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ARTICLE

Changes in DNA methylation induced by multi-walled carbon nanotube exposure in the workplace

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ABSTRACT

This study was designed to assess the epigenetic alterations in blood cells, induced by occupational exposure to multi-wall carbon nanotubes (MWCNT). The study population comprised of MWCNT-exposed workers ($n = 24$) and unexposed controls ($n = 43$) from the same workplace. We measured global DNA methylation/hydroxymethylation levels on the 5th cytosine residues using a validated liquid chromatography tandem-mass spectrometry (LC-MS/MS) method. Sequence-specific methylation of LINE1 retrotransposable element 1 (*L1RE1)* elements, and promoter regions of functionally important genes associated with epigenetic regulation [DNA methyltransferase-1 (*DNMT1)* and histone deacetylase 4 (*HDAC4*)], DNA damage/repair and cell cycle pathways [nuclear protein, coactivator of histone transcription/ATM serine/threonine kinase *(NPAT/ATM)*], and a potential transforming growth factor beta (TGF-β) repressor [SKI proto-oncogene *(SKI)*] were studied using bisulfite pyrosequencing. Analysis of global DNA methylation levels and hydroxymethylation did not reveal significant difference between the MWCNT-exposed and control groups. No significant changes in Cytosine-phosphate-Guanine (CpG) site methylation were observed for the *LINE1* (L1RE1) elements. Further analysis of gene-specific DNA methylation showed a significant change in methylation for *DNMT1, ATM, SKI*, and *HDAC4* promoter CpGs in MWCNT-exposed workers. Since DNA methylation plays an important role in silencing/regulation of the genes, and many of these genes have been associated with occupational and smoking-induced diseases and cancer (risk), aberrant methylation of these genes might have a potential effect in MWCNT-exposed workers.

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Introduction

With the expanding market and increase in production capacity, concerns have been raised regarding human exposure and safety of carbon nanotubes (CNT). Results obtained from *in vitro*, *in silico*, and *in vivo* studies have already confirmed toxicity of several forms of multi-walled carbon nanotubes (MWCNT) (Firme and Bandaru 2010; Liu et al. 2013; Donaldson et al. 2013b; Dong and Ma 2015; Henderson et al. 2016; Kuempel et al. 2017). Oxidative stress (Ye et al. 2009; Reddy et al. 2010; Srivastava et al. 2011), accumulation of inflammatory cytokines (Ye et al. 2009; Yamashita et al. 2010), DNA damage (Muller et al. 2008; Lindberg et al. 2009; Asakura et al. 2010; Jackson et al. 2015), activation of nuclear transcription factors, among others, have been proposed as possible mechanisms behind MWCNT toxicity. Most of these anomalies are considerably similar to that of cancer (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011).

Based on the existing evidences, a working group of the International Agency for Research on Cancer (IARC) classified a particular rigid MWCNT (namely,

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Supplemental data for this article can be accessed here.

Mitsui-7) as 'possibly carcinogenic to humans' (Group 2B) (Grosse et al. 2014). Other MWCNT, including single, multiple, rigid, and flexible MWCNT have been classified as 'not classifiable as to its carcinogenicity to humans' (Group 3) due to differences in toxicity response by CNTs with different physicochemical properties (Grosse et al. 2014). Additionally, based on the physicochemical similarities like fibrous nature, high aspect ratio, carbon nanotubes have been compared to asbestos. These similarities are supported by toxicogenomic evidences in normal human bronchial epithelial cells (Kim et al. 2012). Significant similarities, such as deregulation of signaling pathways including apoptosis, cell cycle, and metabolism have been demonstrated for MWCNT (Pacurari, Castranova, and Vallyathan 2010; Donaldson et al. 2010; Donaldson et al. 2013a; Luanpitpong et al. 2016). A more recent article reported carcinogenic effect of MWCNT, for which the aspect ratio and curvature have been considered determining factors (Rittinghausen et al. 2014). Studies in animal models have reported induction of mesothelioma, lung adenocarcinoma, hyperplasia and fibrosis (Takagi et al. 2008; Nagai et al. 2011; Snyder-Talkington et al. 2015; Suzui et al. 2016), and deletion of cyclin dependent kinase inhibitor 2 A/2B (CDKN2A/2B) tumor suppressor genes (Nagai et al. 2011). These studies provide considerable evidence of similarity between some MWCNT and asbestos in *in vitro* and *in vivo* models.

Over the last decade, exposure to CNT in the workplace has been measured in several studies (Han et al. 2008; Lee et al. 2010; Dahm et al. 2015; Kuijpers et al. 2016; Shvedova et al. 2016). The study by Dahm et al., reported exposure to CNT in 14 sites across United States of America (USA) (Dahm et al. 2015). The inhalable concentrations reported in the personal breathing zone ranged between 0.01 and 79.57 $\mu g/m^3$, with CNT sizes between 2 and 5 μm and at times $>5\,\mu m$. The respirable concentrations were reported between 0.02 and 2.94 $\mu g/m^3$. Overall, 30% of the inhalable elemental carbon concentration in personal breathing zone were found to be $>1\,\mu g/m^3$. In addition, other studies have indicated that in some facilities the exposure to CNT was higher than the National Institute for Occupational Safety and Health (NIOSH) – recommended exposure limit (REL) of '1 $\mu g/m^3$ elemental carbon (EC) as a respirable mass 8-hour time-weighted average (TWA) concentration'. It is important to mention that the NIOSH-REL is not based on health studies; but based on quantification limits, and therefore may not be representative for safe exposure limit. In the facilities with higher levels of CNT exposure, adverse health effect in workers is of prime concern. These concerns have been validated in a limited number of recent studies. Fatkhutdinova et al. found a significant increase in levels of interleukin 1 beta (IL-1β), interleukin 6 (IL6), tumor necrosis factor alpha (TNF-α), and Krebs von den Lungen 6 (KL-6), in workers exposed to MWCNT (Fatkhutdinova et al. 2016). In another study by the same group, the authors observed a significant change in non-coding RNA (ncRNA) and messenger RNA (mRNA) expression in peripheral blood cells in workers exposed to MWCNT (Shvedova et al. 2016). However, interpretation of results from most of these studies is limited by the small population size (usually $n \leq 10$). A more recent study from Vlaanderen et al., investigated lung function, exhaled nitric oxide levels, blood count, and immune markers in a relatively larger population of 22 workers exposed to MWCNT, which indicated effects of occupational exposure to MWCNT (Vlaanderen et al. 2017). In this study, we have used the same population (Vlaanderen et al. 2017).

Although genetic changes are known to play key role in carcinogenicity, advances in toxicology and cancer research indicate significant role of epigenetic alterations in chemically –induced carcinogenesis (Watson and Goodman 2002; Esteller 2008; Chappell et al. 2016). Recently, epigenetic changes were identified to be one of the ten key characteristics of carcinogens identified by IARC (Smith et al. 2016). The epigenetic markers including DNA methylation, histone modification and miRNA play a crucial role in altering gene expression and mechanism of carcinogenesis. Of these, DNA methylation is the most well studied epigenetic mark, which are cell type specific and often used as hallmark of cancer and disease progression (Li, Beard, and Jaenisch 1993; Bird 2002; Robertson 2005; Ting Hsiung et al. 2007; Kim et al. 2010; Kulkarni et al. 2011; Chouliaras et al. 2013). Epigenetic alterations have already been associated with tobacco smoking, smoking associated coronary artery disease and asbestos induced malignant mesothelioma. Hypo/hypermethylation has been observed in several

genes including APC, WNT signaling pathway regulator (*APC),* ATM serine/threonine kinase *(ATM)*, *CDKN2B*, cadherin 1 *(CDH1)*, estrogen receptor 1 *(ESR1)*, solute carrier family 6 member 20 *(SLC6A20)*, *CDKN1A*, SKI proto-oncogene *(SKI)* and spleen associated tyrosine kinase *(SYK)* (Tsou et al. 2007; Christensen et al. 2008; Kobayashi et al. 2008; Fujii et al. 2012; Lyn-Cook et al. 2014; Steenaard et al. 2015; Zhang et al. 2016).

Despite significant evidence of epigenetic changes in diseases including cancer and growing evidence of toxicity and potential carcinogenic effects of some forms of CNTs, not much is known about CNT induced epigenetic alterations. In the literature, a few existing *in vitro* and *in vivo* experiments suggest involvement of epigenetic changes after exposure to CNT. In our study *in vitro*, gene promoter specific hypomethylation was observed in human monocytes exposed to CNT (Öner et al. 2016). In another study, exposure to carbon black, MWCNT and SWCNT increased global (%) DNA methylation levels in human lung cancer cells (Li et al. 2016). *In vivo*, a substantial promoter hypomethylation of *ATM* gene was observed in mice exposed to CNT by our group (Tabish et al. 2017). In another study, mice exposed for 24 h and 7 d to MWCNT by oropharyngeal instillation, revealed a global hypomethylation in the lung tissue, and gene-specific hypomethylation of interferon gamma (IFN-c), tumor necrosis factor (TNF), and hypermethylation of thymus cell antigen 1 (Thy1) (Brown et al. 2016).

Since no or very limited data exist regarding epigenetic modifications (DNA methylation) induced by CNT, in human populations, the hypothesis of the study is, therefore, to identify whether exposure to MWCNT in the workplace induce DNA methylation changes at global and/or gene-specific level in some functionally important genes. Such changes might increase the susceptibility of the population to CNT-induced cardiovascular or lung diseases and play possible role in carcinogenesis. Therefore, we designed the present cross-sectional study to observe changes in DNA methylation (global and sequence specific) in peripheral blood cells collected from a group of workers occupationally exposed to MWCNT. We measured global DNA methylation/hydroxymethylation levels using a validated liquid chromatography tandem-mass spectrometry (LC-MS/MS) method. Additionally, we studied the sequence specific methylation of *LINE-1* elements using bisulfite pyrosequencing. *LINE-1* elements constitute a large part of the genome and are significantly methylated, and thus analyzing methylation of *LINE-1* can be representative/used as surrogate of global DNA methylation (Tabish et al. 2015). We also studied sequence specific methylation of Cytosine-phosphate-Guanine (CpG) sites in promoter regions of few selected functionally important genes associated with epigenetic regulation [DNA methyltransferase-1 (*DNMT1)* and histone deacetylase 4 (*HDAC4*)], DNA damage/repair pathways [nuclear protein, coactivator of histone transcription/ATM serine/threonine kinase *(NPAT/ATM)* and SKI proto-oncogene *(SKI)*]using bisulfite pyrosequencing.

Materials and methods

Study participants and exposure assessment

Exposure measurement and sample collection for this study was conducted in two phases- Phase 1 (June 2013) and Phase 2 (October 2013) as described in earlier publications (Kuijpers et al. 2016; Vlaanderen et al. 2017). In phase 2 of the study however, only a limited number of subjects could be recruited and processed for the epigenetic study (11 exposed individuals and 4 controls). Due to the low sample size in Phase 2 of the study, we will focus primarily on the results of Phase 1. The results of Phase 2 have been briefly discussed in the supplementary file.

From Phase 1, a total of 24 workers (age: 35.87 ± 6.90) were recruited from a factory where MWCNT are produced on a commercial scale and compared to 43 (age: 34.64 ± 8.57) matched control subjects with no history of MWCNT exposure. The workers from the exposed and control group were comparable on socioeconomic status, occupational physical activity, shift work, current smoking status, and age. We included both male (m) and female (f) participants in the control (m:f = 32:11) and exposed groups (m:f = 20:4).

The exposure measurements that formed the basis of the MWCNT exposure groups in this study have been described earlier (Kuijpers et al. 2016). Briefly, the measurements were performed in two areas [production and Research & Development

(R&D)] of a company commercially producing MWCNT (>100 kg MWCNT/d) using chemical vapor deposition (CVD) method. In addition to synthesis, workers were involved in handling activities such as packaging of the material. Measurement of inhalable particle was done in workers from different parts of the production and R&D facility over a period of 7 d. EC concentration was measured in the collected particulate matter. Scanning Electron Microscopy-Energy-Dispersive X-Ray (SEM-EDX) was used for physicochemical characterization of the MWCNT, which revealed agglomerated MWCNT on all the filters also consisting of other elements such as calcium and catalyst metals (i.e. aluminum and iron). The average estimated exposure to MWCNT (200 nm–100 μm agglomerates), based on levels of EC in workplace was determined to be between 4.6 and 42.6 $\mu g/m^3$. Additionally, for gene specific methylation analysis described later; individuals were divided into control and three exposure groups [lab-low ($n = 9$; 1 $\mu g/m^3$ EC), lab-high ($n = 6$; 7 $\mu g/m^3$ EC), and operators ($n = 7$; 45 $\mu g/m^3$ EC)] based on exposure assessment, and individual work patterns described earlier (Kuijpers et al. 2016; Vlaanderen et al. 2017). In Table 1, the variables of the control and exposed groups are summarized. The respirable mass concentrations were reported to be between 0.07 and 4.45 $\mu g/m^3$, with highest in the production area (Kuijpers et al. 2016). We included question pertaining to previous exposure history to chemicals/particulate matters in the workplace, for which we were able to obtain a semi-qualitative response (yes/no and the duration). Since, previous exposures at workplace could impact epigenome, it was controlled for during the statistical analysis, however, this was not based on actual exposure assessment data and could be considered a limitation.

Sample collection and DNA extraction

The study was approved by the Commission for Medical Ethics of UZ Leuven (reference number S54607). All participants were briefed about the purpose of the study and informed consent was obtained from the participants. Participants filled out a general health and lifestyle questionnaire based on a validated questionnaire by ELON ('Europees Luchtweg Onderzoek Nederland'). The questionnaire was used to acquire demographic information, health history, respiratory health, asthma and allergies, complaints of the circulatory system, lifestyle factors including smoking, and alcohol consumption, radiation exposure history, family medical history, and work history. Lung function measurements were performed using the EasyOne electronic spirometer (ndd Medizintechnik, Zurich, Switzerland), the results of which have been described by Vlaanderen et al. (2017). No significant trends in forced vital capacity (FVC), FEV1 (forced expiration volume in 1 s), and FEV1/FVC with exposure to MWCNTs were observed. Additionally, Vlaanderen et al. (2017) also reported significantly lower levels of fractional exhaled nitric oxide (FENO) among operators compared to controls in Phase 1 of the study. For epigenetic analysis, whole blood was collected [Ethylenediaminetetraacetic acid (EDTA) tubes of 4.5 ml] in the morning hours (before midday) by venipuncture of forearm veins and stored in −80 °C till further processing. DNA was extracted from blood using QIAamp DNA Mini Kit (Qiagen, Belgium) according to the manufacturers' instruction.

Table 1. Summary of study population (Phase 1).

Variables		Control ($n = 43$)	Exposed ($n = 24$)
Gender	Male	32 (74.41%)	20 (83.33%)
	Female	11 (25.58%)	4 (16.66%)
Age (years)		34.6 ± 8.57	35.9 ± 6.90
Average weight (kg)		76.0 ± 17.36	85.3 ± 17.52
Smoking	Never smoker	24	13
	Former smoker	7	6
	Current smoker	12	5
Alcohol	Yes/No	35/8	15/9
	Glass/day[b]	1.1 ± 0.91	0.9 ± 0.77
Subjects with previous history of exposure to chemicals[a]		9	8
Duration of exposure to nanoparticle at current job (years)		0	4.25 ± 2.40

[a]as reported by the study subjects.
[b]Alcoholic drinks consumed on average per day over the past 4 weeks.

Global DNA methylation-LC/MS/MS

DNA was analyzed by LC-MS/MS as described previously (Godderis et al. 2015). Briefly, isolated genomic DNA samples (1 μg) were enzymatically hydrolyzed and each sample was then analyzed twice using LC-MS/MS. The absolute concentrations of cytosine (C), 5-methylcytosine (5-mC), and 5-hydroxymethylcytosine (5-hmC) were calculated by interpolation the results on to a calibration curve. The results were expressed as DNA methylation in percentage (%) [calculated as 5-mC/(5-mC +5-hmC + C)], and DNA hydroxymethylation (%) [calculated as 5-hmC/(5-mC +5-hmC + C)].

Sequence specific methylation

Methylation levels (%) of repetitive element (*LINE-1*) and the gene promoters (*DNMT1*, *HDAC4*, *NPAT/ATM*, *SKI*) were analyzed by bisulfite pyrosequencing, as described previously (Tabish et al. 2015). The selection of the genes were based on our *in vitro* whole-genome study in human monocytes (Öner et al. 2016) and human bronchial epithelial cells exposed to CNTs (manuscript under revision) and published literature. Additional reason for the selection of each sequence/gene is as follows. Alterations on *LINE-1*, a transposable element sequences, indicate the majority of retrotransposon activity in the human genome. Generally, alterations on *LINE-1* also indicate DNA methylation changes at the global level since it comprises approximately 17% of the mammalian genome.

DNMT1 gene encodes the enzyme, which functions in methyl-group transfer to CpG residues in DNA. Therefore, the main function of this gene is in regulation and maintenance of the DNA methylation. The sequence selected for the study was located in the promoter region of *DNMT1* at chromosome 19 (chr19:10305774–10305811). Five CpG sites were studied in the sequence, which in turn is part of a larger CpG island (chr19:10304967–10305864; 89 CpG). Based on the ENCODE project, University of California Santa Cruz (UCSC) genome browser and the Ensembl genome browser, transcription factor binding derived from collection of ChIP-seq experiments indicated the regions to be binding sites of early growth response 1 (EGR-1), nuclear respiratory factor 1 (NRF1) among others.

HDAC4 gene plays a role in histone deacetylation, which is an important factor in DNA that alters chromosome structure and binding of transcription factors. Of note, histone acetylation/deacetylation is also a critical factor in developmental processes, cell cycle regulation, and transcriptional regulation. The sequence investigated for this study was located at chromosome 2 (chr2:240323289–240323326). The sequence studied constituted of 7 CpG sites, which is part of a CpG island (chr2:240321778–240323919; 181 CpG) located in the promoter region of *HDAC4,* in proximity of histone mark (H3K27Ac mark). Based on the ENCODE project, UCSC, and the Ensembl genome browser, transcription factor associated with the region include and not limited to RNA polymerase II, CCCTC-binding factor (CTCF) that encodes a zinc finger protein, E2F transcription factor 1 (E2F1), Sp1 transcription factor (SP1).

Previously, an *in vivo* study (in mouse) by our group identified methylation alterations on *ATM* gene promoter region, associated with nanoparticle exposure (Tabish et al. 2017). A bi-directional promoter sequence for genes *ATM* and *NPAT*, located at Chromosome 11 (chr11:108093191–108093221), comprising of 5 CpG sites were studied for methylation changes. The sequence was part of a CpG island spanning chr11:108093212–108093969, comprising of 75 CpG sites. CTCF, zinc finger protein 263 (ZNF263), E2F transcription factor 4 (E2F4), GA-binding protein transcription factor (GABP), transcription factor binding regions were found to be associated with the region as obtained from the ENCODE project, UCSC genome browser, and the Ensembl genome browser. Based on the crucial role of *ATM* (a serine-threonine kinase) in DNA damage repair response including cell cycle arrest, DNA repair, and/or apoptosis; and significant evidences gathered from our *in vitro* and *in vivo* study this was one of ideal choice for the study.

In addition to the above genes, a sequence to study sequence specific methylation in *SKI* gene, located at chromosome 1 (chr1:2161097–2161125), was selected. Methylation changes on *SKI* gene have been associated with the smoking exposure (Steenaard et al. 2015).

We selected sequences in the gene promoter regions, since changes in DNA methylation of the gene promoter regions are generally associated with aberrant transcription factor binding and

altered gene expression. These sequences comprise the gene promoter regions that are important for transcription factor binding activity of CTCF, GABP, and EGR-1 among others.

Briefly, 200 ng of genomic DNA was bisulfite converted using the EZ-96 DNA Methylation-Gold™ Kit (#D5008, Zymo Research, Irvine, CA). Converted DNA was amplified by polymerase chain reaction (PCR), using Qiagen PyroMark PCR Master Mix (#978703, Qiagen). All the PCR and sequencing primers were obtained from Qiagen (*LINE-1*: PyroMark Q24 CpG *LINE-1*, #970042; *DNMT1*: PM00075761; *HDAC4*: PM00007539; *NPAT/ATM*: PM00153622; *SKI*: PM00004011; Supplementary Table 1). Subsequently, PCR products were immobilized onto streptavidin sepharose beads (#17-5113-01, GE Healthcare) and pyrosequencing was performed on the PyroMark Q24 (Qiagen) sequencing platform following the manufacturer's instructions. The results were analyzed using the PyroMark analysis version 2.0.7 software (Build 3, Qiagen, Belgium).

Statistical analysis

At the onset, we determined the effect of MWCNT exposure on DNA methylation (global, *LINE-1* and gene-specific) using logistic regression analysis using IBM SPSS statistics, where control (unexposed) and exposed were used as classifiers. To assess the effect of MWCNT exposure, DNA methylation was selected as response variable, with correction for possible confounding variables (age, gender, smoking habit, and alcohol consumption). Subsequently, we analyzed three categories of exposed workers ['lab-low' ($n = 9$; 1 μg/m^3 EC), 'lab-high' ($n = 6$; 7 μg/m^3 EC), and 'operators' ($n = 7$; 45 μg/m^3 EC)] to non-exposed controls. We assigned values of 0–3 to the exposure categories (Thus 0 being the control, 1 lab low, 2 lab high, and 3 being the operators) based on their exposure ranking, and conducted linear regression using the assigned actual exposure estimates for each category (Vlaanderen et al. 2017). We evaluated the association of the significant genes with other genes using the GeneMANIA plugin in Cytoscape version 3.2.1 (National Institute of General Medical Sciences, Seattle, WA, United States of America).

Results

Global DNA methylation and hydroxymethylation

In this study, no significant difference in the levels of 5-mC (%) and 5-hmC (%) were observed between the control and MWCNT-exposed individuals (Figure 1(a,b)). The levels of 5-mC (%) and 5-hmC (%) were positively correlated ($R = 0.74$), and no effect of confounders (age, gender, smoking, and alcohol consumption) was observed in the study population.

LINE-1 *methylation*

Figure 2 shows no significant differences between *LINE-1* methylation (%) in the control and MWCNT-exposed individuals. We obtained an average *LINE-1* methylation (%) of 74.20 ± 1.08 for MWCNT-exposed group, as compared to 74.37 ± 1.44 for the control group.

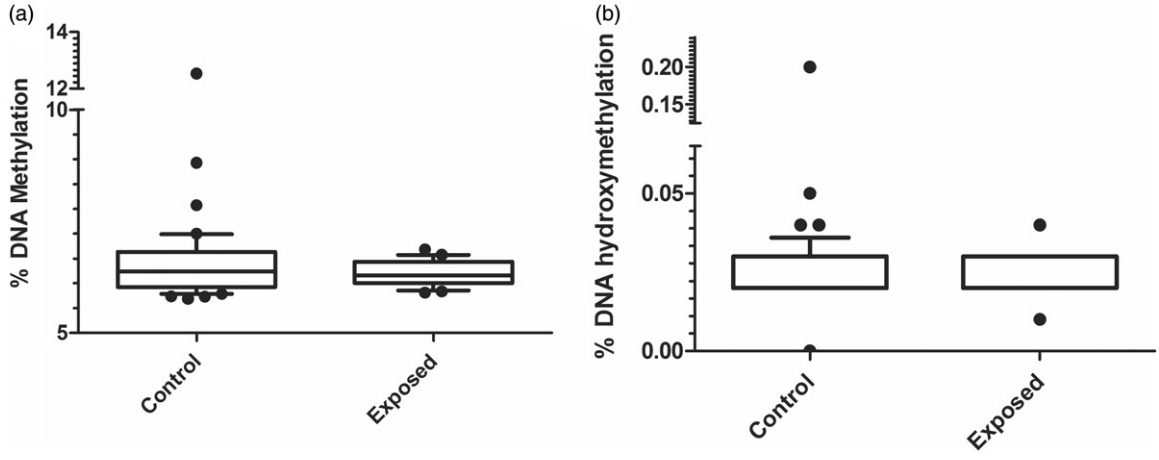

Figure 1. Effect of MWCNT exposure on, a: Global DNA methylation (%) and b: hydroxymethylation (%) in control, and MWCNT exposed population.

Gene-specific DNA methylation

The results of DNA methylation for all the genes have been presented in Supplementary Table 2 and Figure 3.

DNMT1

We observed a significant hypomethylation ($p \leq 0.001$) of CpG 1 (Figure 3(a)), while significant hypermethylation (Figure 3(b,c)) was observed for CpG 3 ($p \leq 0.01$) and CpG 5 ($p \leq 0.001$). On the whole, the promoter site sequenced was significantly hypermethylated ($p \leq 0.05$) for the MWCNT-exposed group.

HDAC4

We investigated the methylation status of a sequence belonging to the promoter region. Here, we found that 4 of the 7 CpG sites in the selected promoter sequence region were differentially methylated (Figure 3(d–f)). Three were significantly hypermethylated (CpG 2, $p \leq 0.05$; CpG 6 & CpG 7, $p \leq 0.01$) and one was significantly hypomethylated (CpG 3, $p \leq 0.01$).

NPAT/ATM

The sequences selected for the study are shared by both *NPAT* and *ATM* genes and act as bi-directional promoter. The overall methylation of the promoter sequence remained unaffected in MWCNT exposed group, except for a significant hypermethylation of CpG position 6 (Figure 3(e); $p \leq 0.05$).

SKI

The methylation of 3 CpG site were significantly altered for *SKI*. While CpG 2 (Figure 3(e)) was significantly ($p \leq 0.001$) hypermethylated, CpG 4

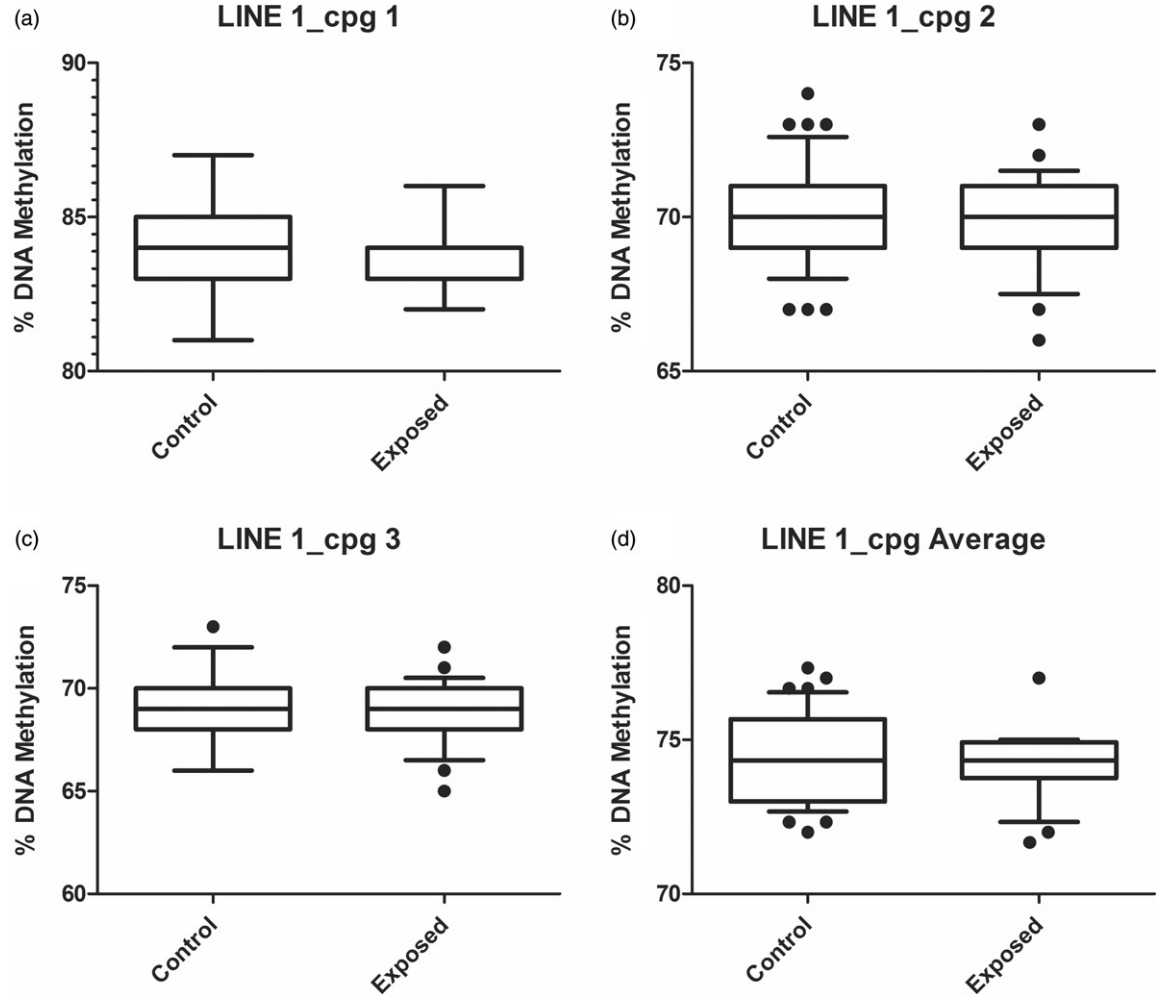

Figure 2. *LINE-1* methylation at a: CpG position 1, b: CpG position 2, c: CpG position 3, and d: average methylation of CpG sites in *LINE-1*.

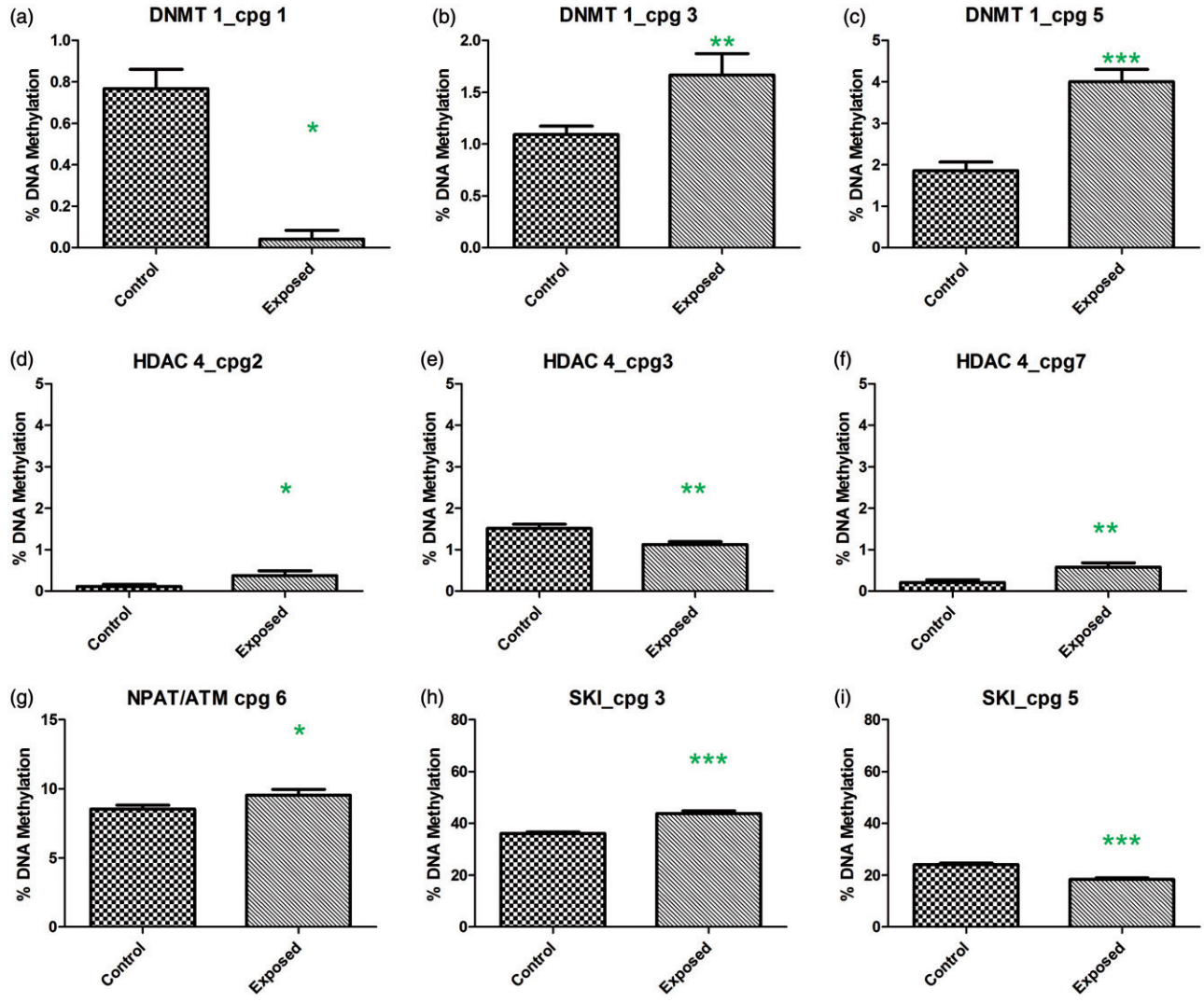

Figure 3. Sequence specific methylation of CpG sites showing significant difference between the control and exposed groups, a: *DNMT1* CpG site 1, b: *DNMT1* CpG site 3, c: *DNMT1* CpG site 5, d: *HDAC4* CpG site 2, e: *HDAC4* CpG site 3, f: *HDAC4* CpG site 7, g: *NPAT/ATM* CpG site 6, h: *SKI* CpG site 3, and i: CpG site 5; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

($p \leq 0.05$) and CpG 5 (Figure 3(i); $p \leq 0.001$) were significantly hypomethylated.

Changes in gene-specific DNA methylation as function of work

To understand the influence of MWCNT exposure levels, the 'exposed group' was further subdivided into 'lab-low' ($n = 9$; 1 μg/m^3 EC), 'lab-high' ($n = 6$; 7 μg/m^3 EC), and 'operators' ($n = 7$; 45 μg/m^3 EC) as previously described (Vlaanderen et al. 2017). Significant differences in methylation percentages were observed (Figure 4) in gene promoter for lab-low (*DNMT1*- CpG1, CpG5; *HDAC4*- CpG7; *SKI*- CpG3, CpG5), lab-high (*DNMT1*- CpG1, CpG2, CpG5; *HDAC4*- CpG7; *SKI*- CpG3, CpG5), and operators (*DNMT1*- CpG1, CpG5; *HDAC4*- CpG2; *SKI*- CpG3, CpG5) when compared to control. A significant difference between methylation (%) of *DNMT1*- CpG2 in lab-high group was observed, when compared to both lab-low and operator groups. However, no exposure response was observed among the MWCNT-exposed group.

Discussion

The mechanism of cancer is recognized as a complex, multi-step and progressive process, in which healthy cells/tissues transform to malignant cells/tissues. Growing evidence suggests that genotoxic or non-genotoxic environmental or occupational exposures may alter epigenome and such alterations

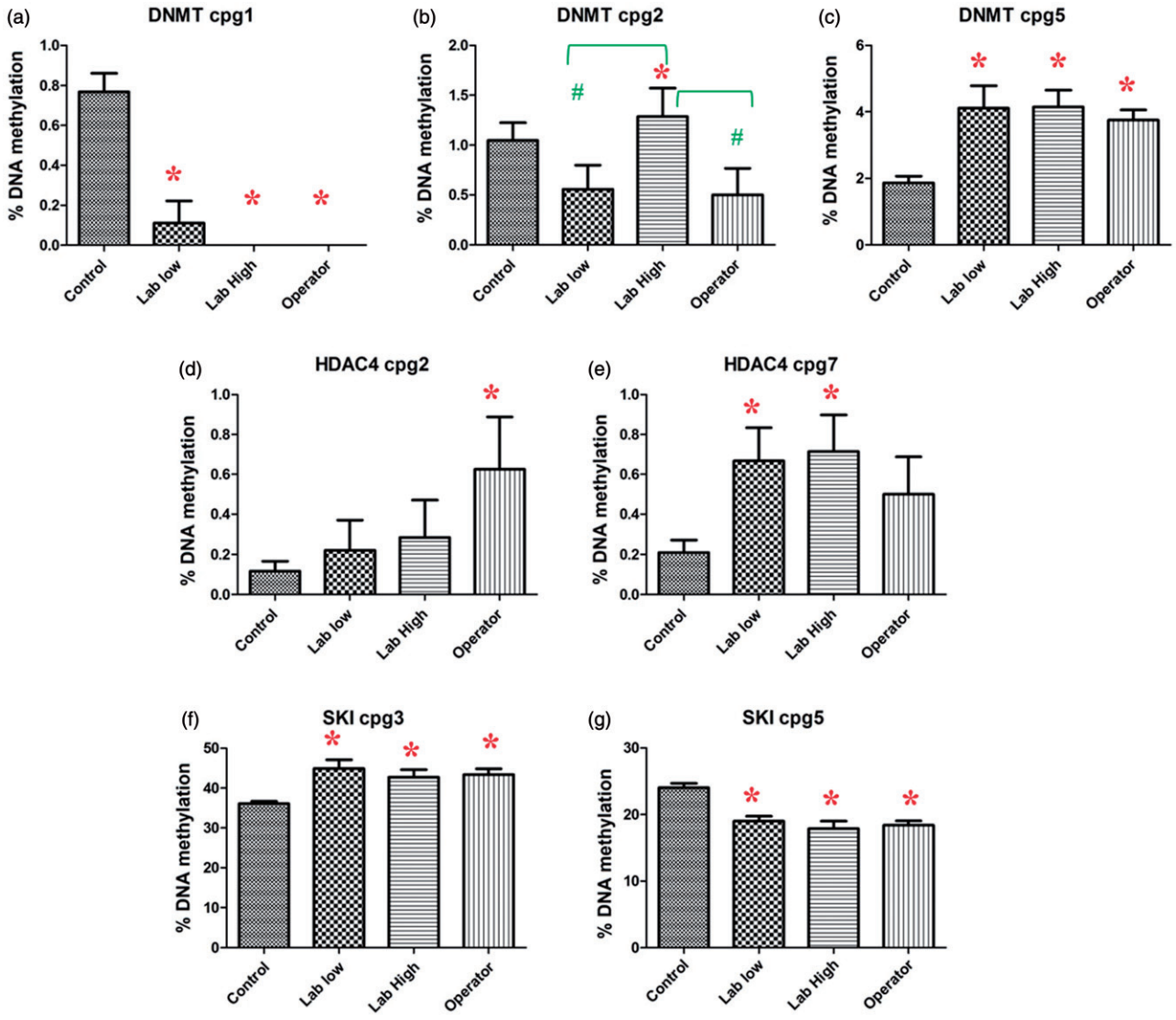

Figure 4. Sequence specific methylation showing significant changes in different exposure groups for a: *DNMT1* CpG site 1, b: *DNMT1* CpG site 2, c: *DNMT1* CpG site 5, d: *HDAC4* CpG site 2, e: *HDAC4* CpG site 7, f: *SKI* CpG site 3, and g: *SKI* CpG site 5; $^{*}p \leq 0.05$, compared to control; $\#p \leq 0.05$ compared to exposure group Lab High.

contribute to the mechanisms of carcinogenesis (Smith et al. 2016). Epigenetics establishes the chromatin structure, homeostasis, and gene expression state of the cells. In healthy cells, epigenetics is balanced and stable, however, in almost all cancer cells; global hypomethylation is seen together with gene-specific hypo/hypermethylation and histone modifications. Identification of early and subtle methylation alterations before the disease occurrence may probably indicate risk of the disease occurrence and can be exploited to identify early biomarkers of the exposure. Epigenetic changes in surrogate tissues, such as blood represent a set of informative class of biomarkers of both exposure as well as effect.

Epigenetic control of gene regulation is primarily governed by DNA methylation, histone modification and ncRNA. Of these changes, DNA methylation has been recognized as a key factor influencing several environmentally induced cancers. Changes in DNA methylation have been observed in human populations environmentally exposed to various agents such as smoking, arsenic, cadmium, PAH, and other heavy metals (Li et al. 2003; Sutherland and Costa 2003; Baccarelli and Bollati 2009).

In this study, we first evaluated global DNA methylation/demethylation changes and gene-specific DNA methylation alterations induced by MWCNT-exposure in workplace. Methylation and demethylation to/of 5-mC by either passive or TET

mediated active mechanisms to 5-hmC has been considered significant in development processes and disease progression (Pfeifer, Kadam, and Jin 2013; Wang et al. 2014). This DNA demethylation (or in other words, hypomethylation), indicates decrease in the number of cytosine methylation levels. Passive or active demethylation can occur. Passive DNA demethylation is due to the lack of methyl donor S-adenosyl-L-methionine (SAM) group and alterations on DNA methyltransferases (i.e. *DNMT1*) which inhibits the binding of methyl groups enzymatically. Active loss of DNA methylation is suggested to be because of impaired function of DNA repair machinery, however, the knowledge regarding the exact mechanism is limited. Over the years, studies have established the association between levels of 5-mC/5-hmC and cancer (Momparler and Bovenzi 2000; Jin et al. 2011; Kudo et al. 2012; Pfeifer, Kadam, and Jin 2013; Yang et al. 2013). In this study however, no significant difference in the levels of 5-mC (%) were observed between the control and exposed subjects. The levels of 5-hmC (%), also remained unaltered in the study population.

Methylation status of repetitive element can provide significant insight into the epigenetic regulation (Jones and Takai 2001). Of these elements, *LINE-1* account for ~17% of human DNA, and *LINE-1* insertions play a significant role regulating gene expression. Both hypomethylation and hypermethylation of *LINE-1* element has been associated with various diseases (Chalitchagorn et al. 2004; Cash et al. 2011; Bollati et al. 2011; Liao et al. 2011; Kitkumthorn et al. 2012). Altered *LINE-1* methylation has been studied for environmental and anthropogenic agents (Valentina Bollati et al. 2007; Andrea Baccarelli et al. 2009; Baccarelli and Bollati 2009; Tarantini et al. 2009). In this study, we used bisulfite pyrosequencing to determine methylation status of *LINE-1* element. However, no change in *LINE-1* methylation (%) was observed between the groups. The result was consistent with that obtained from global methylation analysis.

While global methylation pattern and methylation of repetitive elements provide significant preliminary information of the chromatin state, methylation status of CpG in promoter gene region provides a better understanding of specific gene silencing. In general, hypermethylation of promoter region of genes has been associated with gene silencing. In this study, promoter CpG methylation (%) was studied for four selected genes (*DNMT1, HDAC4, NPAT/ATM*, and *SKI*) by bisulfite pyrosequencing.

DNA methylation, a post-replicative modification, is established and maintained by the DNA methyltransferases (DNMT1, DNMT3A, DNMT3B, and DNMT3L). Among the methyltransferases, DNMT1 plays principal role in maintenance of cytosine methylation. In this study, we observed a significant hypo/hypermethylation of the CpG sites, for the MWCNT-exposed group. Additionally, we observed significant difference in methylation percentage of CpG2 of *DNMT1* gene, between the exposure sub groups (lab-low/lab-high and lab-high/operator). It is well established that differential methylation of CpG islands in the gene promoter region of *DNMT1* is associated with gene silencing and cancer (Herman and Baylin 2003) and, therefore, changes in *DNMT1* promoter methylation might have an impact on disease outcome. Observing hypomethylation for some CpGs and hypermethylation for other CpG in the promoter region of gene make the results however biologically challenging.

In addition to DNA methylation, histone modifications play key role in epigenetic regulation of gene expression. A balance between acetylation and deacetylation of lysine residues in the N terminal domain of histone is critical for DNA replication, repair and transcription (Delcuve, Khan, and Davie 2012; Wang, Qin, and Zhao 2014). The balance in acetylation is brought about by the activities of histone acetyltransferases (HATs) and histone deacetylase (HDAC). HDACs regulate DNA methylation, and at the same time are also recruited by the CpG methylation along with methyl-cpg-binding protein 2 (MeCP2) to repress transcription. Of the four classes HDACs comprising of 18 different types, *HDAC4* plays a critical role (Delcuve, Khan, and Davie 2012; Wilson et al. 2008; Wang, Qin, and Zhao 2014). HDAC4 enzyme has been associated with regulation of cellular growth, proliferation, survival, and aberrations on their activity has been linked with the carcinogenesis. The HDAC4 appears to be one of the key enzymes in control of gene expression. Significant downregulation of histone deacetylases including HDAC2 and HDAC4 have been associated with the cigarette smoking (Sundar and

Rahman 2016). Therefore, we investigated the methylation status of a sequence belonging to the promoter region of *HDAC4* and found that 4 of the 7 CpG sites were significantly differentially methylated. Hypermethylation of the promoter might result in silencing of *HDAC4*. This may potentially lead to an altered expression of *HDAC4* in the exposed workers, thereby affecting the chromatin structure and transcription. Additionally, downregulation of *HDAC4* expression has been known to induce apoptosis, growth inhibition and increase in p21 transcription (Wilson et al. 2008; Abbas and Dutta 2009).

The *ATM* gene codes for serine-threonine kinase and is a key regulator in pathways activated by DNA strand breaks. *ATM* acts by phosphorylating p53, ultimately leading to cell cycle arrest, DNA repair, and apoptosis. *ATM* phosphorylates and thereby regulates the function of many cell cycle checkpoint kinases (CHK1, CHK2), repair factors [RAD9 checkpoint clamp component A (RAD9A, also known as RAD9), RAD50 double strand break repair protein (Rad50), RAD51 recombinase (RAD51)], BH3 interacting domain death agonist (BID), among others. Therefore, proper functioning of *ATM* is critical for normal cellular functioning. Additionally, higher *ATM* methylation has been proposed as suitable biomarker of breast cancer risk (Flanagan et al. 2009; Brennan et al. 2012) and oral squamous cell carcinoma (Rigi-Ladiz, Kordi-Tamandani, and Torkamanzehi 2011). In our study in BALB/c mice (Tabish et al. 2017), intra-tracheal administration of CNTs induced sequence specific differential methylation in promoter region of *ATM* gene. Hence, for this study we investigated the methylation status of the *ATM* promoter region. The sequences selected for the study are shared by both *NPAT* and *ATM* genes and acts as bidirectional promoters. The overall methylation of the promoter sequence remained unaffected in the MWCNT-exposed group, except for a single hypermethylated CpG site. While it is possible that hypermethylation might lead to downregulation of *ATM*, it remains to be validated for the present population.

Significant changes in CpG methylation were also observed in the promoter region of *SKI* in the MWCNT-exposed group. Based on published evidences, it can be suggested that any change in expression would lead to disruption of TGF-β signaling and cell cycle progression (Medrano 2003; Suzuki et al. 2004). Additionally, increased levels of *SKI* expression have been reported in melanoma tissues (Reed et al. 2001). Hypermethylation of *SKI* CpG (cg05603985) has been observed in tobacco smoking associated coronary artery disease risk (Steenaard et al. 2015).

Significant changes in methylation of at least one or more CpG sites were observed for all the genes studied (*NPAT/ATM, SKI, DNMT1*, and *HDAC4*) in MWCNT exposed workers. Except for one CpG site in *DNMT1* (CpG2) promoter region, no significant differences between the exposure sub-groups (lab-low, lab-high, and operator) were observed. It can be speculated that differential methylation of CpG sites in the promoter region of these genes would functionally affect the transcription initiation of these genes. While the extent of the effect on gene expression remains to be determined, changes in expression of the studied genes might have consequences in DNA damage repair and G1/S transition of cell cycle (*NPAT/ATM*; Figure 5); DNA/histone methylation (*DNMT1*; Figure 5); and Chromatin remodeling and transcriptional repression (*HDAC4*, *SKI*; Figure 5).

In this study, we identified alterations on CpG sites in the promoter regions of functionally important genes in peripheral blood cells. Such methylation alterations might inhibit or promote the gene expression of the corresponding gene. An increase in the methylation may inhibit the binding of the transcription factors or demethylation may promote binding of the transcription factors. As discussed earlier, the regions selected for this study function as binding site of several important transcription factors including EGR-1, NRF1, RNA Polymerase II, CCTF, E2F1, SP1, among others, and, therefore, might have significant impact on the gene expression. It is however, critical to assess whether these epigenetic alterations proceed with the phenotypical alterations by investigating aberrant gene transcription or protein translation. Although, in this study, we do not study the gene expression alterations, we provide the first insight in epigenetic modifications attributed to MWCNT-exposure in the workplace. The genes selected in this study implies the most important genes that can lead to changes in epigenetic machinery (i.e. *DNMT1*, *HDAC4*) and DNA damage response and G1/S transition in the cell cycle (i.e. *ATM*), and

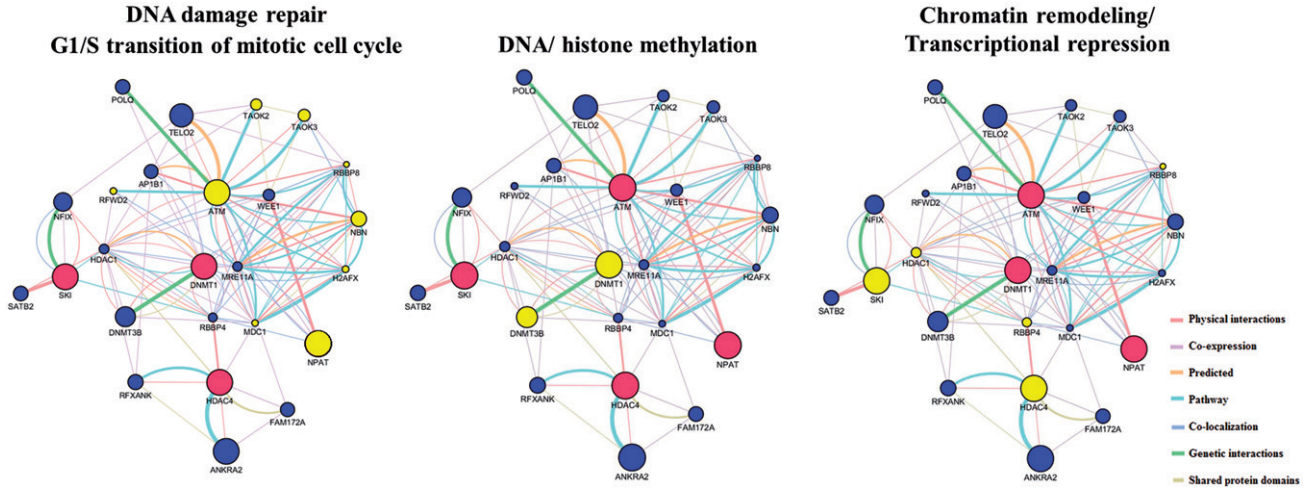

Figure 5. Possible effect of sequence specific promoter methylation of *NPAT/ATM, SKI, DNMT1*, and *HDAC4* on different pathways; the circles in yellow indicate the genes involved in the specific pathway, studied genes not associated with the pathways are indicated in magenta, and blue circles indicates the interacting genes.

oncogenic activity (i.e. *SKI*), which is a potential repressor of TGF- β signaling that plays important role in fibrosis and CNT-exposure. Ideally, this study should be replicated, with a larger number of exposed-workers in order to be used as early biomarker of disease and exposure.

Conclusion

In summary, no difference in global methylation of the genomic DNA or repetitive element (LINE-1) was observed. Significant changes in methylation of CpG sites in promoter region of *DNMT1, HDAC4, NPAT/ATM* and *SKI* were observed for the MWCNT-exposed group. These results provide first evidence of epigenetic alterations because of CNT exposure at the work place. However, investigation on other epigenetic marks (such as histone modifications and noncoding RNAs) remains to be studied. Due to the small sample size these results need to be interpreted with caution. However, it is important to mention that in the present scenario it is difficult to obtain a larger sample size as significant differences in type and procedure of CNT production exist precluding obtaining a larger population size with same/similar exposure scenario. Notwithstanding, we believe that the study provides important insight into the DNA methylation alterations as a result of occupational exposure to MWCNTs and could be used as a stepping-stone toward biomarker development.

Disclosure statement

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