0001$) than controls.

Table III-1. General characteristics of nuclear power plant workers and controls

Variables	Controls	Workers	p-value
Number	30	176	
Age (mean ± SD), years	46 ± 8	47 ± 6	0.17[a]
Current smokers			0.58[b]
No (no. (%))	17 (56.67)	109 (61.93)	
Yes (no. (%))	13 (43.33)	67 (38.07)	
Alcohol drinkers			0.021[b]
No (no. (%))	11 (36.67)	32 (18.18)	
Yes (no. (%))	19 (63.33)	144 (81.82)	
Number of chromosome aberrations per 500 cells (mean ± SD)	3.83 ± 2.18	8.41 ± 3.57	<0.0001 [a]
Number of micronuclei per 1,000 cells (mean ± SD)	9.97 ± 3.02	20.85 ± 11.80	<0.0001[a]
Duration of work (mean ± SD), years	-	20.09 ± 6.02	-
Total radiation dose (mean ± SD), mSv	-	156.00 ± 84.92	-
Recent 5.5-years radiation dose (mean ± SD), mSv	-	29.80 ± 21.73	-
Recent 1.5-years radiation dose (mean ± SD), mSv	-	7.98 ± 6.84	-

Aberration: SD, Standard Deviation.
a, Mann-Whitney U test
b, χ^2 test

3.2. Effect of ionizing radiation on global DNA methylation

3.2.1. Levels of global DNA methylation in workers and controls

Global DNA methylation levels (5-mc(%)) was lower in radiation-exposed workers than in controls (Figure III-1). The median value was 0.53 in workers and 0.65 in controls (Mann-Whitney U test, p=0.035). Global DNA methylation levels of controls did not change throughout the age group, but the levels of workers were increased with age (Table III-2). Smoking and drinking status exhibited no association with DNA methylation levels in controls and workers group (Table III-2).

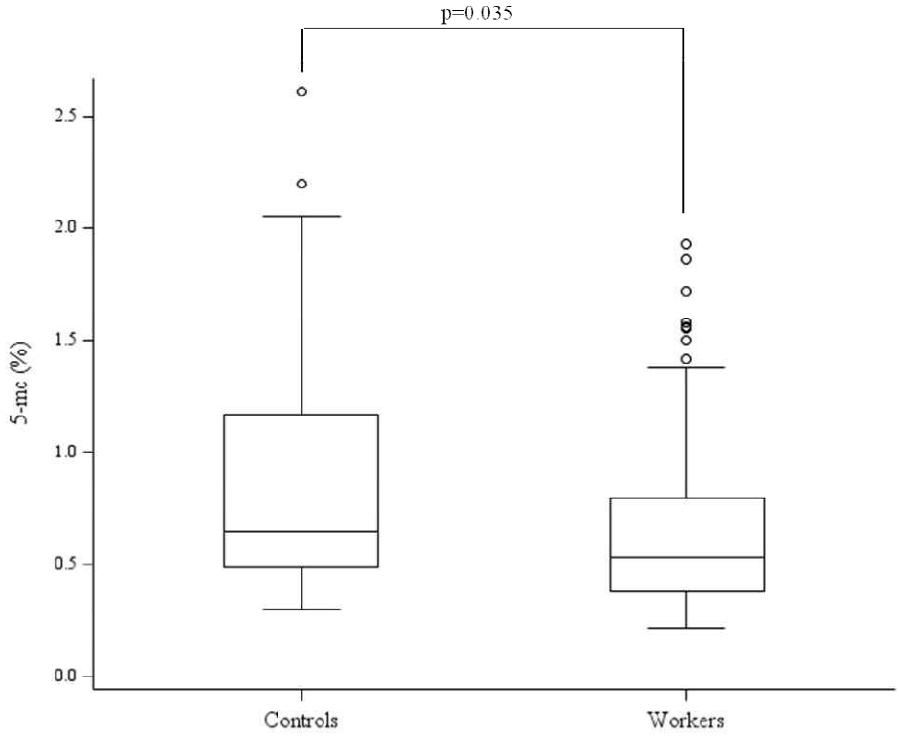

Figure III-1. Comparison of global methylation levels (5-mc(%)) between workers and controls. The nuclear power plant workers had significantly lower levels of global DNA methylation compared to controls (p=0.035; Mann-Whitney U test). Circles represent outliers.

Table III-2. The effects of age, smoking and drinking on global methylation (5-mc(%)) in workers and controls

		Total subjects		Controls		Workers	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age							
	-39	21	0.38 (0.32 - 0.45)	5	0.62 (0.40 - 0.96)	16	0.32 (0.28 - 0.37)
	40-49	99	0.62 (0.55 - 0.68)	15	0.75 (0.52 - 1.08)	84	0.59 (0.54 - 0.66)
	50-	86	0.62 (0.56 - 0.69)	10	0.79 (0.47 - 1.32)	76	0.60 (0.54 - 0.68)
	Kendall's τ		0.15		0.055		0.19
	p		0.0079		0.71		0.0021
Current smokers	No	126	0.61 (0.55 - 0.66)	17	0.73 (0.54 - 0.99)	109	0.60 (0.54 - 0.65)
	Yes	80	0.56 (0.49 - 0.63)	13	0.75 (0.50 - 1.15)	67	0.53 (0.47 - 0.59)
	Kendall's τ		-0.069		-0.0065		-0.086
	p		0.23		0.97		0.17
Alcohol drinkers	No	43	0.58 (0.50 - 0.71)	11	0.61 (0.39 - 0.95)	32	0.57 (0.49 - 0.72)
	Yes	163	0.59 (0.54 - 0.63)	19	0.83 (0.63 - 1.11)	144	0.56 (0.51 - 0.60)
	Kendall's τ		0.15		0.23		-0.029
	p		0.0079		0.14		0.64

Aberrations; GM, Geometric mean; CI, Confidence interval

p, Tested by Kendall rank correlation coefficient (τ)

3.2.2. Levels of global DNA methylation with radiation exposure in radiation exposed workers

Associations between radiation exposure dose and global DNA methylation levels were evaluated. Three different radiation doses, such as total cumulative dose, recent 5.5-year dose, and recent 1.5-year dose were considered. The levels of global DNA methylation by different radiation doses are shown in Table III-3. The trend of dose-dependent change was tested by Kendall rank correlation coefficient. Significant dose-effect relationships were found among different radiation dose groups (total cumulative dose, $p<0.0001$; recent 1.5 year dose, $p<0.0001$, Table III-3). As shown in Figure III-2, global DNA methylation levels were significantly correlated with total cumulative dose and recent 1.5-year dose. The global DNA methylation level was increased with total cumulative dose, while the level was decreased with recent 1.5 year dose (Table III-3 and Figure III-2). When the control group was included the analysis, the similar results were observed (Figure III-3).

Multiple linear regression analysis was performed to evaluate the association of radiation exposure dose with global DNA methylation levels independently of the variables such as age, smoking, and drinking habit. Total cumulative radiation dose and recent 1.5 year radiation dose were significantly associated with the levels of global DNA methylation after adjusting for age, smoking status and alcohol intake (Table III-4 and Table III-6), while no significant association between recent 5.5 year radiation dose and

global DNA methylation levels was found (Table III-5). As shown in Table III-4 and Table III-6, global DNA methylation level was significantly increased with total cumulative radiation dose, but it was decreased with recent 1.5 year radiation dose.

As shown in Figure III-4, the workers with lower total cumulative dose tend to be exposed to more radiation during recent 1.5-year than those with high total cumulative dose. The lowest total cumulative dose group had high levels of recent 1.5-year dose compared to other groups (Figure III-4A), and there was a trend (τ = -0.077, p=0.13, Figure III-4B) for recent 1.5-year radiation dose to be inversely associated with total cumulative dose.

To evaluate the independent association of total cumulative radiation dose and recent 1.5 year radiation dose with global DNA methylation, both total cumulative radiation dose and recent 1.5 year radiation dose were included in the multiple linear regression analysis. Even after adjusting for each radiation dose, similar results were observed. Global DNA methylation levels were positively associated with total cumulative radiation dose after adjusting for recent 1.5 year dose, while the levels were negatively associated with recent 1.5 year radiation dose after adjusting for total cumulative dose (Table III-7).

Table III-3. Geometric mean levels of the 5-mc(%) according to radiation exposure in radiation exposed workers

Radiation exposure	No.	GM (95% CI)
Total cumulative dose (mSv)		
<=100	38	0.40 (0.34 - 0.46)
>100, <=150	61	0.61 (0.54 - 0.69)
>150, <=200	29	0.59 (0.49 - 0.72)
>200	48	0.66 (0.58 - 0.75)
Kendall's τ		0.25
p		<0.0001
Recent 5.5 year dose (mSv)		
<=15	58	0.63 (0.55 - 0.72)
>15, <=30	40	0.49 (0.42 - 0.58)
>30, <=45	23	0.61 (0.50 - 0.75)
>45	55	0.54 (0.47 - 0.62)
Kendall's τ		-0.086
p		0.13
Recent 1.5 year dose (mSv)		
<= 5	76	0.65 (0.58 - 0.73)
>5, <=10	35	0.58 (0.50 - 0.68)
>10, <=15	32	0.57 (0.46 - 0.70)
>15	33	0.39 (0.35 - 0.44)
Kendall's τ		-0.26
p		<0.0001

Aberrations; GM, Geometric mean; CI, Confidence interval

p, Tested by Kendall rank correlation coefficient (τ)

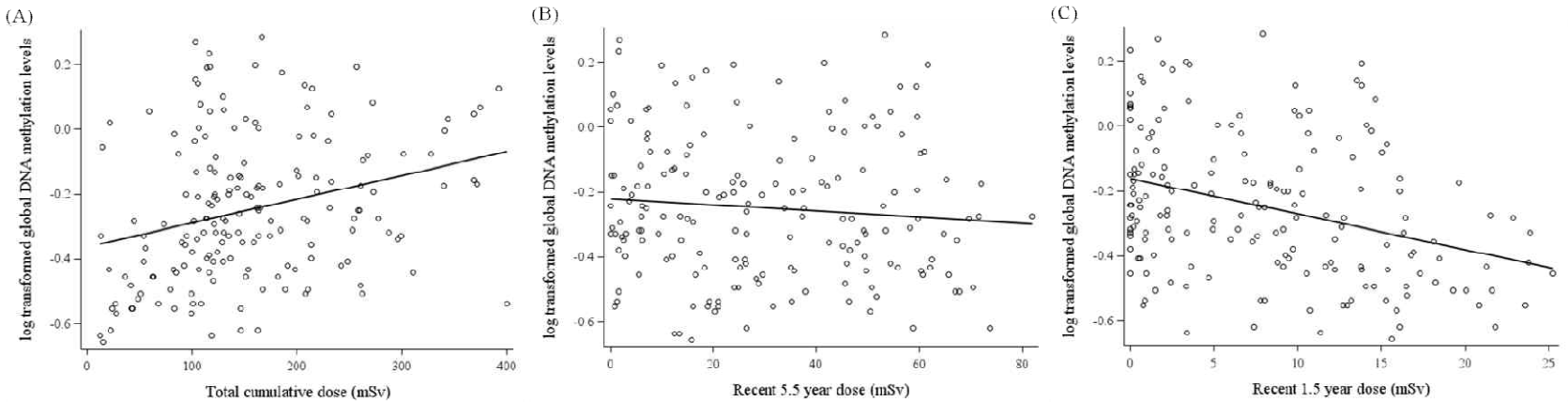

Figure III-2. Correlation between global DNA methylation levels and different radiation doses in radiation exposed workers. A total of 176 workers were analyzed for correlation between the levels of global DNA methylation and different radiation doses such as total cumulative dose (A), recent 5.5 year dose (B), and recent 1.5 year dose (C). The levels of global DNA methylation are log-transformed. (A), Kendall's τ = 0.22, $p<0.0001$; (B), Kendall's τ = -0.069, $p=0.17$; (C), Kendall's τ = -0.23, $p<0.0001$.Circles mean individual data points and line means univariate regression line.

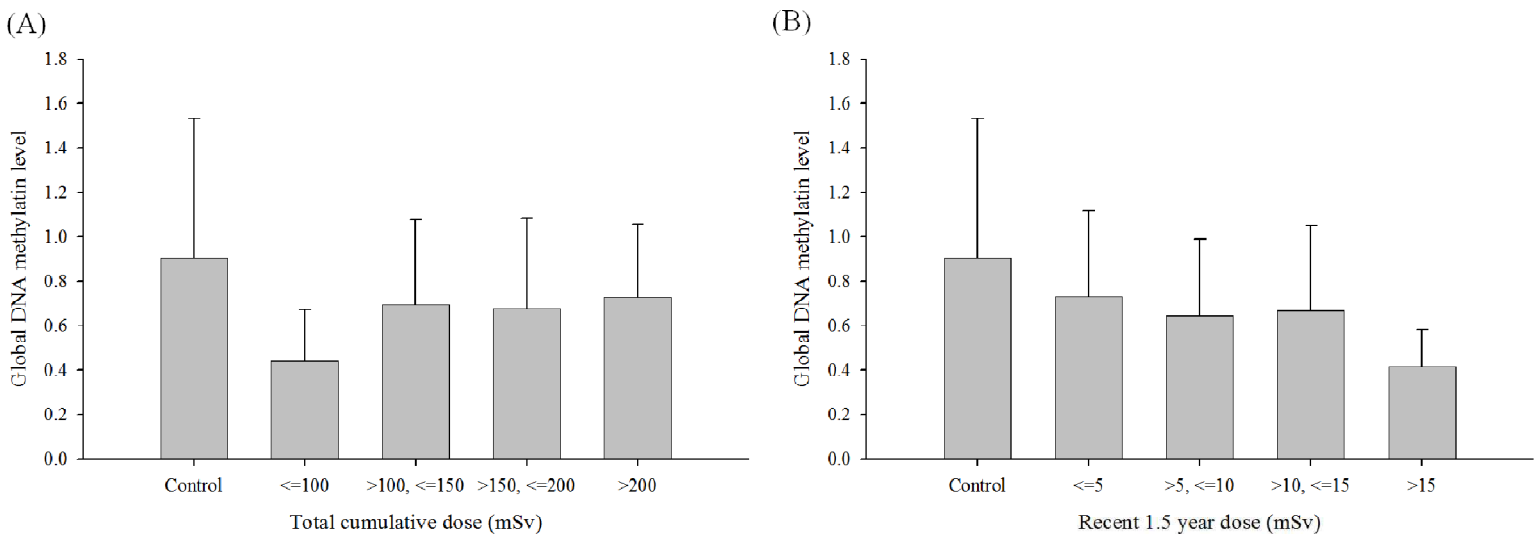

Figure III-3. Associations between radiation dose and global methylation in subjects including workers and controls. A total of 206 subjects including workers and controls were analyzed for association between the global DNA methylation levels and radiation dose such as total cumulative dose (A) and recent 1.5 year radiation dose (B). The relationship between radiation exposure and DNA methylation levels was tested by kendall rank correlation coefficient. Values represent mean ± SD. (A), Kendall's τ = 0.11, p=0.03; (B), Kendall's τ = -0.25, p<0.0001.

Table III-4. Multiple linear regression analysis of total cumulative radiation dose on log-transformed global methylation level in nuclear power plant workers

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	-0.60	-0.88	-0.31	<.0001
Age	0.0061	-0.000059	0.012	0.052
Smoking status (0. 1)[a]	-0.016	-0.084	0.052	0.64
Drinking status (0.1)[b]	-0.015	-0.097	0.067	0.71
Total cumulative radiation dose	0.00052	0.00011	0.00094	0.014

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-5. Multiple linear regression analysis of recent 5.5 year radiation dose on log-transformed global methylation level in nuclear power plant workers

		95% CI		
Variable	β	Low	Upper	p-value
Intercept	-0.67	-0.96	-0.35	<.0001
Age	0.0093	0.0035	0.015	0.0018
Smoking status (0. 1)[a]	-0.014	-0.084	0.055	0.68
Drinking status (0.1)[b]	-0.020	-0.10	0.063	0.63
Recent 5.5 year radiation dose	-0.00026	-0.0018	0.0013	0.73

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-6. Multiple linear regression analysis of recent 1.5 year radiation dose on log-transformed global methylation level in nuclear power plant workers

		95% CI		
Variable	β	Low	Upper	p-value
Intercept	-0.40	-0.71	-0.079	0.015
Age	0.0050	-0.0010	0.011	0.10
Smoking status (0. 1)[a]	-0.011	-0.078	0.056	0.75
Drinking status (0.1)[b]	-0.016	-0.097	0.064	0.69
Recent 1.5 year radiation dose	-0.0089	-0.014	-0.0038	0.0007

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

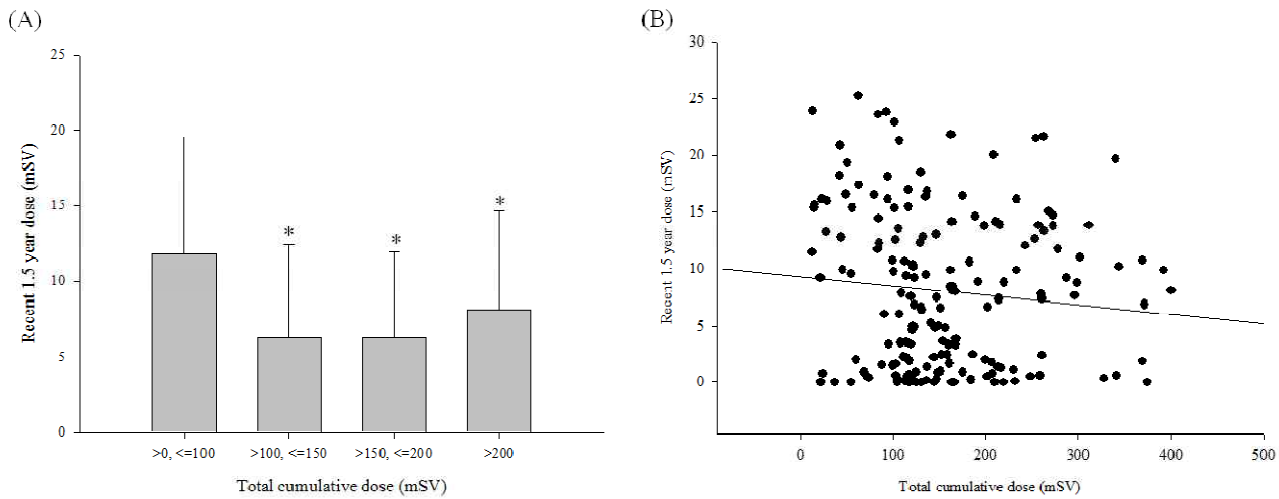

Figure III-4. Association between total cumulative dose and recent 1.5 year dose. A total of 176 workers were analyzed for association between total cumulative dose and recent 1.5-year dose. (A) Levels of recent 1.5 year radiation dose in various total cumulative dose groups. Values represent mean ± SD. Asterisk (*) means statistical significance for $p<0.05$ between the recent 1.5-year dose of each group and the dose of the group with the lowest total cumulative dose (>0, <=100). (B) Correlation between total cumulative dose and recent 1.5 year dose (Kendall's $\tau = -0.077$, $p=0.13$).

Table III-7. Multiple linear regression analysis of total cumulative radiation dose and recent 1.5 year radiation on log-transformed global methylation level in nuclear power plant workers

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	-0.27	-0.59	0.055	0.10
Age	0.00027	-0.0063	0.0069	0.93
Smoking status (0. 1)[a]	-0.013	-0.078	0.053	0.70
Drinking status (0.1)[b]	-0.011	-0.090	0.068	0.78
Recent 1.5 year radiation dose	-0.0010	-0.015	-0.0050	0.0001
Total cumulative radiation dose	0.00063	0.00023	0.0010	0.0023

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

3.2.3. Levels of global DNA methylation with duration of work in radiation exposed workers

I also evaluated the association between duration of work and global DNA methylation levels. The trend of duration-dependent change was tested by Kendall rank correlation coefficient, and significant dose-effect relationships were found (Kendall's $\tau = 0.17$, $p=0.0030$, Table III-8). As shown in Figure III-5, global DNA methylation levels were significantly correlated with duration of work.

Multiple linear regression analysis was performed to evaluate the association between duration of work and global DNA methylation levels independently of the variables such as age, smoking, and drinking habit. Duration of work was slightly associated with the levels of global DNA methylation after adjusting for age, smoking status and alcohol intake (Table III-9).

As shown in Figure III-6, there were significant correlations between duration of work and different radiation doses such as total cumulative dose, recent 5.5 year radiation dose, and recent 1.5 year radiation dose. To evaluate the association of duration of work with global DNA methylation independently of the radiation doses, multiple linear regression analysis was performed. After adjusting for radiation doses, there was no significant association between duration of work and global DNA methylation levels (Table III-10).

Table III-8. Geometric mean levels of the 5-mc(%) according to duration of work

Duration of work (yr)	No.	GM (95% CI)
<=15	31	0.38 (0.33 - 0.43)
>15, <=20	55	0.62 (0.54 - 0.72)
>20, <=25	66	0.64 (0.58 - 0.72)
>25	24	0.53 (0.42 - 0.67)
Kendall's τ		0.17
p		0.0030

Aberrations; GM, Geometric mean; CI, Confidence interval

p, Tested by Kendall rank correlation coefficient (τ)

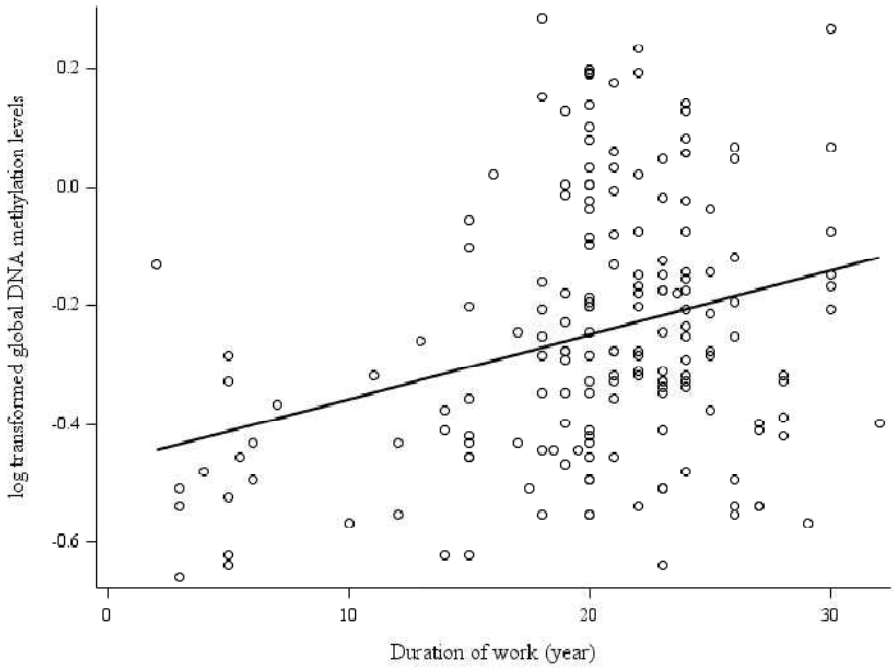

Figure III-5. Association between log transformed global DNA methylation and duration of work. Circles mean individual data points and line means univariate regression line. (Kendall's $\tau = 0.17$, $p = 0.0016$)

Table III-9. Multiple linear regression analysis of duration of work on log-transformed global methylation level in nuclear power plant workers

		95% CI		
Variable	β	Low	Upper	p-value
Intercept	-0.57	-0.87	-0.26	0.00030
Age	0.0037	-0.0047	0.012	0.39
Smoking status (0. 1)[a]	-0.0096	-0.078	0.059	0.78
Drinking status (0.1)[b]	-0.011	-0.094	0.072	0.80
Duration of work	0.0078	-0.00048	0.016	0.065

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

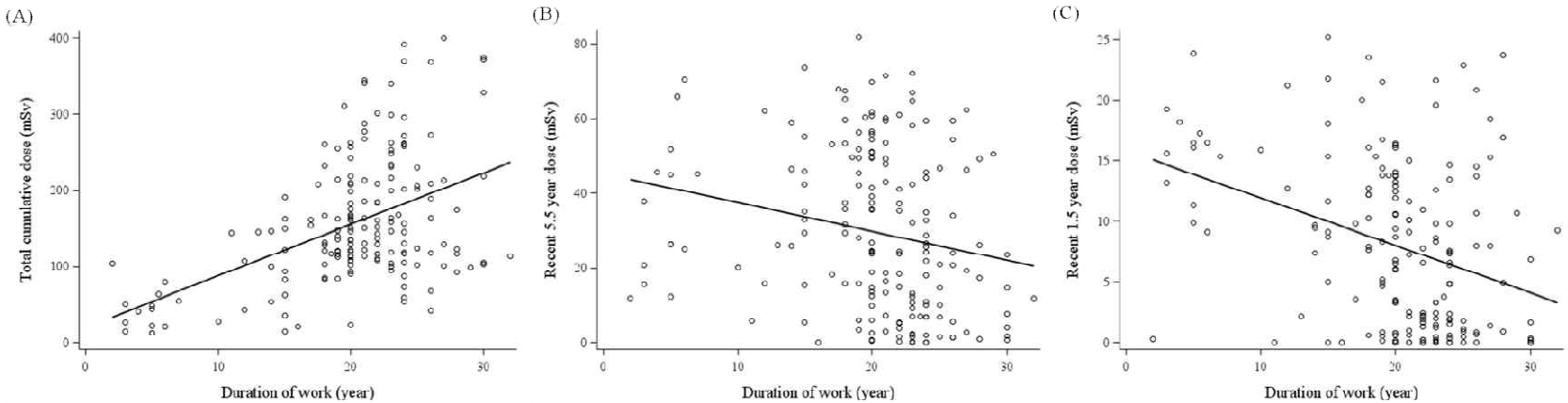

Figure III-6. Correlation between duration of work and different radiation doses in radiation exposed workers. A total of 176 workers were analyzed for association between duration of work and different radiation doses such as total cumulative dose (A), recent 5.5 year radiation dose (B), and recent 1.5 year radiation dose (C). (A), Kendall's $\tau = 0.28$, $p<0.0001$; (B), Kendall's $\tau = -0.20$, $p<0.0001$; (C), Kendall's $\tau = -0.24$, $p<0.0001$.

Table III-10. Multiple linear regression analysis of radiation dose and duration of work on log-transformed global methylation level

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	-0.20	-0.53	0.14	0.25
Age	-0.0035	-0.012	0.0051	0.42
Smoking status (0. 1)[a]	-0.0089	-0.074	0.057	0.79
Drinking status (0.1)[b]	-0.0054	-0.084	0.074	0.89
Total cumulative radiation dose	0.00057	0.00016	0.00098	0.0071
Recent 1.5 year radiation dose	-0.0099	-0.015	-0.0049	0.0001
Duration of work	0.0055	-0.0025	0.014	0.18

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

3.3. Effect of radiation exposure on repeat element methylation

3.3.1. Levels of repeat element methylation in workers and controls

Since methylation status of repeat elements such as LINE-1 and Sat2 is important given that together these elements, which consist of interspersed repeats and tandem repeats comprise approximately 45% of the human genome, the methylation levels of LINE-1 and Sat2 elements were also evaluated. LINE-1 methylation levels were higher in workers than in controls ($p<0.0001$, Figure III-7), while Sat2 methylation levels had no significant differences between workers and controls ($p=0.090$, Figure III-8).

The effects of age, smoking and drinking on repeat element methylation in workers and controls were evaluated. Age, smoking and drinking status exhibited no association with LINE-1 methylation levels in the controls and workers group (Table III-11). Sat2 methylation levels exhibited no association with smoking status and alcohol consumption in controls and workers. The levels were decreased with age in controls and workers, but it was not significant in workers (Table III-12).

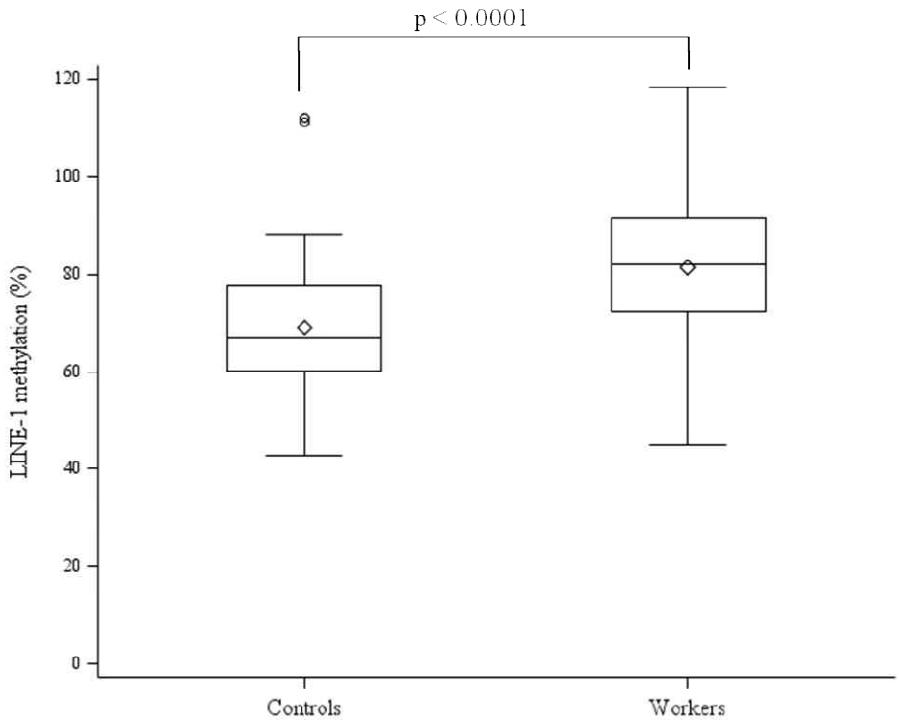

Figure III-7. Comparison of LINE-1 methylation between workers and controls. The nuclear power plant workers had significantly higher levels of global DNA methylation compared with controls ($p<0.0001$; t-test). Circles represent outliers and diamonds represent mean values.

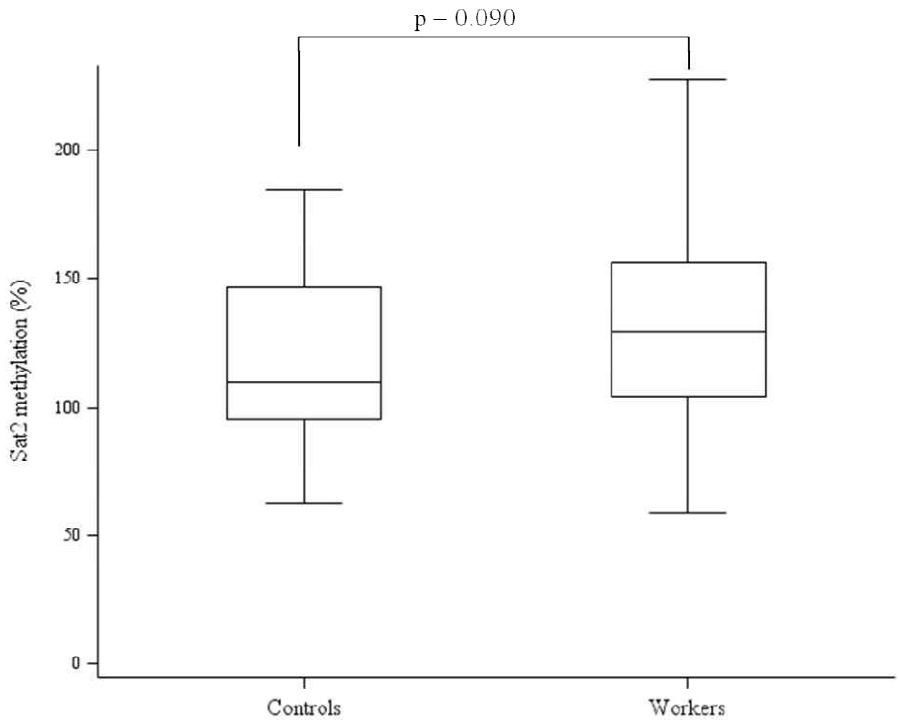

Figure III-8. Comparison of Sat2 methylation between workers and controls. The median (interquartile range) methylation levels were 129.30% (104.00% - 156.55%) in radiation exposed workers and 109.95% (95.20% - 146.85%) in controls. Mann–Whitney U test was used for statistical analysis.

Table III-11. The effects of age, smoking and drinking on LINE-1 methylation in workers and controls

		Total subjects		Controls		Workers	
		N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
Age							
	-39	21	78.37 ± 14.90	5	68.74 ± 13.87	16	81.38 ± 14.29
	40-49	99	81.69 ± 15.47	15	70.78 ± 20.94	84	83.64 ± 13.53
	50-	86	77.44 ± 15.60	10	66.03 ± 10.49	76	78.94 ± 15.59
	Kendall's τ		-0.063		-0.038		-0.082
	p		0.25		0.80		0.17
Current smokers	No	126	79.97 ± 15.38	17	68.25 ± 14.70	109	81.80 ± 14.72
	Yes	80	78.96 ± 15.84	13	69.65 ± 19.50	67	80.77 ± 14.52
	Kendall's τ		-0.033		0.087		-0.047
	p		0.56		0.57		0.45
Alcohol drinkers	No	43	79.16 ± 14.87	11	76.24 ± 20.03	32	80.17 ± 12.89
	Yes	163	79.69 ± 15.74	19	64.58 ± 13.07	144	81.68 ± 14.99
	Kendall's τ		0.024		-0.22		0.037
	p		0.67		0.16		0.55

p, Tested by Kendall rank correlation coefficient (τ)

Table III-12. The effects of age, smoking and drinking on Sat2 methylation in workers and controls

		Total subjects		Controls		Workers	
		N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)
Age							
	-39	20	141.1 (124.7 - 159.6)	5	141.3 (112.9 - 176.6)	15	141.0 (120.0 - 165.8)
	40-49	99	124.7 (117.5 - 132.2)	15	112.4 (94.3 - 134.0)	84	127.0 (119.3 - 135.2)
	50-	82	120.0 (112.9 - 127.6)	10	105.7 (90.3 - 123.8)	72	122.2 (114.3 - 130.6)
	Kendall's τ		-0.11		-0.30		-0.092
	p		0.058		0.043		0.13
Current smokers	No	121	123.9 (117.5 - 130.7)	17	112.4 (95.9 - 131.9)	104	125.9 (119.0 - 133.3)
	Yes	80	124.8 (117.3 - 132.7)	13	117.0 (101.0 - 135.6)	67	126.3 (117.9 - 135.4)
	Kendall's τ		0.0089		0.048		0.0096
	p		0.88		0.75		0.88
Alcohol drinkers	No	43	121.9 (110.7 - 134.3)	11	106.3 (87.6 - 128.9)	32	127.8 (114.2 - 143.1)
	Yes	158	125.0 (119.5 - 130.6)	19	119.4 (104.7 - 136.2)	139	125.7 (119.9 - 131.8)
	Kendall's τ		0.015		0.17		-0.031
	p		0.79		0.27		0.62

Aberrations; GM, Geometric mean; CI, Confidence interval

p, Tested by Kendall rank correlation coefficient (τ)

3.3.2. Levels of repeat element methylation with radiation exposure in radiation exposed workers

Associations with radiation exposure dose and repeat element methylation were evaluated. Three different radiation doses, such as total cumulative dose, recent 5.5-year dose, and recent 1.5-year dose were considered. The levels of repeat element methylation by different radiation doses are shown in Table III-13 and Table III-14. Trend of dose-dependent change was tested by Kendall rank correlation coefficient. Significant dose-effect relationship, which is not adjusted to variables such as age, smoking status and alcohol consumption, was not found among different radiation dose groups (Table III-13, Table III-14, Figure III-9, and Figure III-10).

Multiple linear regression analysis was performed to evaluate the association of radiation exposure dose with repeat element levels independently of the variables such as age, smoking, and drinking habit. Total cumulative radiation dose was significantly associated with the levels of global DNA methylation after adjusting for age, smoking status and alcohol intake (Table III-15), while recent 5.5 year radiation dose and recent 1.5 year dose were not associated with LINE-1 methylation (Table III-16 and Table III-17). Although LINE-1 methylation was decreased with total cumulative radiation dose after adjusting for other variables, LINE-1 methylation level was higher in workers with total cumulative radiation dose below 200 mSv than controls (Figure III-11). However, Sat2 methylation level was not

associated with radiation exposure dose such as total cumulative dose (Table III-18), recent 5.5 year dose (Table III-19), and recent 1.5 year dose (Table III-20).

Table III-13. Levels of LINE-1 methylation according to radiation exposure

Radiation exposure	No.	Mean ± SD
Total cumulative dose (mSv)		
<=100	38	82.6 ± 14.8
>100, <=150	60	83.1 ± 15.9
>150, <=200	29	83.3 ± 76.4
>200	48	76.4 ± 16.5
Kendall's τ		-0.082
p		0.15
Recent 5.5 year dose (mSv)		
<=15	58	81.9 ± 14.4
>15, <=30	40	80.8 ± 15.9
>30, <=45	23	84.4 ± 15.5
>45	54	79.3 ± 17.4
Kendall's τ		-0.068
p		0.24
Recent 1.5 year dose (mSv)		
<= 5	76	81.9 ± 14.7
>5, <=10	35	78.1 ± 17.2
>10, <=15	32	82.9 ± 15.7
>15	33	81.1 ± 16.9
Kendall's τ		-0.047
p		0.41

p, Tested by Kendall rank correlation coefficient (τ)

Table III-14. Levels of Sat2 methylation according to radiation exposure

Radiation exposure	No.	GM (95% CI)
Total cumulative dose (mSv)		
<=100	37	129.8 (117.2 - 143.8)
>100, <=150	58	127.0 (117.9 - 136.8)
>150, <=200	28	126.2 (112.0 - 142.3)
>200	48	122.1 (112.8 - 132.2)
Kendall's τ		-0.074
p		0.20
Recent 5.5 year dose (mSv)		
<=15	55	123.0 (114.6 - 132.0)
>15, <=30	38	123.8 (109.7 - 139.8)
>30, <=45	23	128.5 (114.6 - 144.0)
>45	55	129.9 (121.0 - 139.4)
Kendall's τ		0.052
p		0.37
Recent 1.5 year dose (mSv)		
<= 5	73	123.9 (115.1 - 133.4)
>5, <=10	34	126.2 (116.4 - 136.8)
>10, <=15	32	129.4 (117.4 - 142.7)
>15	32	127.8 (114.7 - 142.4)
Kendall's τ		0.023
p		0.70

Aberrations; GM, Geometric mean; CI, Confidence interval

p, Tested by Kendall rank correlation coefficient (τ)

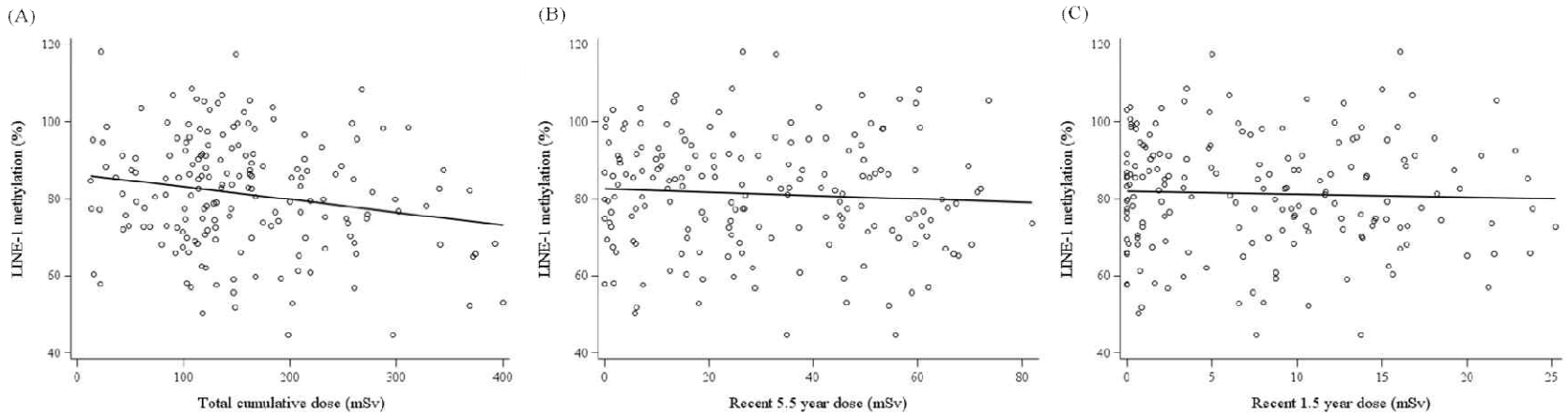

Figure III-9. Correlation between LINE-1 methylation levels and different radiation doses in radiation exposed workers. A total of 176 workers were analyzed for correlation between the levels of LINE-1 methylation and different radiation doses such as total cumulative dose (A), recent 5.5 year dose (B), and recent 1.5 year dose (C). Circles mean individual data points and line means univariate regression line.

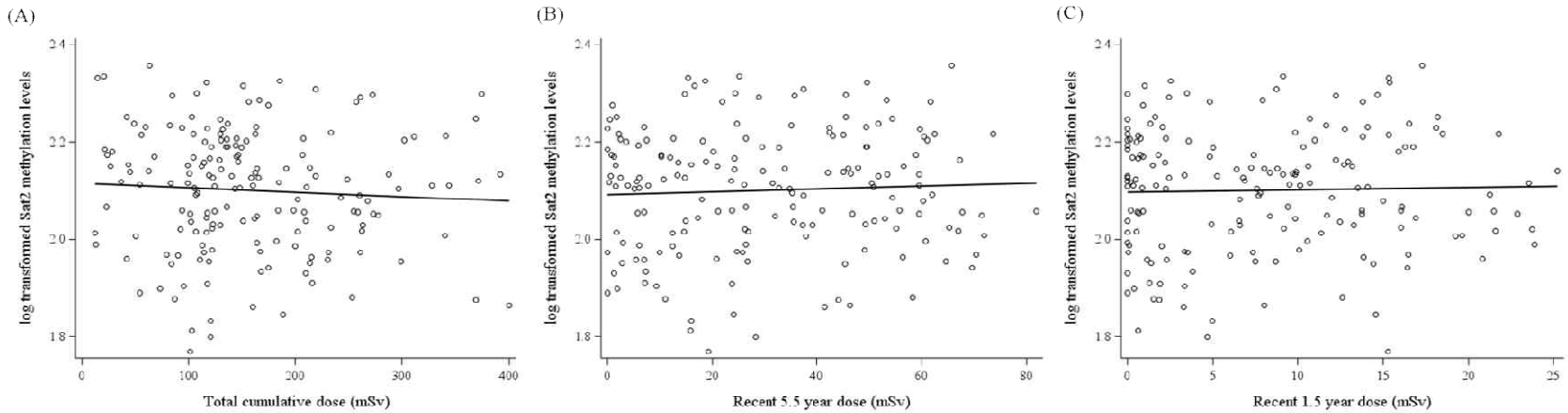

Figure III-10. Correlation between Sat2 methylation levels and different radiation doses in radiation exposed workers. A total of 176 workers were analyzed for correlation between the levels of Sat2 methylation and different radiation doses such as total cumulative dose (A), recent 5.5 year dose (B), and recent 1.5 year dose (C). Sat2 methylation levels were log transformed. Circles mean individual data points and line means univariate regression line.

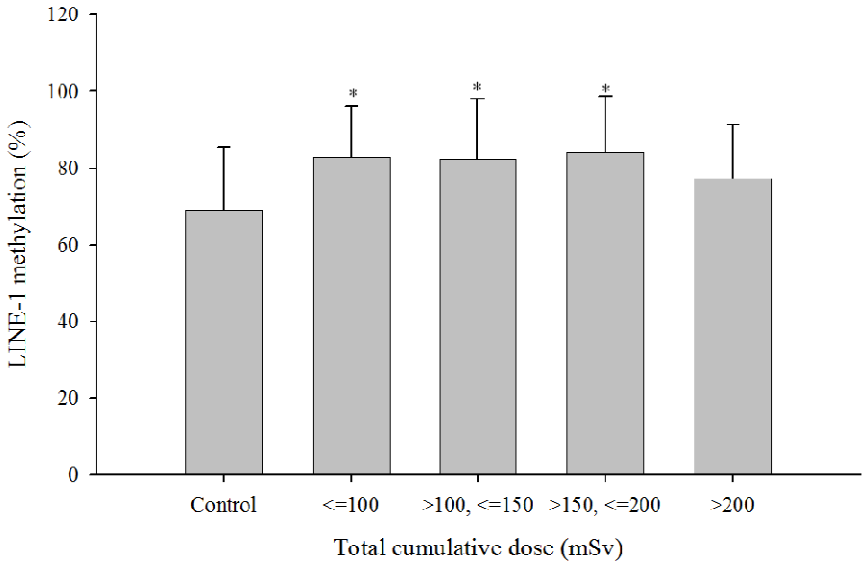

Figure III-11. Associations between radiation dose and LINE-1 methylation in subjects including workers and controls. A total of 206 subjects including workers and controls were analyzed for association between the LINE-1 methylation levels and total cumulative radiation dose. Values represent mean ± SD. Results were statistically analyzed with Tukey's post hoc test after one-way ANOVA test (*, $p < 0.05$ compared to control).

Table III-15. Multiple linear regression analysis of total cumulative radiation dose on LINE-1 methylation in nuclear power plant workers

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	89.92	70.34	109.51	<.0001
Age	-0.085	-0.51	0.34	0.69
Smoking status (0. 1)[a]	-2.16	-6.84	2.52	0.36
Drinking status (0.1)[b]	1.52	-4.11	7.15	0.59
Total cumulative radiation dose	-0.031	-0.060	-0.0027	0.032

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-16. Multiple linear regression analysis of recent 5.5 year radiation dose on LINE-1 methylation level in nuclear power plant workers

		95% CI		
Variable	β	Low	Upper	p-value
Intercept	99.56	78.69	120.44	<.0001
Age	-0.35	-0.75	0.042	0.079
Smoking status (0. 1)[a]	-2.23	-6.95	2.49	0.35
Drinking status (0.1)[b]	1.59	-4.10	7.27	0.58
Recent 5.5 year radiation dose	-0.063	-0.17	0.041	0.23

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-17. Multiple linear regression analysis of recent 1.5 year radiation dose on LINE-1 methylation level in nuclear power plant workers

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	101.22	78.81	123.62	<0.0001
Age	-0.40	-0.82	0.03	0.066
Smoking status (0. 1)[a]	-2.18	-6.91	2.55	0.36
Drinking status (0.1)[b]	1.83	-3.85	7.52	0.53
Recent 1.5 year radiation dose	-0.21	-0.56	0.15	0.26

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-18. Multiple linear regression analysis of total cumulative radiation dose on log transformed Sat2 methylation in nuclear power plant workers

		95% CI		
Variable	β	Low	Upper	p-value
Intercept	2.33	2.16	2.51	<.0001
Age	-0.0049	-0.0086	-0.0011	0.011
Smoking status (0. 1)[a]	-0.015	-0.055	0.026	0.48
Drinking status (0.1)[b]	-0.0062	-0.055	0.042	0.80
Total cumulative radiation dose	0.000049	-0.00020	0.00030	0.69

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-19. Multiple linear regression analysis of recent 5.5 year radiation dose on log transformed Sat2 methylation level in nuclear power plant workers

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	2.33	2.14	2.51	<.0001
Age	-0.0046	-0.0081	-0.0011	0.011
Smoking status (0. 1)[a]	-0.015	-0.055	0.026	0.48
Drinking status (0.1)[b]	-0.0067	-0.055	0.042	0.79
Recent 5.5 year radiation dose	-0.00031	-0.00093	0.00087	0.94

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-20. Multiple linear regression analysis of recent 1.5 year radiation dose on log transformed Sat2 methylation level in nuclear power plant workers

		95% CI		
Variable	β	Low	Upper	p-value
Intercept	2.37	2.17	2.56	<0.0001
Age	-0.0052	-0.0089	-0.0015	0.0062
Smoking status (0. 1)[a]	-0.014	-0.055	0.026	0.49
Drinking status (0.1)[b]	-0.0059	-0.054	0.042	0.81
Recent 1.5 year radiation dose	-0.0013	-0.0044	0.0017	0.39

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

3.3.3. Levels of repeat element methylation with duration of work in radiation exposed workers

I also evaluated the association between duration of work and repeat element methylation levels. The trend of duration-dependent change was tested by Kendall rank correlation coefficient. LINE-1 methylation was not varied with duration of work (Table III-21 and Figure III-12A), while Sat2 methylation level was decreased with working year group (Kendall's $\tau = -0.16$, $p=0.0046$, Table III-22). As shown in Figure III-12B, Sat2 methylation levels were significantly correlated with duration of work.

Multiple linear regression analysis was performed to evaluate the association of duration of work with repeat element methylation levels independently of the variables such as age, smoking, and drinking habit. After adjusting the variables, significant association between LINE-1 methylation and duration of work was not found (Table III-23). There was also no association between Sat2 methylation level and duration of work after adjusting variables such as age, smoking and drinking habit (Table III-24).

Table III-21. Levels of LINE-1 methylation according to duration of work

Duration of work (yr)	No.	Mean ± SD
<=15	31	82.21 ± 15.55
>15, <=20	55	81.47 ± 15.70
>20, <=25	66	83.57 ± 13.41
>25	24	74.27 ± 12.46
Kendall's τ		-0.054
p		0.34

Aberrations; GM, Geometric mean; CI, Confidence interval

p, Tested by Kendall rank correlation coefficient (τ)

Table III-22. Geometric mean levels of Sat2 methylation according to duration of work

Duration of work (yr)	No.	GM (95% CI)
<=15	30	144.3 (132.6 - 157.2)
>15, <=20	55	126.9 (117.1 - 137.4)
>20, <=25	64	120.3 (112.3 - 128.9)
>25	22	118.3 (102.0 - 137.2)
Kendall's τ		-0.16
p		0.0046

Aberrations; GM, Geometric mean; CI, Confidence interval

p, Tested by Kendall rank correlation coefficient (τ)

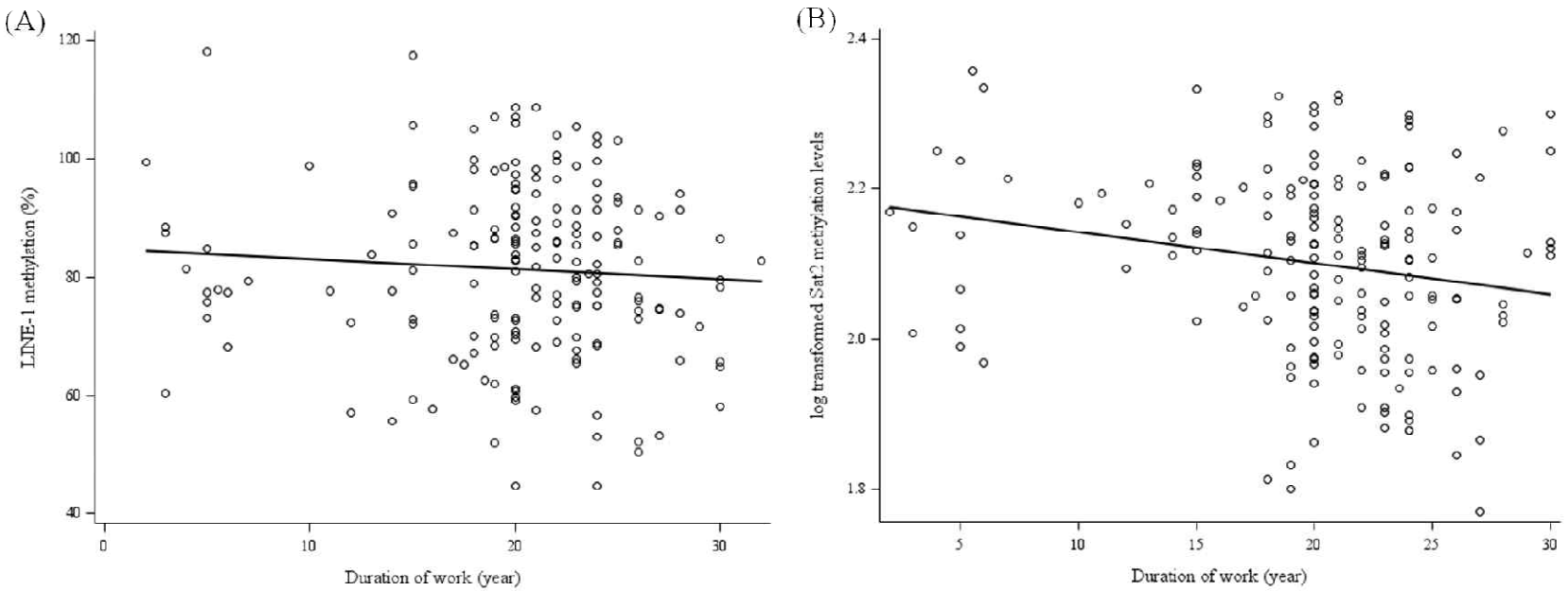

Figure III-12. Association between duration of work and repeat element methylation. (A) LINE-1 methylation, Kendall's $\tau = -0.044$, $p = 0.40$; (B) Sat2 methylation, Kendall's $\tau = -0.15$, $p = 0.0036$. Circles mean individual data points and line means univariate regression line.

Table III-23. Multiple linear regression analysis of duration of work on LINE-1 methylation level in nuclear power plant workers

		95% CI		
Variable	B	Low	Upper	p-value
Intercept	95.12	74.06	116.19	<.0001
Age	-0.32	-0.89	0.26	0.28
Smoking status (0. 1)[a]	-2.25	-7.00	2.51	0.35
Drinking status (0.1)[b]	1.80	-3.94	7.54	0.54
Duration of work	0.030	-0.54	0.60	0.92

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-24. Multiple linear regression analysis of duration of work on log-transformed Sat2 methylation level in nuclear power plant workers

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	2.29	2.11	2.47	<0.0001
Age	-0.0025	-0.0074	0.0025	0.33
Smoking status (0. 1)[a]	-0.016	-0.057	0.024	0.43
Drinking status (0.1)[b]	-0.0099	-0.059	0.039	0.69
Duration of work	-0.0028	-0.0077	0.0021	0.26

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

3.4. Gene-specific promoter methylation changes

To investigate the relationship between the extent of promoter methylation and occupational radiation exposure, I assessed the promoter methylation status of six human genes*: p16*, *RASSF1A*, *MLH1*, *MGMT*, *GSTP1*, and *TERT*. These genes are known to be associated with radiation-induced response. The promoter regions of these genes showed no changes in controls and workers (data not shown).

3.5. DNMTs expression in response to radiation exposure

To investigate the role of DNMT expression on the change of radiation-induced DNA methylation, DNMT expression was evaluated. RNA from 40 subjects in 176 workers was extracted, but only 33 subjects were available to evaluate the expression. Global DNA methylation was correlated with *DNMT1* expression (Table III-25), and the methylation levels were increased with *DNMT1* expression after adjusting age, smoking habit, alcohol consumption and other DNMTs expression (Table III-26). LINE-1 methylation was correlated with *DNMT1*, *DNMT3a*, and *DNMT3b* (Table III-25), but the methylation levels were increased with only *DNMT3b* significantly in the multiple linear model (Table III-27). Sat2 methylation was associated with *DNMT1* expression slightly (Table III-28).

I also evaluated the relationship between these DNMTs expression and radiation exposure. *DNMT1* expression was correlated with recent 1.5 year radiation dose (Table III-29 and Figure III-13), and the expression level was decreased with recent 1.5 year dose after adjusting age, smoking habit, alcohol consumption and total cumulative radiation dose (Table III-30). *DNMT1* expression was also associated with alcohol consumption significantly (Table III-30). Both *DNMT3a* and *DNMT3b* expression was not associated with radiation exposure, but the expression was decreased with smoking status slightly (Table III-31 and Table III-32).

Table III-25. Correlation between DNMTs expression and DNA methylation

		Global DNA methylation (5-mc (%))		PMR of LINE-1		PMR of SAT2	
	n	Kendall's τ	P	Kendall's τ	p	Kendall's τ	p
DNMT1	33	0.30	0.016	-0.38	0.0017	0.14	0.25
DNMT3a	33	-0.076	0.54	0.31	0.0111	-0.11	0.35
DNMT3b	33	-0.072	0.56	0.34	0.0053	-0.091	0.46

p, Tested by Kendall rank correlation coefficient (τ).

Table III-26. Multiple regression analysis of the association between log transformed global DNA methylation and DNMT expression

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	-0.92	-1.85	0.0076	0.052
Age	0.013	-0.0056	0.031	0.17
Smoking status (0. 1)[a]	0.073	-0.12	0.27	0.45
Drinking status (0.1)[b]	-0.049	-0.25	0.15	0.62
DNMT1 expression	0.019	0.00084	0.038	0.041
DNMT3a expression	-0.014	-0.069	0.040	0.59
DNMT3b expression	0.0088	-0.014	0.032	0.43

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-27. Multiple regression analysis of the association between LINE-1 methylation and DNMT expression

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	101.77	25.049	178.48	0.011
Age	-0.43	-1.94	1.08	0.56
Smoking status (0. 1)[a]	1.72	-14.48	17.93	0.83
Drinking status (0.1)[b]	-3.81	-20.11	12.48	0.63
DNMT1 expression	-0.83	-2.36	0.69	0.27
DNMT3a expression	-4.34	-8.80	0.12	0.056
DNMT3b expression	2.20	0.34	4.07	0.023

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-28. Multiple regression analysis of the association between SAT-2 methylation and DNMT expression

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	312.47	160.37	464.56	0.0003
Age	-3.67	-6.67	-0.67	0.019
Smoking status (0. 1)[a]	-24.35	-56.47	7.78	0.13
Drinking status (0.1)[b]	9.14	-23.17	41.44	0.57
DNMT1 expression	3.01	-0.013	6.03	0.051
DNMT3a expression	2.68	-6.16	11.52	0.54
DNMT3b expression	-1.41	-5.11	2.30	0.44

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-29. Correlation between DNMTs expression and radiation exposure

		DNMT1		*DNMT3a*		*DNMT3b*	
	n	Kendall's τ	p	Kendall's τ	p	Kendall's τ	p
Duration of work	33	0.20	0.12	0.036	0.78	-0.0040	0.98
Total cumulative radiation dose	33	0.053	0.66	0.095	0.44	0.087	0.48
Recent 5.5 year radiation dose	33	-0.18	0.13	0.11	0.36	0.16	0.20
Recent 1.5 year radiation dose	33	-0.29	0.018	0.046	0.71	0.11	0.35

p, Tested by Kendall rank correlation coefficient (τ).

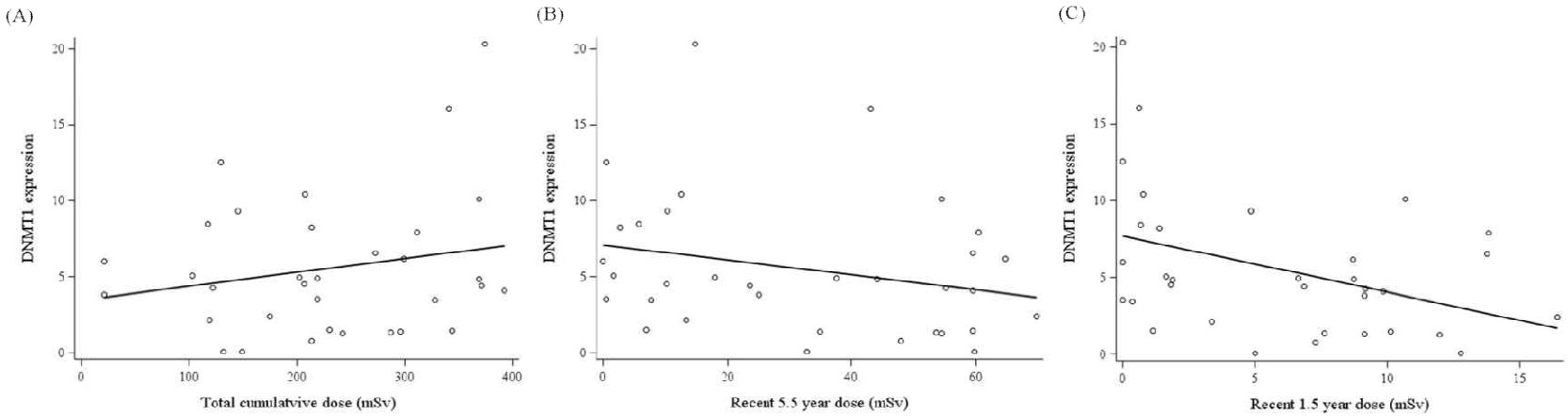

Figure III-13. Correlation between *DNMT1* expression and recent 1.5 year radiation dose in radiation exposed workers. A total of 33 workers were analyzed for correlation between the levels of *DNMT1* expression and different radiation doses such as total cumulative dose (A), recent 5.5 year dose (B), and recent 1.5 year dose (C). (A), Kendall's $\tau = 0.053$, $p = 0.66$; (B), Kendall's $\tau = -0.18$, $p = 0.13$; (C), Kendall's $\tau = -0.29$, $p = 0.018$. Circles mean individual data points and line means univariate regression line.

Table III-30. Regression analysis of radiation exposure on square root transformed DNMT1 expression

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	4.59	-0.76	9.95	0.090
Age	-0.025	-0.13	0.080	0.63
Smoking status (0. 1)[a]	0.28	-0.57	1.14	0.50
Drinking status (0.1)[b]	-1.30	-2.17	-0.42	0.0052
Recent 1.5 year radiation dose	-0.14	-0.22	-0.055	0.0019
Total cumulative radiation dose	0.0027	-0.0012	0.0067	0.16

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-31. Regression analysis of radiation exposure on log transformed DNMT3a expression

		95% CI		
Variable	β	Low	Upper	p-value
Intercept	0.065	-3.94	4.07	0.97
Age	-0.014	-0.093	0.065	0.71
Smoking status (0. 1)[a]	-0.63	-1.27	0.013	0.055
Drinking status (0.1)[b]	0.49	-0.16	1.15	0.13
Recent 1.5 year radiation dose	0.021	-0.040	0.081	0.49
Total cumulative radiation dose	0.0014	-0.0015	0.0043	0.34

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

Table III-32. Regression analysis of radiation exposure on log transformed DNMT3b

Variable	β	95% CI		p-value
		Low	Upper	
Intercept	0.37	-4.42	5.16	0.88
Age	-0.016	-0.11	0.078	0.72
Smoking status (0. 1)[a]	-0.71	-1.48	0.054	0.067
Drinking status (0.1)[b]	0.59	-0.19	1.37	0.13
Recent 1.5 year radiation dose	0.038	-0.034	0.11	0.29
Total cumulative radiation dose	0.0015	-0.0020	0.0050	0.39

a, smoking status: 0, current smoker = no; 1, current smoker = yes.

b, drinking status: 0, alcohol drinker = no; 1, alcohol drinker = yes.

3.6. Relationship between genomic instability and DNA methylation patterns

To investigate the association between DNA methylation changes and genomic instability, I evaluated the relationship between DNA methylation changes and genetic instability markers such as chromosomal aberrations and micronuclei. Global DNA methylation levels were correlated with chromosomal aberrations in workers ($p<0.0001$, Table III-33). The levels were decreased with increasing the number of chromatid type aberrations or chromosome type aberrations (Table III-33 and Figure III-14). However, these associations were not found in controls (Table III-33). LINE-1 methylation levels were negatively correlated with chromosomal aberrations in the only control group ($p=0.021$, Table III-34), and Sat2 methylation was not correlated with any chromosomal aberrations in both groups (Table III-35). In contrary to the results for chromosomal aberrations, the frequency of micronuclei was not correlated with global DNA methylation and repeat element methylation levels (Table III-36).

Table III-33. Correlations between global methylation levels and chromosome aberrations in subjects

Types of chromosome aberration	Workers		Control		Total	
	Kendall's τ	p-value	Kendall's τ	P-value	Kendall's τ	p-value
Chromatid type aberration	-0.14	0.007	0.16	0.23	-0.12	0.013
Chromatid type deletion	-0.13	0.012	0.16	0.25	-0.12	0.019
Chromatid type exchange	-0.21	0.0006	-0.064	0.68	-0.19	0.0012
Chromosome type aberration	-0.24	<0.0001	0.25	0.10	-0.22	<0.0001
Chromosome type deletion	-0.21	0.0001	-0.051	0.74	-0.22	<0.0001
Chromosome type exchange	-0.058	0.32	0.40	0.0084	-0.050	0.35
Total chromosome aberration	-0.23	<0.0001	0.17	0.22	-0.21	<0.0001

p-value, Tested by Kendall rank correlation coefficient (τ).

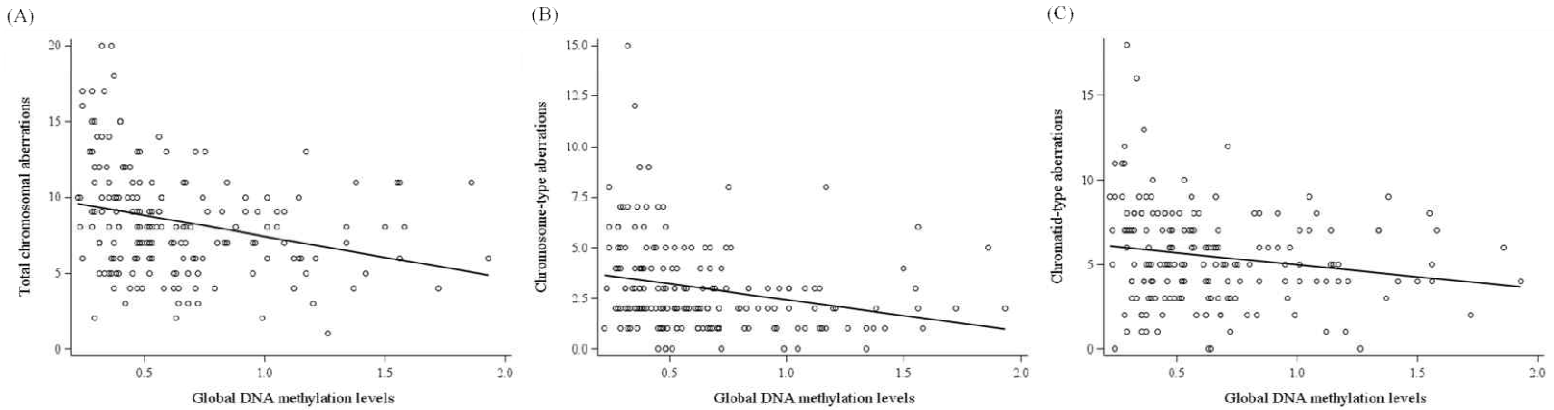

Figure III-14. Correlations between global methylation levels and chromosome aberrations in nuclear power plant workers. Subjects' global methylation levels were associated with total chromosome aberrations (A), chromosome-type aberrations (B), and chromatid-type aberrations (C). (A), Kendall's $\tau = -0.23$, $p < 0.0001$; (B), Kendall's $\tau = -0.24$, $p < 0.0001$; (C), Kendall's $\tau = -0.14$, $p = 0.007$. Circles mean individual data points and line means univariate regression line.

Table III-34. Correlations between LINE-1 methylation levels and chromosome aberrations in subjects

Types of chromosome aberration	Workers		Control		Total	
	Kendall's τ	p-value	Kendall's τ	P-value	Kendall's τ	p-value
Chromatid type aberration	0.084	0.11	-0.22	0.10	0.090	0.066
Chromatid type deletion	0.086	0.11	-0.23	0.092	0.092	0.062
Chromatid type exchange	0.044	0.48	0.14	0.36	0.045	0.43
Chromosome type aberration	-0.021	0.70	-0.29	0.053	0.079	0.11
Chromosome type deletion	-0.016	0.77	-0.27	0.07	0.073	0.15
Chromosome type exchange	-0.023	0.69	-0.11	0.49	0.040	0.45
Total chromosome aberration	0.057	0.28	-0.31	0.021	0.12	0.013

p-value, Tested by Kendall rank correlation coefficient (τ).

Table III-35. Correlations between Sat2 methylation levels and chromosome aberrations in subjects

Types of chromosome aberration	Workers		Control		Total	
	Kendall's τ	p-value	Kendall's τ	P-value	Kendall's τ	p-value
Chromatid type aberration	-0.078	0.15	0.11	0.42	-0.036	0.47
Chromatid type deletion	-0.086	0.11	0.13	0.33	-0.040	0.43
Chromatid type exchange	0.11	0.091	-0.17	0.28	0.059	0.31
Chromosome type aberration	0.049	0.38	0.14	0.36	0.092	0.070
Chromosome type deletion	0.097	0.085	0.11	0.49	0.13	0.015
Chromosome type exchange	-0.049	0.41	0.089	0.56	-0.00025	0.99
Total chromosome aberration	-0.043	0.42	0.16	0.22	0.010	0.83

p-value, Tested by Kendall rank correlation coefficient (τ).

Table III-36. Correlations between DNA methylation levels and the frequency of micronuclei in subjects

	Workers		Control		Total	
	Kendall's τ	p-value	Kendall's τ	P-value	Kendall's τ	p-value
Global DNA methylation levels	0.062	0.23	0.0048	0.97	0.0087	0.86
LINE-1 methylation levels	-0.021	0.68	-0.072	0.59	0.058	0.22
Sat2 methylation levels	-0.0023	0.96	-0.20	0.13	0.023	0.63

p-value, Tested by Kendall rank correlation coefficient (τ).

4. Discussion

The biological and genetic effects of chronic low-dose radiation exposure and its relationship to carcinogenesis have received a lot of attention in recent years, but the long-term effect of chronic exposure to low-dose irradiation on human health is still unknown. Epigenetic mechanisms, such as DNA methylation and histone modification, could be the missing link in understanding low dose radiation-induced genomic instability. In this study, I investigated the effects of low-dose radiation exposure on DNA methylation using peripheral blood DNA from nuclear power plant workers. I also evaluated whether radiation-induced DNA methylation states were in turn associated with the frequency of micronuclei or chromosomal aberrations, indicators of genomic instability.

4.1. Change in global DNA methylation induced by ionizing radiation

Global DNA methylation levels of subjects were measured by DNA methylation quantification kits and the methylation levels were significantly different between radiation-exposed workers and controls. I also evaluated the associations with radiation exposure dose and global DNA methylation levels in radiation exposed workers. Global DNA methylation was significantly decreased with recent 1.5 year dose. Multiple linear regression analysis for

global DNA methylation levels, after adjusting for the potential impact of variables such as age, smoking status and alcohol consumption, also revealed that recent 1.5 year dose was significantly associated with decreased global DNA methylation levels. These results are similar to other previous studies.

Kalinich et al. (1989) reported that ^{60}Co γ radiation led to the reduction of DNA methylation levels in Chinese hamster ovary (CHO) clone K-1, Chinese hamster lung fibroblast (V79) clone A03, HeLa clone S-3 and Mouse neuroblastoma C-1300 clone NIE-115 cell lines. Tawa et al. (1998) described the induction of global hypomethylation in the X-irradiated mice. The global loss of DNA methylation tends to get repackaged in a more open configuration (Pogribny et al. 2005) and can cause carcinogenesis by reactivating retrotransposable elements inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007).

However, the global DNA methylation levels were increased with the total cumulative radiation dose in workers. Multiple linear regression analysis for global DNA methylation levels, after adjusting for the potential impact of variables such as age, smoking status and alcohol consumption also revealed similar results. As total cumulative dose tends to be negatively correlated with recent 1.5 year dose, both radiation dose types were included in the regression analysis. In the model, global DNA methylation levels were still increased with total cumulative dose. In other words, the workers with high total cumulative dose tend to have more high global DNA methylation levels than those with low total cumulative dose, although the workers were similar to recent exposure dose.

The difference between recent exposure and total cumulative exposure can be explained by several points. First, the effect of acute and chronic irradiation on DNA methylation can be different. Kovalchuk et al. (2004) observed different patterns of radiation-induced methylation changes in muscle tissue of chronically exposed C57/Bl mice as compared to the acutely exposed individuals. Second, the stability of the methylation changes over time can affect the results of this study. Radiation-induced DNA hypomethylation in C57/Bl mice subjected to acute radiation exposure faded 1 month after exposure (Koturbash et al. 2005a).

Global DNA hypermethylation by total cumulative radiation was not expected, but similar results were reported in other studies. Ye et al. (2013) found that long-term low-dose radiation resulted in global DNA hypermethylation in human B lymphoblast cell line HMy2.CIR. Genomic DNA of radiation-exposed pine trees near the Chernobyl nuclear power plant was considerably hypermethylated (Kovalchuk et al. 2003). Besides ionizing radiation, sperm DNA of mice exposed chronically to particulate air pollution was hypermethylated (Yauk et al. 2008). These changes might be associated with up-regulation of DNMTs. In HMy2.CIR cells exposed to long-term low dose radiation, the gene expression of *DNMT1* and protein expression of methyl CpG binding protein 2 (*MeCP2*) were increased, and *DNMT1* was critical in maintaining hypermethylation of the genome and increasing of heterochromatin formation (Ye et al. 2013). In my study, *DNMT1* expression had a trend to increase with total cumulative dose, but there was no significance. The precise mechanism of long term radiation-induced increase

in DNMTs expression needs to be studied in the future.

From the result of global DNA hypermethylation, long-term low dose radiation could lead to formation of heterochromatin and make the chromatin more condensed. The condensation of chromatin made the chromatin more compact and more resistant to radiation. In addition, global hypermethylation could be a defense strategy that prevents genome instability because the a primary function of global hypermethylation is known to maintain genome stability (Kovalchuk et al. 2003; Shames et al. 2007). Thus, the results of this study suggest that increase of global methylation levels might be acquired with total cumulative dose as a defense strategy. On the other hand, the action that DNA methylation returns back to the regular level, permitting normal genome function, may also cause the increase of global DNA methylation levels (Kovalchuk et al. 2003).

However, any aberration in DNA methylation can be detrimental to the cell, although global hypermethylation is associated with maintenance of genome stability (Aypar et al. 2011a). In addition, Kumar et al. (2013) showed that the number of hypermethylated spermatozoa were significantly higher in the hospital workers occupationally exposed to ionizing radiation than in non-exposed population. The study suggested that global hypermethylation observed in the exposed workers implies defective chromatin condensation, which can lead to morphologically abnormal spermatozoa (Kumar et al. 2013). Therefore, further studies need to find the role of global hypo-/hyper-methylation by ionizing radiation and to clarify the association between global hypermethylation and cumulative radiation exposure. These studies could be

helpful to use and interpret global DNA methylation as a potential biomarker for radiation exposure.

4.2. Change in repeat elements methylation induced by ionizing radiation

Methylation status of repeat elements such as LINE-1 and Sat2 is important given that these elements, which consist of interspersed repeats and tandem repeats, comprise approximately 45% of the human genome and that they are normally highly methylated (Lander et al. 2001; Jordan et al. 2003). Aberrant repeat element methylation is another common epigenetic change that evolves during carcinogenic process (Chalitchagorn et al. 2004; Estecio et al. 2007) . It has been suggested that hypomethylation of these sequences promotes genomic instability and subsequent tumor progression (Kazazian and Goodier 2002).

In this study, repeat element DNA methylation levels of controls and workers were evaluated. LINE-1 methylation levels were higher in radiation-exposed workers than in controls, while Sat2 methylation levels had no significant difference between workers and controls. While DNA hypomethylation is known to contribute to carcinogenesis by inducing genomic instability and activating proto-oncogenes (Robertson 2002; Weidman et al. 2007), DNA hypermethylation may be linked to structural changes in chromatin, decreased transposon movement and decreased gene

expression (Robertson 2005; Slotkin and Martienssen 2007; Yauk et al. 2008). Specifically, DNA packaging in heterochromatin, which results from LINE-1 hypermethylation in part, appears to delay DNA strand break processing, leading to slow down repair (Rube et al. 2011)

As repeat element DNA methylation levels have used as a surrogate marker for global DNA methylation levels to investigate the epigenetic changes induced by benzene, lead and persistent organic pollutants (Bollati et al. 2007; Rusiecki et al. 2008; Wright et al. 2010), I expected that the associations between the methylation levels and radiation exposure are similar in case of global DNA methylation levels. Unexpectedly, hypomethylation of repeat elements were not found in this study. The different outcomes in radiation-induced changes between global DNA methylation and repeat elements methylation were observed in other studies (Aypar et al. 2011a; Goetz et al. 2011). Thus, the levels of repeat elements methylation may be inappropriate as a surrogate of global DNA methylation to investigate the radiation-induced methylation changes. Therefore, it needs to study the changes and roles of repeat elements methylation changes itself following irradiation.

Although LINE-1 methylation level was higher in workers than in controls, the levels were decreased by total cumulative radiation dose. The levels of the workers with total cumulative dose below 200 mSv were higher than control, while the levels tend to be decreased in workers with dose above 200 mSv. This result is similar to Aypar et al. (2011a) that LINE-1 hypermethylation was observed after 0.5 Gy of low LET radiation relative to

control but LINE-1 methylation levels at 2Gy was lower than those at 0.5 Gy. Increased DNA methylation was observed in the liver of A^{vy} mice male offspring exposed to 1.4 and 3.0 cGy, but it returned to control level at 7.6 cGy (Bernal et al. 2013). The study suggested DNA hypomethylation at high doses of radiation and hypermethylation at low doses are indicative of a hormetic biphasic radiation dose response effect (Bernal et al. 2013). The changes in not only LINE-1 methylation but also global DNA methylation by total cumulative radiation dose in the present study may be associated with the hormesis phenomenon, although it is not clear which methylation changes are beneficial.

As mentioned previously, any changes in DNA methylation have potential for adverse effects, the radiation-induced change itself may be more important than the direction of the change. CpG sites in LINE-1 are usually heavily methylated but global loss of methylation in these sites is common epigenetic event in malignant cells (Chalitchagorn et al. 2004). This pattern of DNA methylation led to genomic instability in cancer (Baylin and Ohm 2006). LINE-1 hypomethylation may cause transcriptional activation and overexpression of LINE-1, resulting in retrotransposable element transposition, chromosomal alteration and genomic instability (Daskalos et al. 2009; Saito et al. 2010). However, LINE-1 hypermethylation can lead to less accessible chromatin that may slow down DNA repair and result in higher frequency of chromosomal aberrations (Baylin and Ohm 2006). Therefore, further study is needed to determine adverse effects induced by alterations of DNA methylation levels.

LINE-1 hypomethylation in exposed workers was associated with total cumulative radiation dose, and the methylation levels were not associated with recent radiation exposure dose. In other research to study the methylation changes induced by particulate pollution, LINE-1 hypomethylation was associated with long-term exposure to black carbon and sulfates (Madrigano et al. 2011). Similar to these study, the results of my study support that chronic low-dose radiation exposure acts as a more potent inducer of LINE-1 hypomethylation than recent radiation exposure in workers.

I also investigated Sat2 methylation in the subjects of this study. LINE-1 methylation levels were higher in radiation-exposed workers than in controls, while any association between Sat2 methylation levels and radiation exposure was not found in this study. From the results, this study can suggest that Sat2 methylation is less sensitive indicator for radiation exposure than other repeat element methylation and global methylation.

4.3. The role of DNMTs in radiation-induced methylation changes

DNA methylation levels are maintained by DNA methyltransferases (DNMTs). DNMTs catalyze the addition of a methyl group from S-adenosylmethionine to cytosine base at CpG sites after DNA synthesis. *DNMT1* is considered as a maintenance DNA methyltrasferase, whereas *DNMT3a* and *DNMT3b* are responsible for *de novo* DNA methylation (Jones

and Baylin 2002; Laird 2003). Thus, these enzymes can generate new DNA methylation patterns and maintain them though cell division.

To investigate the mechanisms of radiation-induced DNA methylation alterations, the expression of these DNMTs was evaluated. Global DNA methylation level was associated with *DNMT1* expression in this study. *DNMT1* expression was also decreased by increase of recent 1.5 year radiation exposure. These findings suggest that recent 1.5 year radiation exposure caused decreased *DNMT1* expression, which resulted in global DNA hypomethylation. These results are similar to other previous studies (Raiche et al. 2004b; Pogribny et al. 2005).

The loss of DNA methylation by radiation exposure is known to be associated with radiation-induced alterations in DNMTs expression. Altered DNA methylation was associated with reduced expression of *DNMT1* and *DNMT3a* in radiation-exposed mice (Pogribny et al. 2005). In chronically exposed female mice liver, decrease in the expression of *DNMT1*, *DNMT3a* and *DNMT3b* led to global hypomethylation (Raiche et al. 2004b). In consistent with these results, global DNA hypomethylation in radiation-exposed workers resulted from a decrease in the expression of *DNMT1* in the present study. Raiche et al. (2004b) suggested the expression of *de novo* methyltransferases *DNMT3a* and *DNMT3b* are most important in radiation-induced DNA methylation alterations. The expression of *DNMT3a* and *DNMT3b*, however, was not changed with radiation exposure dose in the radiation-exposed workers. This discrepancy may be in part due to the different experimental conditions, because radiation-induced DNA

methylation changes and DNMT expression were dependent on dose, sex and tissues (Pogribny et al. 2004; Raiche et al. 2004b).

LINE-1 methylation was associated with *DNMT3a* and *DNMT3b* expression in this study, but DNMTs expression was not affected by radiation exposure. These results suggests that LINE-1 methylation levels changed by *DNMT3a* and *DNMT3b* may be independent of radiation exposure.

In contrary to results in a total of 176 workers, the associations between total cumulative radiation exposure and DNA methylation changes were not found in 33 workers who were subjected to DNMT evaluation. So, it was difficult to investigate the relationship between DNMTs and total cumulative exposure. Future studies, therefore, are required to find the mechanisms of cumulative radiation exposure-induced DNA methylation alterations.

In addition to DNMT expression, oxidative stress may play a role in radiation-induced changes in DNA methylation, although it was not evaluated in this study. Oxidative DNA damages can also induce DNA hypomethylation by blocking DNA from being a substrate of DNA methyltransferases (Valinluck et al. 2004; Franco et al. 2008). Oxidative stress is known to be important in radiation response, and evidences have suggested that reactive oxygen species can affect changes in DNA methylation related to carcinogenesis and other diseases (Franco et al. 2008; Zawia et al. 2009).

4.4. Change in methylation of various genes involved in

cellular responses to irradiation

Promoter methylation status of the six human genes [*p16*, *MGMT*, *GSTP1*, *RASSFF1A*, *hTERT*, and *MLH1*] was assessed by using MethyLight assay. The genes were chosen because changes in their expression have been linked to cancer and it is also involved in cellular responses to radiation (Fritzell et al. 1997; Schuck et al. 2002; Belinsky et al. 2004; Fachin et al. 2009; Rivera et al. 2010). In the present study, no changes in DNA methylation at the CpG sites analyzed for these genes were observed. It may be difficult to measure the methylation levels of the genes in normal blood, because the methylation levels of these genes were relatively low. Further studies, therefore, are needed to confirm the effects of radiation on promoter methylation of these genes by using other assays.

4.5. Association between radiation-induced genomic instability and DNA methylation

DNA global hypomethylation has been linked to the activation of transposable elements, elevated chromosome breakage, aneuploidy, increased mutation rates, and therefore the phenomenon of genomic instability (Weber and Schubeler 2007; Weidman et al. 2007). Recent studies have suggested that radiation-induced global DNA hypomethylation and promoter hypermethylation might play a role in genomic instability (Raiche et al. 2004b;

Kaup et al. 2006b). The correlation between radiation-induced methylation changes and genetic damage markers was evaluated in this study to investigate the association between radiation-induced epigenetic changes and genomic instability.

Global DNA hypomethylation in peripheral blood is strongly associated with chromosomal aberrations, a validated biomarker for genomic instability and risk of several cancers, among radiation-exposed workers. This result indicates a strong link between genome stability and global methylation.

The correlation between chromosome aberrations and global hypomethylation can be explained by two different mechanisms. First, reduced DNA methylation can lead to increase genomic instability. It has been reported that patients with germline mutations in *DNMT3b* have numerous chromosome aberrations (Xu et al. 1999). Murine embryonic stem cells deficient in *DNMT1* also had significantly elevated mutation rates at both the endogenous hypoxanthine phosphoribosyl transferase gene and an integrated viral thymidine kinase transgene (Chen et al. 1998). Genome-wide DNA hypomethylation can induce chromatin decondensation, centromere and telomere abnormalities, and chromosome segregation defects (Rodriguez et al. 2006). Loss of DNA methylation can also favor mitotic recombination, leading to deletions and translocations (Eden et al. 2003).

On the other hand, there is increasing evidence that presence of DNA lesions may result in DNA hypomethylation. DNA lesions were previously reported to interfere with expression and ability of DNA methyltransferases (Panayiotidis et al. 2004; Koturbash et al. 2005a). Furthermore, during

activated DNA repair process by radiation, DNA polymerases incorporate cytosine, but not methylcytosine (Koturbash et al. 2005a; Gehring et al. 2009). James et al. (2003) also found that lesion-containing DNA is less efficiently methylated than lesion-free DNA from rats, and an increase in DNA strand breaks precede DNA hypomethylation. Consequently, the presence and repair of radiation-induced DNA lesions may result in DNA hypomethylation in the present study.

In this study, the association with chromosome aberrations and global hypomethylation was observed only in workers group, and it was not found in controls. Thus, this result can support that radiation-induced DNA lesions led to global hypomethylation. However, the possibility that it may be difficult to investigate the association in control group due to small sample size and low frequency of chromosome aberrations in control group cannot be entirely excluded.

To investigate the association between global hypomethylation and genomic instability, chromosome aberrations and micronuclei were analyzed as markers of genomic instability. The number of chromosome aberrations was correlated with global hypomethylation regardless of the types of chromosome aberrations, but correlation between the frequency of micronuclei and global hypomethylation was not found. A possible explanation of this finding is that micronuclei tests are not specific to the biological effects of low dose radiation. Micronuclei formation is known to be affected by factors such as age, gender, diet, alcohol intake and smoking status (Fenech et al. 1999). In addition, Thierens et al. (1991) reported that

significant inter-individual variability of spontaneous MN exists in human lymphocytes. This finding suggests that global DNA hypomethylation is associated with radiation-induced double strand breaks, and it needs to investigate involved mechanisms.

In contrast to global methylation, LINE-1 methylation levels were negatively correlated with chromosomal aberrations in only control group. Hypomethylation of LINE-1 promoter region may cause transcriptional activation and overexpression, resulting in retrotransposable element transposition and chromosomal alteration (Daskalos et al. 2009; Saito et al. 2010). Duan et al. (2013) demonstrated that LINE-1 hypomethylation in peripheral lymphocytes correlates with high micronuclei frequencies. Daskalos et al. (2009) also found that LINE-1 hypomethylation correlated with microsatellite instability. In consistent with these findings, LINE-1 hypomethylation was correlated with the number of total chromosome aberrations in controls, but the association was not found in radiation-exposed group. That may be because radiation-induced LINE-1 methylation changes may result in weakening of correlation between LINE-1 methylation and chromosome aberrations in radiation-exposed workers.

On the other hand, LINE-1 hypermethylation can result in less accessible chromatin that may slow down DNA repair and result in higher frequency of chromosomal aberrations (Baylin and Ohm 2006). It is also reported that more LINE-1 methylation is associated with higher frequency of chromosomal aberrations and micronuclei in peripheral blood (Pavanello et al. 2009; Li et al. 2013). Although LINE-1 methylation level was decreased with

total cumulative radiation dose in workers, the level was lower in workers than controls. The workers had higher frequencies of chromosomal aberrations than control. In the analysis for total subjects including the control group, more LINE-1 methylation was correlated with higher frequency of chromosomal aberrations. Thus, this finding needs to be interpret cautiously, and further studies should clarify the association between LINE-1 methylation and chromosomal aberrations.

4.7. Association between radiation-induced methylation changes and other variables known to affect methylation

Some studies have reported on the potential associations between DNA methylation changes and age, smoking status or alcohol consumption. Aging is known to affect global DNA methylation in mammals (Fuke et al. 2004). In this study, global DNA methylation levels were increased with age, but it was not significant after adjusting for other variables. The study of Jenkins et al. (2013), which measured DNA methylation levels by using same method in this study, showed that global DNA methylation levels in sperm were increased with age. However, the global sperm DNA methylation patterns were relatively stable up to approximately 55 to 60 years of age. In my study, the age ranged from 40 to 65 in most (90%) of the subjects. Due to the age range, the significant association between global methylation levels and age was not found in this study. It may also be difficult to investigate the

effect of age on other DNA methylation because of the narrow age ranges in this study. Cigarette smoking is considered as a potential environmental modifier of DNA methylation (Breitling et al. 2011). Smoking has been associated with global hypomethylation in head and neck squamous cell carcinoma (Smith et al. 2007). Prenatal exposure to tobacco smoke was associated with decreased global methylation level in children (Breton et al. 2009). However, the association between global DNA methylation and smoking was not found in my study. In consistent with this result, there was no significant differences in global DNA methylation for smoking status such as current smokers, former smokers and non-smokers in the North Texas Healthy Heart study (Zhang et al. 2011). However, it is difficult to conclude that cigarette smoking did not affect on global DNA methylation, because smoking status of the subjects were classified into just two groups: current smokers and non-current smokers including former smokers and non-smokers. Chronic alcohol consumption produces genomic DNA hypomethylation in the colonic mucosa from rats (Choi et al. 1999). However, I also found no consistent association between global DNA methylation and drinking status in this study. Since any information about pack-year of smoking, time since quitting cigarettes and the amount of alcohol consumption was not available in this study, it may be a too crude classification to evaluate a possible effect of smoking or drinking status on DNA methylation. Further studies are needed to investigate the effects of these confounding variables in well-designed population study.

4.8. Summary and Limitation

In conclusion, the results of this study support the hypothesis that exposure to ionizing radiation may influence DNA methylation. These results suggest the possible use of DNA methylation as a potential biomarker to monitor radiation exposure for subjects exposed to ionizing radiation. The findings of this study also support a link between global hypomethylation and genomic instability.

Limitation of this study is that DNA extracted from peripheral blood leukocytes was used to investigate the effect of radiation on DNA methylation. Some studies have suggested that source of DNA is critical components in the interpretation of global DNA methylation patterns (Terry et al. 2011; Wu et al. 2011; Adalsteinsson et al. 2012). For this reason, I cannot exclude the possibility that differences in methylation observed in this study might reflect differential distribution of various types of leukocytes. To correct for this confounding effect in analyses, Adalsteinsson et al. (2012) suggested an approach to use a proportional number of white blood cell type. However, the confounding effect was not considered because the composition of leukocytes was not available in this study. The potential for confounding to variation in white blood cell subtypes should be addressed in future research. As differential methylation patterns can be observed in different blood cell subsets, using the same source of blood DNA is important when measuring global methylation (Wu et al. 2011). However, the isolation of pure

lymphocytes or specific blood cell subpopulations requires additional steps such as flow cytometric separation, thus it has rarely done in epidemiologic studies (Terry et al. 2011). Therefore, the findings of the present study and other studies should be interpreted carefully. In addition, future studies using specific blood cell subsets need to evaluate exact relationships between the methylation levels and radiation exposure.

Chapter IV. Overall discussion and conclusion

Epigenetic changes could be a missing link among radiation exposure, radiation-induced genomic instability, and radiation-induced carcinogenesis. This study evaluated DNA methylation changes, one of the epigenetic alterations, in normal human blood irradiated with gamma rays *in vitro* and investigated the effects of low-dose radiation exposure on DNA methylation using peripheral blood DNA from nuclear power plant workers.

Although ionizing radiation-induced DNA methylation changes have been studied recently, few epidemiologic studies have examined the effects of exposure to ionizing radiation on DNA methylation. To date, DNA methylation levels in adenocarcinomas from plutonium-exposed workers at Russian nuclear facility and in spermatozoa from occupationally exposed workers at hospitals were only studied (Belinsky et al. 2004; Lyon et al. 2007; Kumar et al. 2013).

The present study provided the first evidence on the effects of occupational low dose radiation exposure on DNA methylation alterations in peripheral blood leukocytes of healthy workers. It was observed that global DNA methylation levels of radiation-exposed workers was lower than those of controls. Consistent with the results from the population study, I found that radiation induced a decrease of global DNA methylation levels in gamma irradiated human blood. Thus, alteration of global DNA methylation can be a potential biomarker for assessing risk of ionizing radiation.

However, there was some differences among the results of this study. The population study found that global DNA methylation level was lower in radiation-exposed workers than in controls. The methylation levels were

negatively associated with recent 1.5 year radiation dose, while the levels were increased with total cumulative dose in radiation-exposed workers group. The *in vitro* study observed that γ-irradiation led to decrease global DNA methylation levels in normal human leukocytes, but only 2 Gy irradiated blood cells had significant association.

There can be several explanations for this finding. First, the effect of acute and chronic irradiation on DNA methylation can be different. Kovalchuk et al. (2004) suggested that chronic low dose radiation exposure may be a more potent inducer of epigenetic effects than acute exposure. The study observed different patterns of radiation-induced methylation changes in muscle tissue of chronically exposed C57/Bl mice as compared to the acutely exposed individuals. The tendency for hypomethylation upon chronic irradiation was found in the muscle tissues, while no significant changes in global DNA methylation patterns were observed upon acute exposure. The human blood leukocytes were acutely exposed to gamma ray in my *in vitro* study, because truly normal cells cannot be propagated long enough to observe radiation-induced changes. Whereas the workers in my population study was chronically exposed to ionizing radiation during whole working period. These facts could make the differences between the results of *in vitro* study and those of population study.

Second, the stability of the methylation changes over time can affect the results of this study. Koturbash et al. (2005a) reported that radiation-induced DNA hypomethylation was persistent in the thymus tissues of the C57/Bl mice subjected to acute and fractionated exposure 1 month after the

irradiation treatment. In the same study, DNA methylation changes in muscle tissues completely faded 1 month after exposure. Whereas Kuhmann et al. (2011) observed a considerable loss of DNA methylation in MCF7 cells after fractionated radiation exposure with a recovery period of 14 to 24 day compared to irradiated cells without a recovery period. Human blood cells were harvested 72 h after irradiation in my *in vitro* study, while the cells of the subjects in my population study might be treated for the various period of radiation exposure and recovery. These different radiation exposure conditions may cause the difference of the results including global DNA methylation, repeat element methylation changes and DNA methylation related gene expression between *in vitro* and population study. In addition, the fact that DNA methylation patterns can be different over time may result in the different association with DNA methylation changes between recent 1.5 year radiation and total cumulative dose.

Depend on the stability of DNA methylation changes over time, it is possible that radiation-induced DNA methylation alterations are transient, and the alterations eventually return to control levels after population doublings. However, if DNA methylation alterations remain stable or increase in severity over time, an increased risk for radiation carcinogenesis by alterations of DNA methylation should be considered. Although the time course of DNA methylation changes was not examined due to difficulty of long-term culture of normal cells in this study, additional studies will be required to test the stability of the changes.

Global DNA hypomethylation may have an important role in human

tumorigenesis by increasing genomic instability (Daskalos et al. 2009). To the best of our knowledge, this is the first study that global DNA hypomethylation in peripheral blood is strongly associated with chromosome aberrations, a validated biomarker for genomic instability, among radiation-exposed workers.

There can be several explanations for this finding. First, global DNA hypomethylation can induce chromatin decondensation, centromere and telomere abnormalities, and chromosome segregation defects (Rodriguez et al. 2006). Loss of DNA methylation can also favor mitotic recombination, leading to deletions and translocations (Eden et al. 2003). Second, DNA lesions were previously reported to interfere with expression and ability of DNA methyltransferases (Panayiotidis et al. 2004; Koturbash et al. 2005a). In addition, during activated DNA repair process by radiation, DNA polymerases incorporate cytosine, but not methylcytosine (Koturbash et al. 2005a; Gehring et al. 2009).

Radiation-induced DNA methylation changes were markedly represented and the changes were associated with chromosome aberrations, which is the representative marker for ionizing radiation. No associations with DNA methylation alterations and the frequency of micronuclei, which is known to be affected by factors such as age, gender, diet, alcohol intake and smoking status. Therefore, the results of this study support that DNA methylation patterns can be used as a potential marker for ionizing radiation, though DNA methylation can be affected by other variables such as dietary factors and environmental pollutants. Further studies are required to examine additional gene-specific methylation modification in larger cohorts in order to

use the methylation alterations as a specific marker for radiation.

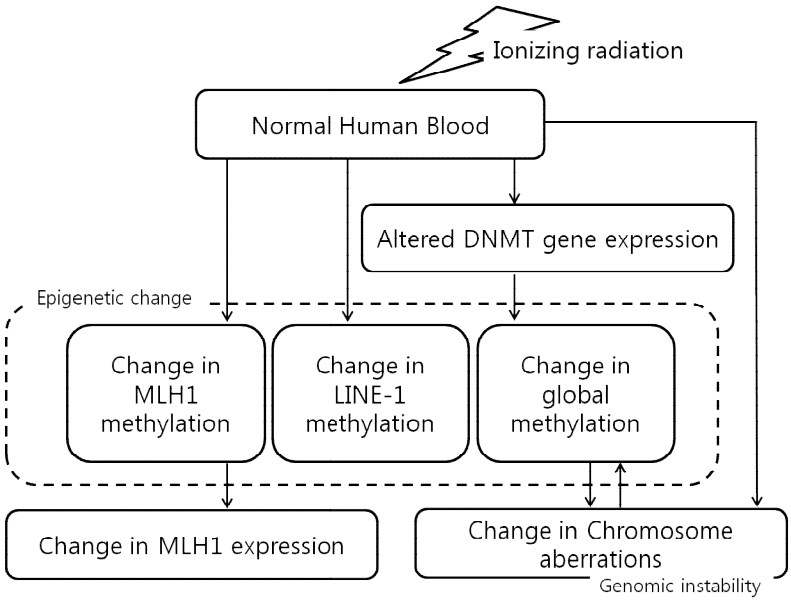

Figure IV-1. Scheme of the effects of ionizing radiation on DNA methylation changes and genomic instability in normal human blood.

The present study provided the evidence on the effects of radiation exposure both *in vivo* and *in vitro* on DNA methylation alterations and related mechanisms in normal human blood and supported the hypothesis that global DNA methylation is linked to RIGI (Figure IV-1). The association between ionizing radiation exposure and global DNA methylation may have implications for the mechanisms of action of radiation on health outcomes, and also suggests that changes in DNA methylation may represent a potential biomarker of past radiation exposure. In addition, the findings of this study support a link between global hypomethylation and genomic instability.

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Abstract in Korean (국문초록)

후성유전학적 변화는 DNA 염기서열의 변화 없이 유전자의 발현을 조절하는 기작으로, 대표적인 후성유전학적 변이로는 DNA 메틸화, 히스톤 변형 등이 있다. 특히, 암세포의 분화과정에서 나타나는 일부 유전자의 과메틸화와 유전체의 광범위한 영역에서의 저메틸화가 유전체의 불안정성을 유도하여 암발생과정에 기여하는 것으로 알려져 있다. 최근에는 방사선에 의한 발암과정과 유전체의 불안정성 유도과정에 있어서도 이러한 후성유전학적 기전의 역할이 클 것으로 기대됨에 따라 방사선에 의한 건강영향을 이해하는데 방사선 노출로 인한 후성유전학적 변화를 파악하는 것을 필요로 하고 있다.

본 연구에서는 감마선을 조사한 사람 정상혈액세포와 원자력발전소에서 방사선에 노출되는 작업자들의 혈액세포를 이용하여 방사선이 DNA 메틸화에 미치는 영향을 평가하였다. 그리고 DNA 메틸화 형성에 관여하는 DNA 메틸기전이효소의 발현을 함께 평가하였으며 유전체 불안정성 마커를 활용하여 방사선에 의한 DNA 메틸화 변화와 유전체 불안정성과의 연관성을 연구하였다.

감마선을 조사한 사람 혈액세포에서는 글로벌 DNA 저메틸

화가 나타났고 DNA 메틸기전이효소 역시 함께 감소하였다. LINE-1 반복서열은 0.5, 1, 2 Gy 를 조사한 세포에서 메틸화 수준이 증가하였고 Sat2 반복유전자는 메틸화 수준의 유의한 변화가 관찰되지 않았다. 그리고 DNA 손상 회복 유전자 중 하나인 *MLH1* 유전자의 메틸화 수준이 2 Gy를 조사한 세포에서 높아졌으며, 동시에 *MLH1* 유전자의 발현이 유의하게 감소하였다.

원자력 발전소에서 근무하는 방사선 작업종사자를 대상으로 한 연구에서는, 글로벌 DNA 메틸화 수준이 비노출군에 비해 방사선 작업종사자에서 유의하게 감소하였다. 그리고 작업자의 글로벌 DNA 메틸화 수준은 작업자의 최근 1.5년 동안 노출된 방사선 양이 증가함에 따라 감소되었고, 반면에 총 누적 노출 양이 증가함에 따라 함께 증가되었다. 글로벌 DNA 메틸화 수준은 DNA 메틸기전이효소 1의 발현과 관련있었고, 그 유전자의 발현은 최근 1.5년 동안 노출된 방사선 양이 증가할수록 감소되었다. LINE-1 반복서열 유전자의 메틸화 수준은 비노출군보다 작업종사자에서 높았으며, 반면에 그 메틸화 정도는 작업종사자들의 총 노출된 방사선 양이 증가함에 따라 감소하는 경향을 보였다.

또한 본 연구에서는 유전체 불안정성의 마커로서 염색체 이상이나 소핵 형성이 방사선에 의해 유도된 DNA 메틸화와의 연관성을 연구하였다. 작업종사자에서는 염색체이상의 유형과 상관없이 염

색체 이상과 글로벌 DNA 메틸화와 역의 상관관계를 보였으며, 소핵의 빈도는 이러한 메틸화 변화와 관련이 없었다.

이와 같이, 본 연구를 통해서 이온화 방사선의 노출이 사람 정상 혈액세포에 DNA 메틸화 변화를 일으킬 수 있음을 확인함으로서, DNA 메틸화 변화가 방사선에 노출되는 인구집단의 노출 및 건강 영향 지표로서 유용하게 활용될 수 있을 것으로 기대된다. 뿐만 아니라, DNA 메틸화와 방사선에 의해 유도되는 유전체 불안정성과의 연관성을 파악함으로서 방사선에 의한 유전체 불안정성과 암발생과정의 이해를 돕는데 기여할 수 있다.

주요어: 이온화 방사선, DNA 메틸화, 유전체 불안정성, 염색체이상, DNA 메틸기전이효소

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