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Exposure to environmental toxicants reducesglobal N6-methyladenosine RNA methylation and alters expression of RNA methylation modulator genes

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Abstract

The epitranscriptome comprises more than 100 forms of RNA modifications. Of these, N6-methyladenosine (m^6A) is the most abundantform of RNA methylation, with roles in modulating mRNA transcript processing and regulation. The aims of the study weretoexamine changes inm^6A RNA methylation in A549 lung epithelial cells in response to environmental toxicants, anddifferential gene expression of m^6A modulator genes ('readers', 'writers' and 'erasers') in human subjects exposed toparticulate matter (PM) and in lung cancer tissueusing publicly-available microarray datasets.Global m^6A methylation levelsweremeasured in total RNA after exposuretotwo carcinogens (PM and sodium arsenite) for 24- and 48-hours, and totwo endocrine disruptors (bisphenol A and vinclozolin)for 24-hours.Global m^6A methylation level significantly decreased with exposure to >62 µg/mlPM, >1 µM sodium arsenite, >1µM bisphenol A (BPA), and0.1µM vinclozolin.In an analysis of a published dataset derived from a population study, we observed that m^6A writers (*METTL3* and *WTAP*), erasers (*FTO* and *ALKBH5*) and readers (*HNRPC*) showed significantly higher expression among participants in the high-$PM_{2.5}$exposure group compared to those in the low-exposure control group(all $p<0.05$).Further, the m^6A writer *METTL3*shows reduced expression in lung tumors in comparison to normal lung epithelia ($p<0.0001$).Our findings reveal that m^6A RNA methylation can be modified by exposure to environmental toxicants, and exposure to particulate matter is associated with differential expression level of m^6A RNA methylation modification machinery.

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Abbreviations

m^6A: N6-methyladenosine, 5mC: 5-methylcytidine, 5hmC: 5-hydroxylmethylcytidine, mRNA: messenger RNA, rRNA: ribosomal RNA, tRNA: transfer RNAs, snoRNA: small nucleolar RNAs, A549: Human adenocarcinomic human alveolar basal epithelial, BPA: Bisphenol A, PM: Particulate Matter, UTRs: Untranslated regions, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, SDS: Sodium dodecyl sulfate, HCl: Hydrochloric Acid

Keywords: RNA methylation, m^6A, particulate matter, environmental exposure.

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1. Introduction

The epitranscriptome refers to the modification of messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and small nucleolar RNAs (snoRNAs). Since their first discovery in 1957(Davis and Allen, 1957), more than 100 different RNA modifications have been reported in a range of organisms(Gilbert et al., 2016). Several modifications of mRNAs in eukaryotic organisms have been reported, including 6-methyladenosine (m^6A), N6,2'-O-dimethyladenosine, 5-methylcytidine (5mC), 5-hydroxylmethylcytidine (5hmC), inosine, pseudouridine and N1-methyladenosine (Dominissini et al., 2012; Li et al., 2017; Meyer et al., 2012; Squires et al., 2012). There isevidence pointing to a critical role of RNA modifications in tRNA stability (Alexandrov et al., 2006; Motorin and Helm, 2010), cellular stress responses (Begley et al., 2007; Netzer et al., 2009; Thompson and Parker, 2009), regulation of cell growth (Emilsson et al., 1992). and post-transcriptional regulation of gene expression through modulation of alternative pre-mRNA splicing, 3'-end processing, nuclear export, translation, and mRNA decay (Roignant and Soller, 2017).

The m^6A modification is the most common form of RNA methylation, accounting for more than 80% of all RNA modifications(Niu et al., 2013). It is particularly abundant atstop codons, 3′ untranslated regions (3′UTRs) and within internal long exons in mRNA transcripts(Meyer et al., 2012; Perry and Kelley, 1974), and more than 7000 mRNAs and 300 non-coding RNA transcriptshave been identified as containing m^6A in human cells (Dominissini et al., 2012). This modificationserves to modulate transcript stability, splicing, export, and translation, and thereby is implicated in the control of tissue development, circadian rhythms, and DNA damage response (Deng et al., 2018; Fustin et al., 2013), and serves as an important regulatory mechanism in embryonic stem cell maintenance(Wang et al., 2014). It is most abundant in the brain (Meyer et al., 2012; Perry and Kelley, 1974)where

it serves in theregulation of neurogenesis(Yoon et al., 2017) and behavioural adaptation(Widagdo et al., 2016). Furthermore, m^6A is the first modification of RNA that has been characterizedas being modified by 'readers', 'writers' and 'erasers'.The 'writer' methyltransferase enzymes (*METTL3*, *METTL14*, *WTAP*, and *KIAA1429*) add a methyl group to the 6^{th}position of adenosine in RNA, which can be recognized and utilized by 'reader' proteins (*HNRNPC, HNRNPA2B1, YTHDF2, YTHDF1,* and *eIF3*) and removed by 'eraser' enzymes (*FTO/ALKBH9* and *ALKBH5*) (Cao et al., 2016). RNA modification machineries are therefore directly involved in the regulation of biological functions (Gilbert et al., 2016), such as *WTAP* in mRNA splicing (Little et al., 2000; Ping et al., 2014; Zheng et al., 2013), *YTHDF1* and *eIF3* in translation (Wang et al., 2015)(Meyer et al., 2015), and *YTHDF2* in mRNA decay (Cao et al., 2016).Subsequently, disruption of their normal function has implications for human health, withmutations in m^6A RNA methylation machinery genes associated with obesity (Dina et al., 2007; Frayling et al., 2007; Scuteri et al., 2007) and neurological diseases (McGuinness and McGuinness, 2014). Furthermore, alterations in normal RNA modification patterns have been reported in a range of cancers (Cui et al., 2017; Geula et al., 2015; Jaffrey and Kharas, 2017; Wang et al., 2017). However, to date there has been no study on whether these RNA epigenetic marks are modifiable through exposure to environmental toxicants, such as carcinogens and endocrine disruptor chemicals. The role of m^6A in tissue development and its disruption in cancers may imply an effect of such toxicants, potentially through altered expression of RNA methylation modulators through oxidative stress-associated pathways.

In this study, we examined the effect of environmental toxicants upon m^6A RNA modifications and the expression ofm^6A modulator genes (writers, erasers, and readers). We analyzed RNA m^6A methylation level in human lung epithelial cells after exposure to two

carcinogens (particulate matter (PM) and sodium arsenite) and two endocrine disruptor chemicals (bisphenol A (BPA) and vinclozolin). These four represent common toxicants with key relevance to public health.Air pollution is a global health problem linked with many diseases including lung cancer (Vineis et al., 2006), chronic obstructive pulmonary disease (Faustini et al., 2013), asthma (Kelly and Fussell, 2011), and cardiovascular diseases (Watson, 2006). $PM_{2.5}$ exposure is associated with increased risk of lung cancer(Hamra et al., 2014) and lung cancer-associated mortality(Turner et al., 2011), and has been shown to impact upon the epigenome(Byun et al., 2013).Along with PM, arsenic is classified as a carcinogen to humans by the International Agency for Research on Cancer (IARC) and is associated with lung cancer, skin and bladder cancers (IARC, 2004). BPAis associated with the development of type 2 diabetes (Shankar and Teppala, 2011), cardiovascular diseases (Lang et al., 2008), cancer (Hiroi et al., 2004), reproductive disorders (Galloway et al., 2010), birth defects, developmental disorders (Bloom et al., 2011; Chou et al., 2011; Miao et al., 2011), and other chronic disorders (Rezg et al., 2014). Vinclozolin is a pesticide that has been implicated in disorders of the male reproductive system through affecting sperm production (Stouder and Paoloni-Giacobino, 2010).There has been particular interest in vinclozolin on account of evidence from animal models of apparent transgenerational inheritance of both exposure-associated aberrant DNA methylation marks (Nilsson et al., 2018) and increased retention of histone proteins associated with sperm DNA that are normally replaced by protamines during spermatogenesis(Ben Maamar et al., 2018).Such studies may offer mechanistic insight into transgenerational inheritance of disease or phenotypes followingtransient exposure of ancestral generations, such as altered development and functionof the kidneys and testes (Anway et al., 2006; Uzumcu et al., 2004).We selected PM exposure for further analysis by examining the expression of RNA methylation modulator genes in a published dataset derived from a population study with high levels of PM exposure and in lung tumor tissue, using

publicly-available gene expression microarray datasets. Our findings reveal that exposure to these four carcinogens and endocrine disruptor chemicals decrease global m^6A levels, and PM exposure is associated with differential expression of RNA methylation modulator genes.

2. Methods

2.1. Exposure of A549 cells to environmental toxicants

The A549 adenocarcinomic human alveolar basal epithelial cell line was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were exposed to PM (15-500 µg/ml) and sodium arsenite($NaAsO_2$,0.25-50µM)for 24- or 48-hours, and exposed to BPA (0.01-50 µM) and vinclozolin (0.1-100 µM) for 24-hours. The PM mixture was purchased as a standard reference material (1648a) from the National Institute of Standard Materials (NIST, Gaithersburg, Maryland, USA). The 1648a mixture is atmospheric particulate matter collected in an urban area that is intended to provide a standardized example of the atmospheric particulate samples obtained from industrialized urban areas, including respirable materials [polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), 16 nitro-PAHs, and pesticides], lead, and inorganic metals. Sodium arsenite was purchased from ChemService (West Chester, PA, USA), while BPA (98.5% purity) and vinclozolin (99.5% purity) were purchased from LGC Standards (DRE-C10655500 and DRE-C17920000;Teddington, UK).PM was suspended and sodium arsenite were dissolved in cell culture medium while BPA and vinclozolin were dissolved in DMSO. Untreated control cells were exposed to an equal volume of cell culture medium or DMSO (as appropriate) as the treated cells.The range of concentrations of the toxicantswere initially identified through the literature before experimental testing, and all concentrations used were

in line with other published studies (Gawda et al., 2018; Jiang et al., 2013; Sanderson et al., 2002; Song et al., 2017). All experiments were performed in duplicate.

2.2. Cell cytotoxicity assays

Cell viability was determined by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay, using the Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 10^4 cells were seeded into each well, washed after 24hours, and the toxicants added. Cells were exposed to each toxicant dosage in duplicate, with the MTT assay then performed in triplicate for each biological replicate. Cell viability was calculated by optical density (OD) as follows:(OD treated well [−blank]) / (OD control well [−blank]) × 100.

2.3. RNA m^6A methylation measurement

Global RNA m^6A methylation was quantified using the EpiQuik™ m^6A RNA Methylation Quantification Kit (# P-9005, Epigentek, Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, total RNA (100 ng) wasbound to each well and detected via a two-antibody colorimetric system. m^6Aabundance was quantified by measuring the absorbance at 450nm, with the m^6A methylation level proportional to the OD intensity measurement. Samples were analyzedin triplicate with standard curves used for relative quantification of m^6A methylation levels, calculated as below:

m^6A %= ((Sample OD – Negative Control OD)/S)/((Positive Control OD – Negative Control OD)/P) x100. S is the amount of input sample RNA in nanogram. P is the amount of input positive control in nanogram.

2.4. Publicly-available gene expression microarray datasets

To examine the effect of PM exposure upon the expression of RNA methylation modulator genes, gene expression microarray data from a study of chronic PM exposure (GSE60767)(Rossner et al., 2015) was utilized. Briefly, blood samples were obtained from healthy male non-smokers aged 22-63 who were residents of Ostrava (high-$PM_{2.5}$;133 participants) or Prague(control; 49 participants) at 1-3 timepoints in winter 2010. Personal PM exposure levels were calculated using data from samplers worn by the participants. The personal $PM_{2.5}$exposure levels inthe high exposuregroup ranged from 6.0 to119.0 $\mu g/m^3$, while in the control group they ranged from 7.6 to 55.1 $\mu g/m^3$.

To examine the same genes in malignancies of the lung, we utilized publicly available gene expression data from lung adenocarcinoma (LUAD),squamous cell carcinoma (LUSC)andnormal lung epithelial tissue from the Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) (Carithers et al., 2015; Tang et al., 2017). A total of 483 samples of LUAD, 347 LUAD-paired adjacent normal tissues, 486 samples of LUSC and 338 LUSC-paired adjacent normal tissues were analyzed.

2.5. Data analysis

Analyses of data obtained after exposure of A549 cells to environmental toxicantswere performed by one-way ANOVA, with Bonferroni's multiple comparison post-hoc tests. Statistical significance was defined as $p<0.05$. We applied the Benjamini& Hochberg correction for false discovery rate (FDR) method to adjust p values using the Limma (Linear Models for Microarray Analysis) R package to analyse gene expression microarray data from a study of chronic PM exposure (GSE60767). Considering the different stratifications of sex, age, ethnicity in tumor and normal samples, multivariable analysis, (MANOVA) was applied using sex, age, ethnicity and diseases state (tumor or normal) as variables for calculating

differential analysis using the TCGA and GTEx data using GEPIA which is a newly developed interactive web server for analyzing the RNA sequencing expression data of TCGA and the GTEx projects.

Gene expression~sex + age + ethnicity + diseases state

The expression data was first $\log_2$ (TPM+1) transformed and the $\log_2$FC was defined as median (tumor)-median (normal). The Benjamini&Hochberg FDR method was used to adjust the pvalues (Tang et al., 2017).Data analyses were performed using GraphPad Prism (version 5.02, GraphPad Software Inc.) and R (version 3.4.4).

3. Results

3.1. Alterations in m^{6}A RNA methylationfollowing exposure to carcinogens

Global m^{6}A RNA methylation levels were measured in adenocarcinomic human alveolar basal epithelial (A549) cells after exposure to PM (0-500 µg/ml) and sodium arsenite (0-50 µM) for 24h and 48h. Exposure to PM at concentrations of >62 µg/ml induced significant changes in m^{6}A methylation, decreasing from 100% in unexposed cells to between 46% (62 µg/ml) and 31% (500 µg/ml) following 24h of exposure (Fig. 1A). The same pattern was observed after 48h of exposure, with significant decreases following exposure to 62, 125, 250 and 500 µg/ml PM. A dose-response effect was observed, with m^{6}A levels significantly and negatively decrease with PM concentration (R^2=-0.56, $p<0.05$ for 24h exposure).No dose-effect was observed for 48h exposure ($p >0.05$, linear regression analysis).

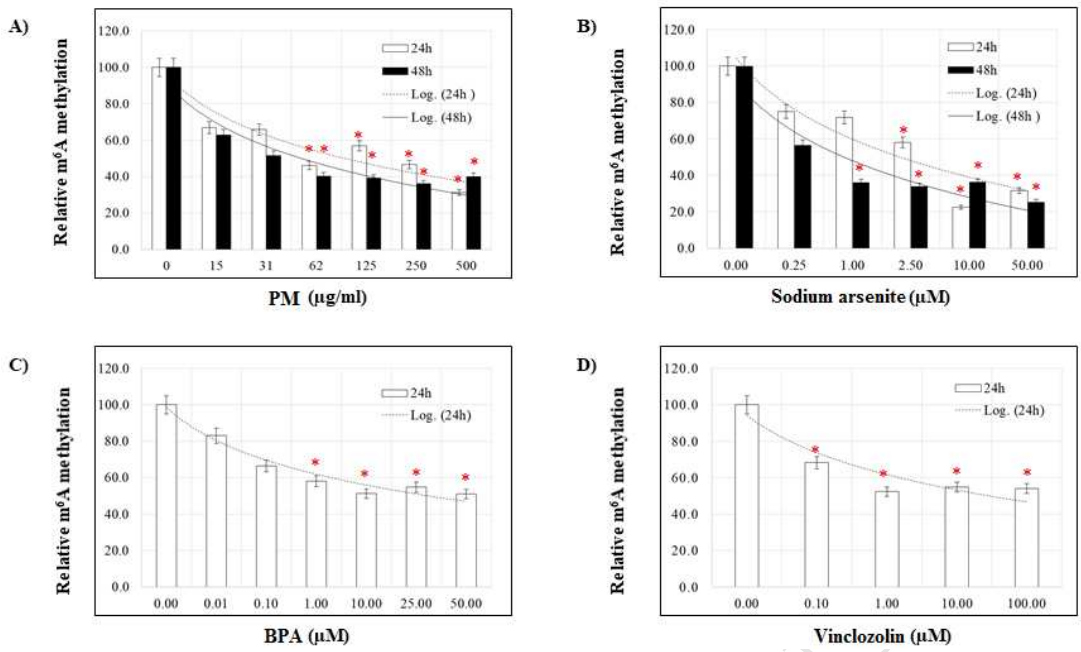

Figure 1. RNA m^6A methylation levels following exposure to PM (A), and sodium arsenite (B) for 24h and 48h, and to bisphenol A (C) and vinclozolin (D) for 24h (Mean±SD).*=$p<0.05$

Exposure to >2.5µM sodium arsenite for 24h significantly decreased m^6A methylation levels to between 58% (2.5µM) and 23% (10µM) of levels observed in untreated cells (Fig. 1B). Exposure to 1.0, 2.5, 10 and 50µM for 48h significantly decreased m^6A levels to 25-36% of normal levels. Linear regression analysis results showed that there was no dose□dependent decrease in the m^6A levels ($p>0.05$)

3.2. Alterations in RNA m^6A methylation following exposure to endocrine disruptor chemicals

Global m^6A RNA methylation levels were measured in A549 cells afterexposureto BPA and vinclozolin for 24h at concentrations of 0-50 µM and 0-100 µM, respectively.Exposure to >0.1 µM BPA resulted in significantly decreased m^6A methylation levels, from 100% in unexposed cells to 51% in those exposed to 50 µM BPA. No dose-effect was observed for 24h

exposure (p >0.05, linear regression analysis) (Fig. 1C). Similarly, exposure to vinclozolin at all concentrations (0.1-100 µM) resulted in significantly decreased m^6A methylation levels (Fig. 1D).The greatest reduction was observed with 1µM vinclozolin, which decreased m^6A levels to 52% of that observed in untreated cells. However, no dose-effect was observed(p >0.05, linear regression analysis).

3.3. Cell viability in responseto exposure to carcinogens and endocrine disruptor chemicals

Cell viability remained high with the lower concentrations of toxicants used, but was substantially reduced at the highest levels of exposure. The level of PM exposuresfor 24h and 48h were negatively associated with cell viability (r=-0.98, p =0.005 and r=-1.0, p =0.0001, respectively;Spearman's rho)(Fig. 2A). Similarly, the level of exposure to sodium arsenite was also negatively associated with cell viability at 24 (r=-0.94, p=0.005) and 48h (r=0.94, p =0.005) (Fig. 2B), as was exposure to BPA (r=-1.0, p =0.01) and vinclozolin for 24h (r=-1.0, p =0.01)(Fig. 2C and D).

Figure 2

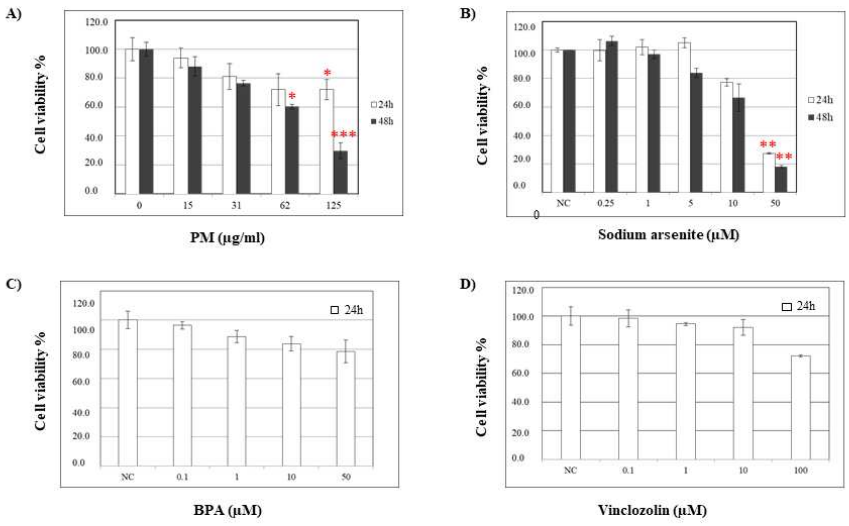

Figure 2. Cell viability following PM (A), and sodium arsenite (B) treatment for 24h and 48h, bisphenol A (C) and vinclozolin (D) treatment for 24h (Mean±SD).*=p<0.05, **=p<0.01, ***=p<0.001

3.4. Expression of RNA methylation modulator genes in in response to PM exposure

The expression of RNA methylation modulator genes (readers, writers and erasers) were analyzed in a published dataset derived from a population study of human participants with high and low exposure to fine PM (PM diameter >2.5 microns; $PM_{2.5}$). Expression of the writers`*METTL3* and *WTAP* were 1.27- and 2.11-fold higher in the high-$PM_{2.5}$ group in comparison to the control group (p<0.05 for *METTL3* and p<0.01 for *WTAP*; Fig. 3A and B). Similarly, expression of the erasers *FTO* and *ALKBH5* were 1.31- and 1.29-fold higher in the high-PM2.5 group in comparison to the control group (p<0.05 for *FTO* and p<0.001 for *ALKBH5*; Fig. 3C and D), while the reader gene *HNRNPC* was 1.52-fold higher in the high-$PM_{2.5}$ group (p<0.05; Fig. 3E). However, expression of the writers *METTL14* and *KIAA1429* showed no significant difference between the groups (data not shown).

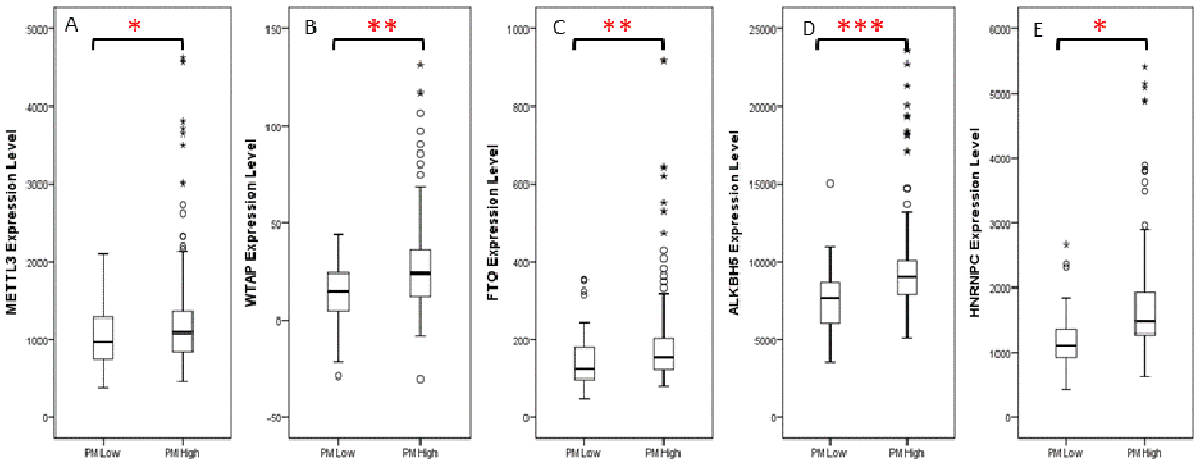

Figure 3. Expression of RNA methylation modulator genes by PM exposure. Expression of the *METTL3* (A), *WTAP* (B), *FTO* (C), *ALKBH5* (D), and *HNRHPC* (E) genes in subjects

exposed to high levels of $PM_{2.5}$ ('PM High') and controls ('PM Low'). * = p<0.05; *** = p<0.01**; p<0.001.

3.5. Expression of RNA methylation modulator genes in lung cancer patients

To investigate the relevance of PM exposure-associated changes inm^6A RNA methylationand RNA methylation modulator gene expressionto the development of malignancies, we analyzed the expression of the RNA methylation writers, erasers and readers in publicly-available LUAD and LUSC expression microarray datasets(Carithers et al., 2015; Tang et al., 2017). Our analysis revealedthat expression of the m^6A writer*METTL3*was 2.1-fold lower in patients with LUAD (*n*=483) than in normal lung tissues (*n*=347) (24.0 and 50.5 Transcripts Per Million; TPM, respectively)(ANOVA, p<0.0001)(Fig. 4A). In patients with LUSC (*n*=486), *METTL3* expression was 2.4-fold lower than in normal lung tissues (*n*=338) (21.3 and 50.8 TPM, respectively)(ANOVA, p<0.0001)(Fig. 4B).With the exception of*METTL3*, wedid not identifysignificantly differentexpression ofany other m^6A RNA methylation modulatorgenes in either LUAD or LUSC in comparison to normal tissue.

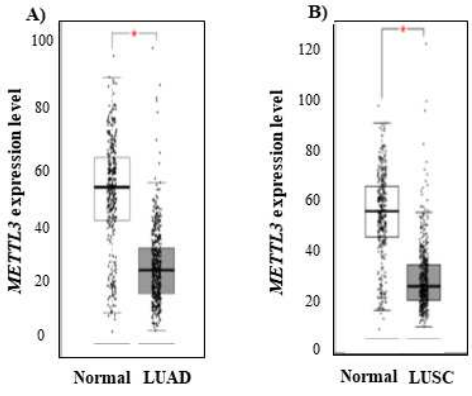

Figure 4. Expression of *METTL3*inlung adenocarcinomas(A; LUAD) and squamous cell carcinomas (B; LUSC)in comparison to normal tissues.* = p<0.05; ** = p<0.0001.

4. Discussion

In this study, we observedthat global m^6A RNA methylation decreased after exposure to carcinogens and endocrine disruptor chemicals in a lung adenocarcinoma cell line. Concordantly, human subjectsexposed to high levels of $PM_{2.5}$showed significantly higher expression of m^6A methylation erasers.Further, we found that them^6A writer gene*METTL3*is more highly expressed in lung tumors than in normal lung epithelia.Together, our findings indicate that exposure to endocrine disruptors and carcinogens are associated with dysregulation of normal m^6A RNA methylation marks. To the best of our knowledge, our study isthe first to identify alterations in m^6A RNA methylationin response to environmental exposures.

Our observation ofdecreasedm^6A following exposure to environmental toxicants is concordant with other studies that have identified alterations in the expression of RNA methylationwriterand eraser genes in response to exposure. Arsenic exposure is associated with decreased expression of the erasers*METTL3*(Zheng et al., 2005), and *WTAP*(Clancy et al., 2012), while expression of readers such as *HNRNPC*, *HNRNPA2B1*, and *YTHDF2*are also affected(Liu et al., 2010; Mattingly et al., 2009; Udensi et al., 2014; Wang et al., 2003). Endocrine disruptor chemicals have similarly been shown to affect the expression of RNA methylation machinery genes including *METTL3*(Ali et al., 2014), *METTL14*, *ALKBH5*(Villeneuve et al., 2011), *WTAP*, *YTHDF1*(Ali et al., 2014; Villeneuve et al., 2011), *FTO*(Ali et al., 2014; Tait et al., 2015), *HNRNPC*, *HNRNPA2B1*(Ali et al., 2014; Guerrero-Bosagna et al., 2013; Ljunggren et al., 2016; Nilsson et al., 2012; Skinner et al., 2008) and *YTHDF2*(Ali et al., 2014; Verbanck et al., 2017). Such observations are supported by evidence for the epigenetic regulation of these genes changing following environmental exposures, including to arsenic (Rojas et al., 2014). In our study, we similarlyobserved

increased expression of m^6Aerasers (*FTO* and *ALKBH5*) with high levels of $PM_{2.5}$exposure,thereby supporting our *in vitro* observation of decreased global m^6A following exposure. Interestingly, we also observed decreasedexpression of the writer *METTL3* in lung tumors,supporting a role for m^6A dysregulation in lung carcinogenesis that could produce disrupted regulation of mRNA transcript turnover.

However, our finding of decreased m^6A RNA methylation following short-term exposure to arsenite is in contrast to findings elsewhere of increased m^6A in bronchial epithelial cells exposed to arsenite for 13 weeks (Gu et al., 2018) and a neuronal cell line exposed for six months(Bai et al., 2018). Neurons display altered sensitivity to arsenite by differentiation status in an*YTHDF2*-mediated manner(Li et al., 2018), which may therefore imply a cell type-specific effect of arsenite exposure upon RNA methylation, which we speculate may be the basis for the discrepancy in our observations.Nonetheless, with the field of epitranscriptomics at a nascent stage, further studies are required to examine the interaction between RNA methylation modulators and response to environmental exposures by tissue type, in conjunction with longitudinal studies to examine the dynamics of m^6A in response to chronic exposures.Furthermore, findings using *in vitro* and *in vivo* models require validation in human population studies, as there are inherent difficulties in the extrapolation of observations in such models to the 'real world' effects of environmental exposures upon humans, not least in regard to accurately modelling dosage.

Decreased global m^6A RNA methylation has been reported in cancers and type 2 diabetes (Shen et al., 2015) among others, and it is frequently associated with alterations in the expression or functions of RNA methylation readers, writers and erasers (He et al., 2018). In concordance with this, we have identified decreased global m^6A and increased expression of

the writers, erasers, and readers in response to PM exposure, and decreased expression of the writer*METTL3* in lung tumors. Such alterations may result in expansive changes in the epitranscriptome, and the subsequent dysregulation of mRNA processing is likely to have wide-ranging effects upon normal cellular processes, which cannot be easily predicted.Importantly, dysregulation of *FTO* promotes lung squamous cell carcinoma via decreased m^6A RNA methylation, in part through decreased stability of tumor-suppressing *MZF1* transcripts (Liu et al., 2018)while conversely *METTL3* promotes the translation of oncogenes (Ljunggren et al., 2016). Our findings may therefore support a role for m^6A dysregulation in promoting PM-associated transformation of lung epithelia.

We note that cell viability decreased markedly at the highest level of toxicant exposures. Mitkus *et al* (Mitkus et al., 2013) reported highly similar findings of a marked decrease in cell viability (<75%) with 24-hour exposure to >62 µg/ml $PM_{2.5}$ (standard 1648a) in the A549 lung adenocarcinoma cell line, and A549 cells are also highly sensitive to sodium arsenite exposures of >20 µM(Jiang et al., 2013). Importantly, we observed significant changes in global m^6A content at doses that did not affect cell viability(62 µg/ml PM for 24h, 1-10 µM sodiumarsenite, all concentrations of BPA and vinclozolin), thereby supporting the hypothesis that changes in the epitranscriptome are not the product of cell death. Indeed, changes in m^6A may in fact lead to alterations in the processing and turnover of mRNA transcripts that regulate apoptosis, as has been observed in response to arsenite exposure(Gu et al., 2018).

We acknowledge several limitations to our study. Firstly, we could not measure the expression of the RNA methylation modulator genes in the exposed cell line that could have helped to elucidate the mechanisms by which environmental toxicants affect m^6A levels. Secondly, we measured total m^6A levels in response to exposure and we were therefore

unable to identify transcript-specific changes that may again provide important insight into dysregulation of key cell processes. This work and the cell viability assays were also performed in duplicate, rather than triplicate, which may have inhibited the obtainment of statistically significant results. Further, it should be noted that the tissues differed between our*in vitro* study (lung epithelial cells) and the human PM study (leukocytes), and the effect of PM exposure and inherent expression of RNA methylation modulators may differ between these. Although we identified complementary effects (that is, loss of m^6A methylation and increased expression of m^6A methylation erasers), which may support the use of blood as a surrogate tissue to study the effects of PM exposure, our observations required validation in further studies.Finally, our *in vitro* system utilized a lung adenocarcinoma cell line. Therefore, while directly relevant to the effects of PM exposure, caution must be taken in extrapolating our results to healthy human tissues.

5. Conclusions

In summary, our study has revealed thatm^6A RNA methylation is modified by exposure to carcinogens and endocrine disruptor chemicals. In addition,exposure to particulate matter is associated with differential expression of m^6A RNA methylation machinery, which mayprovide insight intothe mechanisms by which m^6A is altered. Further work is required in population studies to validate our *in vitro* observations and to examine the impact of m^6A alterations in the mediation of environmental exposures and development of disease.

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Authors' contributions

AC designed and performed the experiments and analyzed the data. TB, LG and HMB aided in the analysis and interpretation of the data and provided project guidance. AC and TMB wrote the manuscript, which HMB and LG revised. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Highlights

- Alterations in m^6A RNA methylation in A549 cells with environmental exposures.
- Global m^6Adecreasedwithparticulate matter, arsenite, BPA & vinclozolin exposure.
- Expression of *METTL3*, *WTAP*, *FTO*, *ALKBH5*, and *HNRPC* increased with particulate matter exposure.
- Expression of the m^6A writer*METTL3* is lower in lung tumours than normal tissue.

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