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Carbon nanotube and asbestos induced DNA and RNA methylation changes in bronchial epithelial cells

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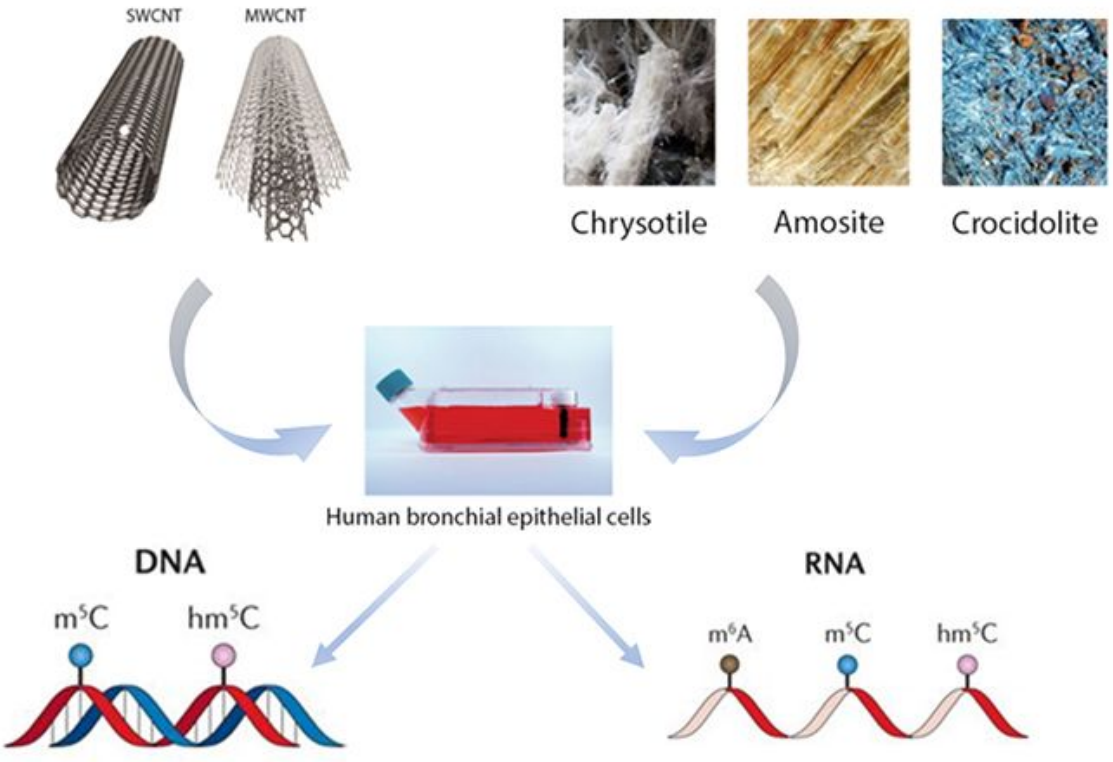

ABSTRACT

Carbon nanotubes (CNTs) are nanoscale tube-shaped carbon materials used in many industrial areas. Their fiber shape has caused concerns about their toxicity given its structural similarity with asbestos. The aim was to elucidate the effect of CNTs and asbestos exposure on global DNA and RNA methylation as well as on the methylation of genes associated with cell cycle, inflammation and DNA damage processes in human lung cells. Human bronchial epithelial cells (16HBE14o-) were exposed for 24 hours to 25 and 100 μg/ml of CNTs (single-walled; SWCNTs and multi-walled; MWCNTs) and 2.5 μg/ml of asbestos (chrysotile, amosite, crocidolite) Global DNA and RNA (hydroxy)methylation to cytosines were measured by a validated liquid chromatography tandem-mass spectrometry method (LC-MS/MS). Global RNA methylation to adenines were measured by colorimetric ELISA-like assay. Gene specific DNA methylation status at certain Cytosine-phosphate-Guanine (CpG) sites of cyclin dependent kinase inhibitor 1A, *CDKN1A;* serine/threonine kinase, *ATM*; and TNF receptor associated factor 2, *TRAF2* were analyzed using bisulfite pyrosequencing technology. Significant global DNA hypomethylation on cytosine and global RNA hypomethylation on

adenosine was observed in MWCNTs exposed cells only. SWCNTs, MWCNTs, and amosite exposure were related to decrease DNA methylation in *CDKN1A* and *ATM* genes. On the other hand, DNA hypermethylation of *TRAF2* gene was observed for SWCNTs. These findings contribute to the understanding of the influence that CNTs could on different carcinogenic pathways.

INTRODUCTION

Carbon nanotubes (CNTs) are increasingly used in different industries because of their unique physico-chemical properties. CNTs are cylinders of graphene with open or closed ends and were classified as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) based on the number of graphene layers.[1] There are raising concerns that exposure to CNTs may present similar health risks to those seen for asbestos, which is a well-known human lung carcinogen.[2]

Despite uncertainty about mechanisms of toxicity, there is considerable number of studies indicating that CNTs induce oxidative stress, acute and chronic pulmonary inflammation, collagen deposition, fibrosis, and granuloma formation on the lungs in animals and humans.[3-10] International Agency for Research on Cancer (IARC Monographs-111; 2014) has classified MWCNT-7 as possibly carcinogenic to humans (Group 2B), whereas other MWCNTs and SWCNTs are not classifiable as carcinogenic to humans (Group 3) due to lack of evidence and data. Furthermore, Kuempel et al. have discussed potential carcinogenic mechanisms of CNTs but also highlighting key data gaps, which should be assessed in the future.[11] In this sense, given the emerging evidence on the role of epigenetic alterations in development of several diseases, it is important to study these endpoints in relation to CNTs and asbestos exposure. Recently, Wong et al. in their review discussed the role of epigenetic changes associated with nanoparticle toxicity.[12] The authors highlighted the fact that the role of CNT's physicochemical properties is largely unexplored.

DNA methylation is the most studied epigenetic modification. Besides 5-methylcytosine (m^5C-DNA), 5-hydroxymethylcytosine (hm^5C-DNA) has also been detected in various cells, and represents part of the DNA demethylation pathway.[13] Methylation alterations of gene promoter or regulatory regions are associated with switching genes on/off, which is

often observed in malignant diseases by activating oncogenes and silencing tumour suppressor genes (TSGs).[14] DNA methylation changes induced by environmental exposures has been broadly examined in the past two decades.[15-30]

Although the majority studies involve methylation changes in DNA, RNA also undergo epigenetic modifications which has recently gained scientific interest. RNA methylation has been observed in both noncoding and coding RNAs.[31] As is generally known, RNA methylation mainly affects the regulation of post-transcriptional gene expression. Therefore, RNA methylation can impact directly protein production.[32] RNA methylation may change microRNA expression and mediate cancer cell migration.[33] While, N6-methyladenosine (m^6A) is the most abundant type of modifications, methylation of cytosines are common. Although there are no *in vivo* evidences, it was suggested that occurrence of m^6A are related to mRNA processing, such as pre-mRNA splicing, mRNA stability, translation, turnover and nuclear export.[34] After determination of demethylation process of m^6A in RNA[35], m^6A related studies, recently, have been focused on its functional relevance in carcinogenesis.[36,37] It was shown that m^6A elevations might predispose to cancer particularly in hematologic malignancies.[37] Recent study discovered that m^6A methylation regulates the ultraviolet-induced DNA damage response.[38] Cytosine methylation (m^5C) in tRNA, which carries amino acids to the ribosome, seem to stabilize tRNA secondary structure, to affect aminoacylation and codon recognition, and to confer metabolic stability.[39] These modifications in rRNA are playing a role in translational fidelity and tRNA recognition.[40] Until now, there is no RNA methylation data related to the exposure to asbestos and CNTs. On the other hand, DNA hypermethylation in TSGs and specific gene-loci in mesothelioma has been associated with asbestos exposure. The epigenome is identified one of the main targets for asbestos in mesothelioma.[41-43] Yu et al. have shown that asbestos exposure causes global DNA hypomethylation, which is linked with genome instability.[44] For the CNTs, no global DNA methylation or hydroxymethylation

alterations in human monocytic cells and mouse lungs have been observed.[45,46] In contrast, A549 cells treated with short MWCNTs displayed significant elevation of global DNA methylation.[47] There are also studies observing that MWCNTs lead to global DNA hypomethylation.[48,49]

Besides global DNA and RNA methylation status, understanding methylation characteristics on certain genes playing roles in CNTs or asbestos induced toxicity, gives insight into the molecular basis of related toxicities. Considering that both asbestos and CNTs exposure leads to DNA damage and apoptosis[50-55], genes involved in these processes (cyclin dependent kinase inhibitor 1A, *CDKN1A;* Ataxia–telangiectasia mutated serine/threonine kinase, *ATM*; and TNF receptor associated factor 2, *TRAF2*) were chosen for further evaluation in terms of DNA methylation. *ATM* is essential in DNA damage, repair and cell cycle checkpoints activation. In response to DNA damage, ATM is activated and it phosphorylates *p53* and other downstream proteins involved in cell cycle checkpoint regulation.[56] Previously, *ATM* gene was analyzed in mice exposed to low level of CNTs and it was found that SWCNTs caused DNA hypomethylation.[45] *CDKN1A (p21)* is a main target regulated by *p53* and *CDKN1A* plays role in cell cycle checkpoint regulation, apoptosis, and gene transcription.[57] On the other hand, *TRAF2* plays role in TNF-alpha-mediated activation of MAPK8/JNK and NF-kappa B[58,59] and plays a role as an important mediator of anti-apoptotic signals.[60] It was showed that *TRAF2* is essential for the proliferation of many epithelial cancers.[61]

In this context, the present study aimed to investigate whether CNTs (SWCNTs and MWCNTs) and asbestos (serpentine and common amphibole types) exposure to human bronchial epithelial cells could lead to epigenetic changes, like global and gene-specific DNA methylation and RNA methylation. Subsequently, the patterns of methylation alterations of asbestos and CNTs were further compared.

EXPERIMENTAL PROCEDURES

Test substances

The carbon nanotubes used for the present study are reference materials, which have been widely used in our previous studies[46,62,63] and in projects like the NANOGENOTOX. SWCNTs were purchased from US National Institute of Standards and Technology (NIST-SRM2483) and MWCNTs were purchased from the European Commission Joint Research Centre (JRC-NM400). While, reference materials are very well characterized, additional characterization of CNTs were performed and has been published previously.[63] Union Internationale Centre le Cancer (UICC) standard reference samples were used for asbestos, which has been used in our study previous study.[64] Asbestos of amphibole (Amosite South African, NB #4173-111-4 and Crocidolite South African, NB #4173-111-3) and serpentine (Chrysotile "A" Rhodesian, NB #4173-111-2) type were supplied from SPI Supplies (Structure Probe Inc., West Chester, USA).

Sample preparation for exposure

In order to disperse CNTs, the standard CNT suspension protocol, published by the Engineered Nanoparticle Risk Assessment (ENPRA; European Union Project) was used.[65] Briefly, CNTs were weighed and diluted in sterile Baxter water containing 2% fetal bovine serum (FBS) for stock CNTs solution (2.5 mg/ml). The stock solutions were sonicated for 16 minutes, placed in an ice bath, at 400 W and 10% amplitude by controlling that the probe does not touch the walls of the vial. Then dilutions were prepared from stock solutions with serum-free cell medium. An amount of 0.2% FBS was obtained at final CNT concentrations. On the other hand, the stock solutions (1 mg/ml) of the asbestos were diluted with the dispersion medium containing 2% of serum and solved in ultrasonic bath for 10 min. All experiments were performed under the HEPA-filter laminar flow with safety clothing. All contaminated materials were discarded as required by Belgian law.

Cell culture and exposure condition

Cell culture solutions were obtained by Gibco-Life Technologies. Human bronchial epithelial cell line (16HBE14o-) kindly provided by Dr. Gruenert (University of California, San Francisco, USA) was used for experiments. The cells were cultured in T25 cell culture flasks in Dulbecco's modified eagle medium: Nutrient mixture F-12 (DMEM/F-12) supplemented with 1% antibiotics (10000 U/ml penicillin and 10 mg/ml streptomycin), 1% L-glutamine, 1% amphotericin B, and 5% fetal bovine serum (FBS) at 37°C in 5% CO_2. Until cells were confluent, media was changed every 2 days. After reaching confluence, cells were split using trypsin-EDTA solution (0.05%). All experiments were conducted at passage 4 to avoid the effect of cell age on epigenetic results. Untreated cells and vehicle (dispersion media) treated cells were used as negative and vehicle controls. Decitabine (5-aza-2′- deoxycytidine at 1 μg/ml) a known DNA demethylating drug[66] was used as positive control.

The concentrations of CNTs for the 24 hours exposure were chosen based on our previous experience with human monocytic cells[46] and bronchial epithelial cells.[63] The 25 and 100 μg/ml were found to be non-cytotoxic and non-genotoxic for MWCNTs and weakly genotoxic for SWCNTs. For amosite, crocidolite, and chrysotile; a final concentration of 2.5 μg/ml (non-cytotoxic but genotoxic) was selected based on our previously published study.[64] For CNTs and asbestos, three independent experiments performed in duplicate were used for each concentration.

RNA and DNA Isolation

AllPrep® DNA/RNA/miRNA Universal Kit by Qiagene was used for simultaneous RNA and DNA isolation from cells. Approximately 3 x 10^6 cells were homogenized in lysis buffer (Buffer RLT Plus). Total RNA and DNA were purified according to kit manual. Quantification and purity assessment of RNA and DNA samples were determined using a NanoDrop spectrophotometer (Thermo Scientific 2000c). The A_{260}/A_{280} ratios were expected as ~1.8 for

DNA and ~2.0 for RNA in purity assessment. The A_{260}/A_{230} purity ratio was also used for DNA within the range of 1.8-2.2. Extracted RNA and DNA samples were stored at -80 ^{0}C until further processing.

Cytosine methylation and hydroxymethylation in DNA and RNA

The isolated DNA/RNA (2 µg) was enzymatically hydrolyzed to individual nucleosides by a simple one-step hydrolysis procedure.[67] A digest mix was prepared by adding phosphodiesterase I, alkaline phosphatase and benzonase® Nuclease to Tris-HCl buffer. Extracted DNA / RNA was spiked with [15N3]-2′-deoxycytidine as internal standard, dried and then hydrolyzed at 37°C for at least 8 h in presence of 10 µL digest mix. After hydrolysis, 490µL ACN : H2O (90:8, v/v) was added to each sample and centrifuged 5 min at 6300 rpm. Supernatant were transferred to vial. In each digested DNA/RNA sample, both DNA and RNA (hydroxy) methylation at the C5 position of cytosine (m^5C and hm^5C) was determined using a HILIC-UPLC-MS/MS method that was previously published elsewhere.[23,24] Briefly, the analysis was conducted on a Waters Acquity UPLC, coupled to a Waters Micromass Quattro Premier Mass Spectrometer using electrospray ionization (ESI). A 20 µL aliquot was injected on a hydrophilic interaction liquid chromatography (HILIC) column (Phenomenex Kinetex 2.6 µm Hilic, 50 mm x 4.6 mm), held at 60°C. Chromatographic separation was achieved using as solvents 20mM ammonium formate buffer pH3 (A) and acetonitrile (B) following the next gradient: starting at 13% A, increasing linearly to 20% A from 0.1 to 2.2 min, then was held from 2.2 to 2.4 min at 20% A, brought back to the initial status from 2.4 to 2.6 min and finally allowed to stabilized for another minute before the following injection. A flow rate of 0.4 mL/min was applied. The analyses were performed using electrospray ionization (ESI) in positive mode and the compounds were determined using multiple reactions monitoring (MRM), with argon as the collision gas.

Global DNA/RNA methylation was determined as a percentage of m^5C versus the sum of m^5C, hm^5C and C, on the other hand, global DNA hydroxymethylation was determined as a percentage of hm^5C versus the sum of m^5C, hm^5C and C.

m^6A-RNA methylation analysis

The EpiQuik m^6A RNA Methylation Quantification Kit (Epigentek Group Inc., NY) provides to detect m^6A-RNA methylation status measuring by colorimetric ELISA-like assay. Analysis were performed according to kit manual. Briefly, 200 ng of isolated total RNA for each sample is bound to strip wells using RNA high binding solution. m^6A is detected using capture and detection antibodies. The detected signal is enhanced and then quantified colorimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer. After generating a standard curve with positive controls, the slope of the standard curve is determined by linear regression. The amount of m^6A-RNA is calculated from equation defined in the kit manual.

Sequence-specific DNA methylation measurements

Bisulfite Conversion and PCR

Bisulfite treatment was used for analysis of DNA methylation. The bisulfite conversion process is based on converting unmethylated cytosine residues to uracil while 5-methylcytosine residues stay unaffected. 200 ng of genomic DNA was converted using the EZ-96 DNA Methylation-Gold™ Kit (Shallow well format) (Zymo Research, USA) according to kit manual. Converted DNA was stored at -80 ^{0}C until used. Converted DNA was amplified by PCR in a final volume of 25 µl containing 0.2 µM of primers, 2x PyroMark PCR master mix, 10x CoralLoad and RNAse free water (PyroMark PCR kit, #978705, Qiagen).

The genes selected for the present study were based on the enriched pathways from our previous studies[25,46,62-64] and that presented by Mossman.[68] Primers for *CDKN1A*, *ATM*, and *TRAF2* were ordered from Qiagen (PyroMark CpG Assays, #PM00025711, #PM00153622, #PM00141309). PCR amplifications for the genes were performed with the following thermal profile: an initial

activation step of 15 min at 95^{0}C, 45 cycles of: 30 s at 94^{0}C, 30 s at 58^{0}C and 30s at 72^{0}C and a final elongation step at 72^{0}C for 10 min. Table 1 shows primer information of the genes.

Pyrosequencing

Pyrosequencing was performed using PyroMark Gold Q24 Reagents (#970802, Qiagen) on the PyroMarkQ24 instrument (Qiagen) according to manufacturer's instructions. Briefly, 20 µl of biotinylated-amplified PCR product was immobilized onto streptavidin sepharose high performance beads (GE Healthcare). Separation of biotinylated PCR strands from non-biotinylated strands was conducted using the vacuum workstation as indicated in the PyroMark user manual. Samples were transferred to a PyroMark Q24 plate, containing sequencing primer (0.3 µM), and following annealing (at 80^{0}C for 2 min, followed by 10 min cooling); plate was run for pyrosequencing analysis. Pyrosequencing results were analyzed using the PyroMark analysis 2.0.7 software (Qiagen).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism Software, version 7. Data was obtained from three independent experiments with duplicate per condition (n=3). Alterations in methylation levels (%) were expressed as mean ± SD. The differences in levels of global DNA methylation/hydroxymethylation or RNA methylation/hydroxymethylation of CNTs and asbestos were determined by repeated measures one-way ANOVA. DNA methylation changes at CpG islands of *CDKN1, ATM,* and *TRAF2* genes were analyzed using paired student-t test compared to the vehicle control. $P<0.05$ was considered to be statistically significant.

RESULTS

Global DNA Methylation and Hydroxymethylation

None of asbestos types affected global DNA methylation and hydroxymethylation levels when compared to vehicle control ($p>0.05$). SWCNTs did neither alter global DNA hydroxymethylation levels, whereas 25 and 100 μg/ml of MWCNTs induced significant dose-dependent DNA hypomethylation when compared to vehicle control ($p<0.05$). The results are shown in Fig. 1.

Global RNA methylation

RNA methylation was determined at adenine (m^6A-RNA) and cytosine (m^5C-RNA) bases. m^6A-RNA levels were not affected by amosite, crocidolite, chrysotile and SWCNTs ($p>0.05$). Only at the highest dose (100 μg/ml) of MWCNTs, significant RNA hypomethylation for adenine bases were observed (Fig. 2a). No changes were observed in global methylation levels of m^5C-RNA for asbestos and CNTs exposures.

Concerning the hydroxymethylation of cytosine in RNA (hm^5C-RNA), statistically significant differences were induced by 2.5 μg/ml chrysotile. There was a significant increased hydroxymethylation at 25 μg/ml of SWCNTs, which was borderline non-significant ($p=0.05$) at 100 μg/ml. MWCNTs at 100 μg/ml, also significantly increased the levels of hm^5C-RNA (Fig 2b).

Impact of asbestos and CNTs on gene specific DNA methylation

CpG #3 site methylation in *CDKN1A* gene was significantly lower in amosite exposed cells (2.04% ± 0.75) compared to vehicle controls (2.94% ± 0.47; $p<0.05$). Amosite also reduced significantly the CpG #4, #7, and total methylation levels. The other asbestos types did not induce differences in DNA methylation of the CpG sites of *CDKN1A* gene (Table 2). Exposure to MWCNTs (25 and 100 μg/ml) decreased the average methylation levels of *CDKN1A* gene.

On the other hand, 100 μg/ml of SWCNTs induced hypomethylation at CpG #4 site and average of the CpGs in *CDKN1A* gene.

Methylation levels of CpG #6 site and average of all CpGs in *ATM* gene were significantly reduced by chrysotile compared to vehicle control (Table 3). CpG #3 and CpG #6 sites of *ATM* gene were hypomethylated by 100 μg/ml of MWCNTs and 25 μg/ml of SWCNTs, respectively.

For *TRAF2* DNA methylation results, no differences were observed for the asbestos treatments. Instead, consistent increase in DNA methylation at all CpG sites except CpG #1 and #2 were observed in SWCNTs exposed cells (Table 4). Although for both 25 and 100 μg/ml concentrations of SWCNT significantly higher levels of DNA methylation were observed, no dose dependent differences were noticed ($p>0.05$). When MWCNTs exposures were assessed, an increase in DNA methylation at the CpG sites of *TRAF2* was observed, however was not statistically significant.

DISCUSSION

In the current study, we assessed methylation changes of DNA and RNA induced by carbon nanotubes and asbestos. We observed that MWCNTs significantly altered DNA methylation, resulting in global DNA hypomethylation. These results indicate that shape and characteristics of CNTs may have an influence in epigenetic effects. Different groups of CNTs have diverse geometries, structural properties and mechanical behaviour that might influence the interaction with biological tissues. For instance, the length of CNTs has been suggested as a critical geometric parameter for toxicity.[69] These findings are in line with numerous studies that have reported the high-aspect-ratio of CNTs can induce pulmonary toxicity.[70,71]

Decreased DNA methylations are consistent with the earlier report displaying global DNA hypomethylation in blood and lung of mice by MWCNTs (FA-21).[48] Sierra et. al. also

observed global DNA hypomethylation by MWCNTs (NM-401) in BEAS-2B) cells.[49] On the contrary, a previous report described unchanged global DNA methylation pattern in human monocytes and human bronchial epithelial cells.[46,62] Also, exposure to low and high concentrations of SWCNTs and MWCNTs (NM-400) in lung of mice did not affect m^5C-DNA and hm^5C-DNA levels.[45] Contrary to the studies showing unchanged and decreased DNA methylation, Li et al. showed an increase in global DNA methylation in A549 lung cells exposed to low-dose carbon-based nanoparticles (SWCNTs and short/long MWCNTs).[47] These differences might be explained by the fact that DNA methylations are cell-type and cell-state dependent. In addition, differences may be due to the used CNTs and their concentrations. In terms of asbestos, we did not find any statistically significant changes in global DNA methylation, consistent with our previous finding.[64] Results obtained from one epidemiological study showed that global DNA hypomethylation was observed in blood of asbestos-exposed workers compared to control group.[44] Recently, Kettunen et al. have performed genome-wide DNA methylation study to understand the effect of asbestos. They found that DNA hypomethylation was characteristic of lung cancer tissue from asbestos exposed individuals and same study has showed methylation in some of genes.[72] Inherent to epidemiological studies, the obtained biological materials from patients or workers that are more likely to be exposed to mixtures of several asbestos types as well as with other chemicals. Thus, in these studies, asbestos induced methylation effects were not attributed to a certain type of asbestos. In our in vitro study, cells were exposed to different asbestos types (amosite, chrysotile, and crocidolite) in a well-controlled milieu. In addition, cell type differences might affect the methylation levels considering response to asbestos of normal and cancer cells.

Assessment of DNA methylation in specific genes that constitutes the other part of the study, provides insight in toxicity mechanisms related to CNTs and asbestos. Cell cycle checkpoints and DNA repair systems maintains genome integrity. When genes involved in the

sustenance of genome integrity are inactivated, genome instability, cancer predisposition, and early aging might occur. In this study, we observed hypomethylation in cells exposed to chrysotile at averages methylation of all CpG sites in *ATM* gene. After exposure to chrysotile and 25 μg/ml of SWCNTs, CpG #6 sites also showed DNA hypomethylation. Recently, it was found that 16HBEo- cells exposed to MWCNTs showed differential methylation and expression on *ATM* gene while SWCNTs exposure showed downregulation of *ATM* gene.[63] In addition, Ghosh et al. observed that MWCNT-exposed workers have aberrant methylation in CpGs, belonging to the same region of *ATM* promoter.[25]

When DNA methylation levels in *CDKN1A* gene were examined, it was observed that amosite, SWCNTs, and MWCNTs lead to hypomethylation. Öner et al. also reported differential methylation and expression of *CDKN1A* for MWCNT exposure.[63] As *CDKN1A* gene promoter sites contain high density of CpG sites, gene transcription level is regulated by methylation. Therefore, hypomethylation in DNA of *CDKN1A* gene may accelerate its transcription level. Also, as we know that a wide range of stress factors induce *CDKN1A* expression[73], it might be assumed that amosite, SWCNTs and MWCNTs cause *CDKN1A* overexpression which can inhibit two critical cell cycle checkpoints; G1 and G2.[74] These processes can induce senescence through inhibition of cell proliferation in normal cells.

The other gene (*TRAF2)*, which was assessed for sequence specific methylation changes, is a critical member of the TRAF family. *TRAF2* dysregulation is reported in malignant mesothelioma cell lines.[75] In addition, dysregulation of *TRAF2* and associated pathways have been observed for asbestos induced apoptosis and mesothelioma.[51,68,76] In this study, SWCNTs caused DNA hypermethylation in *TRAF2* gene. This abnormal methylation could lead to decreased *TRAF2* gene expression and other proteins associated with *TRAF2*

signal pathways. Öner et al. also found that exposure to CNTs in 16HBEo- cells resulted in differential methylation (MWCNTs) and expression (SWCNT and MWCNTs) of *TRAF2*.[63]

One of the key goals of this study was to determine global RNA methylation levels for assessment of CNTs and asbestos exposures in human lung cells. Determination of global RNA methylation status is an emerging investigation area for toxicity mechanisms as a link between changes in RNA methylation and cancer has been demonstrated.[37] Therefore, we analyzed methylation in both adenine and cytosine bases in RNA. Our results revealed that CNTs and asbestos did not cause any significant changes of m^5C-RNA levels. In contrast, SWCNTs, MWCNTs and chrysotile exposure led to increased hm^5C-RNA. In most of organisms, ten-eleven translocation (tet) methyldioxygenases catalyzes the formation of hm^5C-RNA from m^5C-RNA. And it was thought that this dynamic m^5C metabolism in RNA might play important roles in RNA function.[77] Delatte et al. found that RNA hydroxymethylation can promotes mRNA translation.[78] Until now, reports have also showed that hm^5C is most abundant in brain tissue and affects transcriptional regulatory activity.[79] On the other hand, we found that high dose MWCNTs have induced hypomethylation in m^6A-RNA. These aberrant changes were consistent with DNA methylation results. Although knowledge on the function of m^6A-RNA is limited, it is now known that these modifications mediate post-transcriptional regulation of gene expression[32,80] and X-inactive specific transcript (*XIST*) mediated transcriptional silencing of genes on the X chromosome.[81] Relationship between m^6A-RNA modification and certain cancer related long noncoding RNAs has also been reported.[82] Furthermore, m^6A is believed to play key role in modulation of the p53 signaling pathway and apoptosis.[80]

Our results demonstrate a relatively acute response to CNTs and asbestos. However, the abrasion and weathering of consumer products containing CNTs are probably the main exposure source of the general population. In addition, biomedical devices might cause CNTs

exposure internally. As these exposures may display chronic pattern, our results might change in chronic human exposure scenario. *In vivo* and epidemiological studies are required to understand the epigenetic toxicity of CNTs further. Thus, we have been conducting further assessments on the animals to verify our hypothesis.

From our findings, asbestos and CNTs exposed bronchial epithelial cells have not shown similar epigenetics patterns. Only MWCNTs changed global DNA methylation status. In addition, CNTs and certain type of asbestos lead to sequence specific epigenetic changes in the *ATM* and *CDKN1A* genes. In addition, as far as we are aware, it is the first report to show changes in RNA methylation status in relation to these toxicant exposures. MWCNTs exposure resulted in an increase of m^6A-RNA level. These findings suggest a novel mechanism of action of CNTs, particularly with respect to RNA methylation and may contribute to the understanding of how CNTs exposure influences the etiology of carcinogenesis. Our findings provide further justification for determining causational link between nanomaterials exposure and epigenetics modifications.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ATM, serine/threonine kinase; *CDKN1A,* cyclin dependent kinase inhibitor 1A; CNTs, carbon nanotubes; hm^{5}C-DNA, 5-hydroxymethylcytosine in DNA; hm^{5}C-RNA, 5-hydroxymethylcytosine in RNA; m^{5}C-DNA, 5-methylcytosine in DNA; m^{6}A-RNA, N6-methyladenosine in RNA; m^{5}C-RNA, 5-methylcytosine in RNA; MWCNTs, multi-walled carbon nanotubes; SWCNTs, single-walled carbon nanotubes; *TRAF2,* TNF receptor associated factor 2.

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Table 1. Primer Information of *CDKN1A*, *ATM*, and *TRAF2* from Qiagen

Gene	Product Number	Sequence to Analyze (Chromosomal location)	Number of CpG Sites Included
CDKN1A	Hs_CDKN1A_03_PM (PM00025711)	CCRC**G**GC**G**GTTC**G**C**G**CC**G**GCCAGCCCCACTCC**G**C **G**GGA (Chr 6: 36,648,429-36,648,466)	7
ATM	Hs_NPAT/ATM_01_PM (PM00153622)	C**G**C**G**GAC**G**C**G**GGAWGGAGGGTTATTGGACCC**G**G C (Chr 11: 108,093,191-108,093,221)	5
TRAF2	Hs_TRAF2_02_PM (PM00141309)	C**G**CCCC**G**CTCC**G**AGC**G**C**G**CCTGAC**G**GA (Chr 9: 139,780,813-139,780,836)	6

Table 2. *CDKN1A* Specific DNA Methylation (%) Results for CNTs and Asbestos Treatments

CDKN1A	Vehicle Control	Amosite	Crocidolite	Chrysotile	SWCNT 25 µg/ml	SWCNT 100 µg/ml	MWCNT 25 µg/ml	MWCNT 100 µg/ml	Decitabine
CpG #2	3.71 ± 0.61	3.05 ± 0.79	3.82 ± 0.90	3.45 ± 1.43	3.88 ± 1.02	3.91 ± 0.83	2.92 ± 0.83	3.32 ± 1.14	**3.3 ± 0.53***
CpG #3	2.94 ± 0.47	**2.04 ± 0.75***	2.55 ± 1.03	2.30 ± 1.08	2.37 ± 0.48	2.48 ± 0.45	2.33 ± 0.30	2.31 ± 0.29	**1.74 ± 0.28***
CpG #4	2.78 ± 0.53	**2.22 ± 0.74***	3.00 ± 0.70	2.47 ± 0.68	2.80 ± 1.22	**2.12 ± 0.79***	2.30 ± 0.69	2.76 ± 1.31	2.26 ± 0.69
CpG #5	1.13 ± 0.36	1.22 ± 0.61	0.89 ± 0.22	1.15 ± 0.72	1.47 ± 0.91	1.31 ± 0.33	1.11 ± 0.41	1.13 ± 0.25	1.07 ± 0.40
CpG #6	2.08 ± 0.85	1.80 ± 0.55	2.16 ± 0.60	1.67 ± 0.57	2.25 ± 0.58	1.96 ± 0.37	1.34 ± 0.24	1.48 ± 0.68	1.62 ± 0.80
CpG #7	2.87 ± 0.53	**2.16 ± 0.26***	2.60 ± 0.78	2.23 ± 0.56	2.69 ± 0.57	2.47 ± 0.54	2.61 ± 0.41	2.75 ± 0.48	2.28 ± 0.22
CpG #8	0.61 ± 0.29	0.39 ± 0.03	0.48 ± 0.06	0.52 ± 0.20	0.61 ± 0.33	0.43 ± 0.09	0.71 ± 0.11	0.62 ± 0.12	0.51 ± 0.24
Average of CpGs	2.31 ± 0.32	**1.84 ± 0.39***	2.21 ± 0.43	1.97 ± 0.45	2.29 ± 0.55	**2.10 ± 0.22***	**1.90 ± 0.21***	**2.05 ± 0.33***	1.83 ± 0.30

Mean of % of methylation values of individual CpG sites and average of all CpG sites ± SD (*p<0.05 compared to the vehicle control with student t test)

Table 3. *ATM* Specific DNA Methylation Results for CNTs and Asbestos Treatments

ATM	Vehicle Control	Amosite	Crocidolite	Chrysotile	SWCNT 25 µg/ml	SWCNT 100 µg/ml	MWCNT 25 µg/ml	MWCNT 100 µg/ml	Decitabine
CpG #1	0.41 ± 0.12	0.49 ± 0.11	0.45 ± 0.08	0.60 ± 0.51	0.58 ± 0.43	0.69 ± 0.34	0.62 ± 0.49	0.61 ± 0.34	0.47 ± 0.25
CpG #2	0.46 ± 0.14	0.42 ± 0.19	0.39 ± 0.10	0.32 ± 0.05	0.44 ± 0.31	0.65 ± 0.32	0.56 ± 0.43	0.54 ± 0.22	0.55 ± 0.38
CpG #3	1.08 ± 0.50	1.03 ± 0.56	0.76 ± 0.47	0.58 ± 0.03	1.05 ± 0.64	0.75 ± 0.21	0.81 ± 0.58	**0.67 ± 0.28***	0.83 ± 0.51
CpG #4	0.84 ± 0.39	0.65 ± 0.11	0.56 ± 0.08	0.45 ± 0.13	1.03 ± 0.53	1.20 ± 0.50	0.88 ± 0.61	1.03 ± 0.58	0.49 ± 0.34
CpG #6	2.34 ± 0.38	1.78 ± 0.51	1.76 ± 0.45	**1.69 ± 0.26***	**1.85 ± 0.44***	1.83 ± 0.32	1.77 ± 0.93	1.88 ± 0.58	**1.56 ± 0.24***
Average of CpGs	1.03 ± 0.20	0.88 ± 0.17	0.78 ± 0.18	**0.73 ± 0.12***	0.99 ± 0.22	1.02 ± 0.23	0.93 ± 0.57	0.95 ± 0.28	**0.78 ± 0.14***

Mean of % of methylation values of individual CpG sites and average of the all CpG sites ± SD; (*p<0.05 compared to the vehicle control with student t test)

Table 4. *TRAF2* Specific DNA Methylation Results for CNTs and Asbestos Treatments

TRAF2	Vehicle Control	Amosite	Crocidolite	Chrysotile	SWCNT 25 µg/ml	SWCNT 100 µg/ml	MWCNT 25 µg/ml	MWCNT 100 µg/ml	Decitabine
CpG #1	0.57 ± 0.21	0.64 ± 0.67	0.98 ± 0.62	0.87 ± 0.35	0.68 ± 0.15	0.79 ± 0.58	0.55 ± 0.37	0.88 ± 0.78	0.45 ± 0.17
CpG #2	1.33 ± 0.59	0.96 ± 0.14	1.92 ± 0.99	1.83 ± 1.35	1.51 ± 0.54	1.43 ± 1.02	1.37 ± 0.24	2.25 ± 0.95	1.33 ± 0.66
CpG #3	3.74 ± 2.58	2.51 ± 1.14	4.19 ± 3.66	4.05 ± 3.57	**7.99 ± 3.23***	**8.31 ± 3.81***	4.94 ± 2.32	5.12 ± 2.56	3.57 ± 1.57
CpG #4	3.97 ± 3.65	2.61 ± 1.80	4.72 ± 5.06	4.94 ± 4.70	**9.33 ± 2.99***	**9.97 ± 5.05***	6.07 ± 2.74	5.56 ± 2.74	4.38 ± 2.32
CpG #5	2.28 ± 1.56	1.48 ± 0.89	3.07 ± 1.86	2.91 ± 1.69	**4.57 ± 1.03***	**4.80 ± 1.47***	3.39 ± 1.23	3.75 ± 1.51	2.52 ± 0.78
CpG #6	4.68 ± 3.23	3.22 ± 1.95	5.38 ± 3.84	5.45 ± 3.87	**9.66 ± 2.87***	**10.57 ± 3.99***	7.30 ± 3.03	7.54 ± 3.85	5.28 ± 1.85
Average of CpGs	2.76 ± 1.76	1.90 ± 1.02	3.38 ± 2.35	3.34 ± 2.18	**5.63 ± 1.68***	**5.98 ± 2.20***	3.94 ± 1.51	4.18 ± 1.72	2.92 ± 1.04

Mean of % of methylation values of individual CpG sites and average of the all CpG sites ± SD; (*p<0.05 compared to the vehicle control with student t test)

FIGURE LEGENDS

Figure 1. Global DNA methylation and hydroxymethylation results for CNTs and asbestos. m^5C-DNA / hm^5C-DNA measured by LC-MS/MS in 16HBE14o- cells after 24 h exposure (**p<0.05 compared to the vehicle control with repeated measures ANOVA*)

Figure 2. Global RNA methylation levels for CNTs and asbestos.

a) m^6A-RNA measured by EpiQuik m^6A RNA Methylation Quantification Kit (Epigentek) in 16HBE14o- cells after 24 h exposure (**p<0.05 compared to the vehicle control with repeated measures ANOVA*)

b) m^5C-RNA/hm^5C-RNA measured by LC-MS/MS in 16HBE14o- cells after 24 h exposure to 25 and 100 µg/ml of CNTs and 2.5 µg/ml of Asbestos (**p<0.05 compared to the vehicle control with repeated measures ANOVA*)

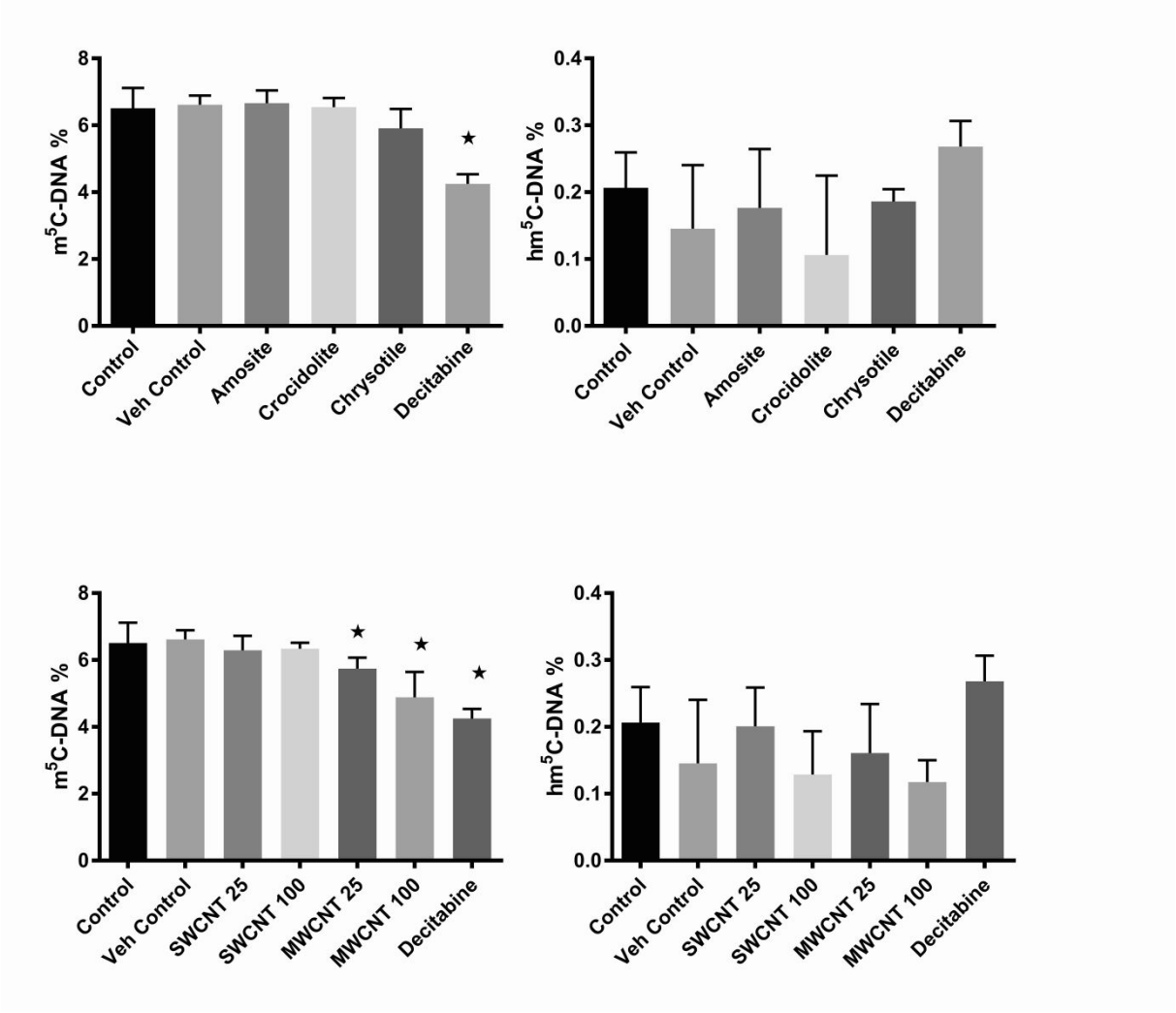
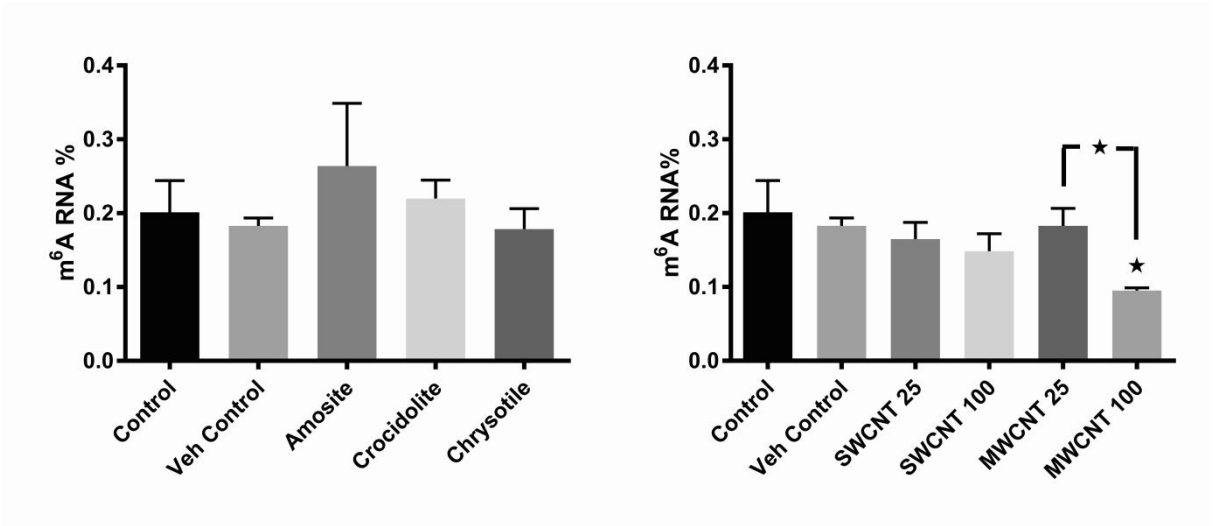
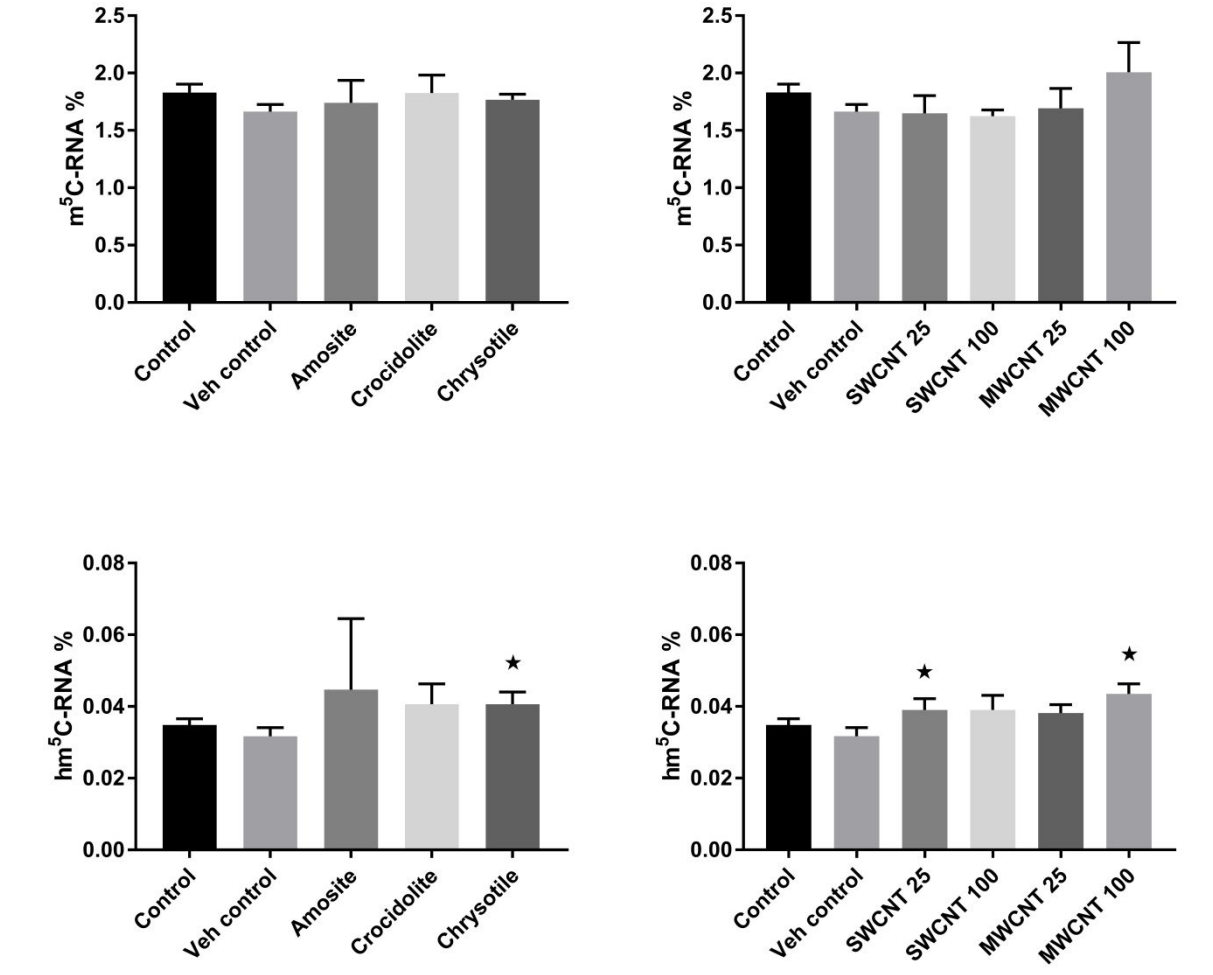


Figure 1.

2a)


m6A RNA %
0.4
0.3
0.2
0.1
0.0
Control
Veh Control
Amosite
Crocidolite
Chrysotile
m6A RNA%
★
★
Control
Veh Control
SWCNT 25
SWCNT 100
MWCNT 25
MWCNT 100

2b)


m5C-RNA %
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2.0
1.5
1.0
0.5
0.0
Control
Veh control
Amosite
Crocidolite
Chrysotile
Control
Veh control
SWCNT 25
SWCNT 100
MWCNT 25
MWCNT 100
hm5C-RNA %
0.08
0.06
0.04
0.02
0.00
★
★
★

Figure 2.