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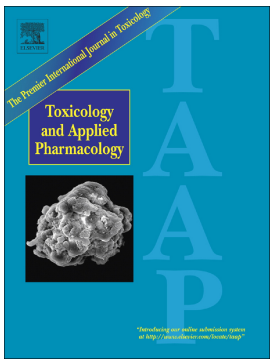

Effects of heated tobacco product aerosol extracts on DNA methylation and gene transcription in lung epithelial cells

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Effects of heated tobacco product aerosol extracts on DNA methylation and gene transcription in lung epithelial cells

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Abbreviations: 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine; CDKN1A, cyclin-dependent kinase inhibitor 1A; CGI, CpG island; CS, Cigarette smoking; CYP1A1, cytochrome P450 family 1 subfamily A member 1; DKK1, dickkopf WNT signaling pathway inhibitor 1; DMC, differentially methylated cytosine; DMR, differentially methylated region; DNAm, DNA methylation; DNMT, DNA methyltransferase; GDF15, growth differentiation factor 15; HMOX1, heme oxygenase 1; HTP, heated tobacco product; IL-6, interleukin 6; LDH, lactate dehydrogenase; PM, particulate matter; RASSF9, Ras association (RalGDS/AF-6) domain family (N-terminal) member 9; RC, reference cigarette; RRBS, reduced representation of bisulfite

sequencing; TET, ten-eleven translocation; TGFB2, transforming growth factor beta 2; TPM, total particulate matter; VEGFA, vascular endothelial growth factor A.

Abstract

Aims: Smoking causes DNA methylation (DNAm) alterations that lead to lung cancer development. Although the use of heated tobacco products (HTPs) has recently increased, their impact on health remains unclear. This study aimed to evaluate the effects of HTPs on DNAm and gene transcription in human lung epithelial cells *in vitro*.

Main methods: Human lung adenocarcinoma (A549) cells with type II alveolar epithelial characteristics were treated with aerosol extracts of two HTPs or a smoke extract of combustible reference cigarette (RC). Global 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) levels were quantified using dot blot analysis. Furthermore, reduced representation of bisulfite sequencing, DNA microarray, and quantitative PCR analyses were performed to determine CpG methylation and gene transcription changes induced by HTP and RC.

Key findings: Global 5-mC and 5-hmC levels were decreased by the RC extract but not the HTP extracts. However, an HTP extract altered the CpG methylation pattern, and Gene Ontology enrichment analysis of the differentially methylated regions of the RC and HTP groups showed a similar pattern. The HTP extract affected gene expression, albeit to a lesser extent than the RC extract. In particular, the HTP extract markedly affected the mRNA expression and promoter

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methylation of cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*), which is associated with carcinogenic risk.

Significance: The study results suggest that HTPs as well as conventional combustible cigarettes can alter CpG methylation and gene transcription in lung epithelial cells.

Keywords: Heated tobacco products, Cigarette smoking, DNA methylation, RRBS, CYP1A1

Introduction

Lung adenocarcinoma is the leading cause of cancer-related deaths worldwide [1]. Cigarette smoking (CS) is the most preventable cause of such pulmonary diseases, and its exposure is associated with alterations in DNA methylation (DNAm) [2]. DNAm plays a critical role in the regulation of gene expression by controlling the accessibility of DNA to the transcription machinery. DNA methyltransferases (DNMTs) are enzymes involved in the DNAm processes. DNMTs add a methyl group to the cytosine nucleotide in DNA, whereas the ten-eleven translocation (TET) family of DNA demethylation enzymes converts 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC).

Aberrant DNAm patterns are thought to silence tumor suppressor genes, activate proto-oncogenes, and decrease chromosomal stability [3]. Clinical studies have reported that CS-associated DNAm markers can predict lung cancer incidence [4, 5]. Changes in DNAm following CS exposure have been extensively documented for numerous genes, including aryl

hydrocarbon receptor repressor (*AHRR*), coagulation factor II receptor-like 3 (*F2RL3*), death-associated protein kinase (*DAPK*), and cyclin-dependent kinase inhibitor 2A (*p16*) [2]. In addition, it has been suggested that cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*) is highly expressed via promoter methylation alterations induced by CS in the lungs [6, 7]. CYP1A1 is involved in the metabolic activation of polycyclic aromatic hydrocarbons into mutagenic and carcinogenic derivatives that bind to DNA and subsequently induce neoplastic transformation [8]. Along with CS, particulate matter (PM) air pollution has also been associated with genome-wide differences in DNAm in lung and bronchial epithelial cells [9, 10]. Thus, DNAm can be affected by environmental factors and is implicated in pulmonary diseases.

Recently, heated tobacco products (HTPs) have been developed as less harmful alternatives to traditional combustible cigarettes. In Japan, a study reported that one in three current smokers used HTPs in 2020 [11]. However, toxic compounds are not completely removed from HTPs [12], and the actual impact of HTPs on the health of users and their overall impact on public health have not been fully investigated. Currently, various types of HTPs are used worldwide, and it is necessary to assess their effects. A previous study suggested that HTP aerosol exposure induces oxidative stress and the secretion of the inflammatory cytokine IL-6 in mice [13]. Toxicity among HTPs could differ and may depend on the cigarette heating temperatures of these devices as well as the amount or composition of the generated PM [14]. The reduced impact of HTPs relative to CS has been previously reported in aged aortic cells and 3D airway tissues *in vitro* [15, 16]. Notably, several reports have only studied

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the effects of low-temperature (30–40°C) heating devices and showed low risks [17-19]. However, the effects of high-temperature (≥ 200°C) heating devices should also be evaluated.

This study investigated the effects of aerosol extracts of two HTPs on global DNAm in human lung epithelial cells and compared them with those of combustible reference cigarette (RC) smoke extract. Reduced representation of bisulfite sequencing (RRBS) and DNA microarray analyses were performed to determine CpG methylation and gene transcription changes induced by the HTP and RC extracts.

Materials and methods

Preparation of aerosol extracts

An RC and two types of HTPs were used in this study. The reference material 1R6F cigarette was purchased from the University of Kentucky, Kentucky Tobacco Research and Development Center (Lexington, KY, USA). Two commercial HTPs, Ploom S (HTP1) and Ploom TECH+ (HTP2), were obtained from Japan Tobacco Inc. (Tokyo, Japan). Details of the tobacco heating systems have been described previously [20]. Briefly, HTP1 comprises two parts: a tobacco stick and battery with a heater. HTP1 is a high temperature (200°C) heating product. HTP2 comprises three parts: a tobacco capsule, cartridge, and battery. HTP2 is a low temperature (40°C) heating product. Mainstream 1R6F cigarette smoke and aerosols from HTPs were generated using smoking machines under the Health Canada Intense puffing regimen (55 mL puff volume, 2 s duration, 30 s puff interval, and bell-shaped

puff profile). The 1R6F cigarettes were smoked until the butt length measured 35 mm (ISO 4387). For HTP1, the total number of puffs per tobacco stick was set to 10 for aerosol collection. For HTP2, the total number of puffs per tobacco capsule was set to 65 for aerosol collection. Total particulate matter (TPM) was collected on a 44 mm Cambridge filter pad. TPM was dissolved in dimethyl sulfoxide (DMSO) (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) at a concentration of 40 mg/mL for the 1R6F extract and 100 mg/mL for the HTP extracts. These aerosol extracts were dispensed and stored at −80°C until use.

Cell culture

Human lung adenocarcinoma (A549) cells with type II alveolar epithelial characteristics were used in this study. The cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were grown in Eagle's minimum essential medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Cosmo Bio, Tokyo, Japan), non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin-streptomycin solution (Gibco) at 37°C in a humidified atmosphere with 5% CO_2. Exponentially growing cells were used for all experiments. Cells were seeded in culture plates and incubated for 24 h to allow cell adhesion. After attachment, the cells were treated with an extract-containing medium for indicated treatment times, and various assays were subsequently performed.

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Cytotoxicity

A549 cells (7×10^3) were seeded in 96-well plates and cultured for 24 h. Extracts were diluted with DMSO to the indicated TPM concentrations and added to the culture medium at 2% (v/v) before extract treatment. After the incubation with media containing different concentrations of RC, HTP1, and HTP2 extracts for 24 and 48 h, intracellular dehydrogenase activity (cell metabolic activity) was evaluated using the WST-8 assay. The level of lactate dehydrogenase (LDH), a marker of cytotoxicity, was detected in the medium using the Viability/Cytotoxicity Multiplex Assay kit (Dojindo, Kumamoto, Japan). Absorbance was measured at 450 and 490 nm for the WST-8 and LDH assays, respectively, using a VICTOR Nivo Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA).

DNA dot blot analysis

A549 cells (6×10^5) were seeded in 6-well plates and cultured for 24 h. After the incubation with media containing different concentrations of RC, HTP1, and HTP2 extracts for 12 h, genomic DNA was isolated using the QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and then adjusted to 50 ng/μL. DNA samples were denatured with 0.2 M NaOH and 20 mM EDTA at 95°C for 10 min. After the addition of saline

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sodium citrate buffer (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0), the denatured DNA samples were loaded on a Hybond-N+ nylon membrane (GE Healthcare, Buckinghamshire, England) using a 96-well dot blot apparatus (Bio-Rad, Hercules, CA, USA). Then, the membrane was irradiated with 120 mJ/cm^2 UVB using FUNA UV Crosslinker FS-1500 (Funakoshi, Tokyo, Japan). After blocking with 5% skim milk for 1 h, the membrane was incubated with polyclonal anti-5-mC (1:500, Active Motif, Carlsbad, CA, USA, 61255) and polyclonal anti-5-hmC (1:10,000, Active Motif, 39769) antibodies at 4°C overnight. Immunoreactive 5-mC and 5-hmC were visualized using the ECL Select^{TM} Western Blotting Detection Reagent (GE Healthcare). Chemiluminescent signals were detected using the ChemiDoc^{TM} MP Imaging System (Bio-Rad) and quantified using the Image Lab software (Bio-Rad). To ensure equal loading, the membrane was stained with methylene blue (Sigma-Aldrich) after immunoblot detection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

A549 cells (6×10^5) were seeded in 6-well plates and cultured for 24 h. After the incubation with RC and HTP1 extract-containing media for 12 h, total RNA was extracted using ISOGEN (FUJIFILM Wako Pure Chemical Co.), according to the manufacturer's instructions. The RNA concentration was quantified using NanoDrop. Then, cDNA was synthesized from RNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed using the QuantStudio™ 3 System (Thermo Fisher Scientific) and THUNDERBIRD

SYBR® qPCR Mix (Toyobo, Osaka, Japan), according to the manufacturers' instructions. Primers were purchased from Eurofins Genomics (Tokyo, Japan), and primer sequences are listed in Table S1. The amplification protocol comprised denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression was normalized to that of 18S rRNA.

RRBS

A549 cells (6×10^5) were seeded in 6-well plates and cultured for 24 h. After the incubation with 0.1 mg TPM/mL RC extract- or 2 mg TPM/mL HTP1 extract-containing medium for 12 h, genomic DNA was isolated using the QIAamp DNA Micro kit (Qiagen), according to the manufacturer's instructions. After DNA quantification using NanoDrop, equal amounts of DNA were pooled from six samples of each group. DNA quality was confirmed using TapeStation (Agilent Technologies, Santa Clara, CA, USA). Subsequently, bisulfite sequencing was performed by Rhelixa (Tokyo, Japan). RRBS libraries were prepared using the Zymo-Seq RRBS Library Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Briefly, genomic DNA was digested with the MspI restriction enzyme, which recognizes and cleaves the C^CGG sequence. Samples were incubated with DNA ligase and RRBS adapters for adapter ligation. Taq DNA polymerase and 5-mC dNTP mix were added and then the samples were incubated at 74°C for 30 min. After bisulfite treatment, index primer amplification was performed. The bisulfite reaction chemically converts the unmethylated cytosine bases to uracil. Index primer amplification with standard dNTPs and

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uracil-tolerant Taq DNA polymerase generated libraries with thymines instead of the originally unmethylated cytosines. Sequencing was performed using NovaSeq 6000 (Illumina, San Diego, CA, USA) in 150 bp 2 paired-end mode with a sequencing read of 60 M (30 M pairs) reads per sample.

Bioinformatics analysis of RRBS data

The read quality of RRBS data was checked using the FastQC (v0.11.7) software. The reads were trimmed using Trim Galore (v0.5.0, q=20, Phred scores 33) to remove adapter sequences and low-quality bases and mapped using Bismark (v0.20.0). Read counts are shown in Table S2. MethylKit (v1.16.0) was used to calculate the read count in each region and methylation rate for comparative analysis, and the resulting data were annotated using in-house software. MethylKit was also used to normalize the read count, and the differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) were identified using Fisher's exact test. A significant difference in the methylation rate was set at ≥ 25%. A heatmap illustrating DMC/DMR of 32,000 randomly chosen CpG sites was generated. DMRs were annotated according to the type of CpG and genomic regions. Gene Ontology (GO) terms were determined using DAVID Bioinformatics Resources (v103.1).

DNA microarray

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A549 cells (6×10^5) were seeded in 6-well plates and cultured for 24 h. After the incubation with 0.1 mg TPM/mL RC extract- or 2 mg TPM/mL HTP1 extract- containing medium for 12 h, total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. After RNA quantification using NanoDrop, equal amounts of RNA were pooled from six samples of each group. RNA quality was confirmed using the Agilent 2100 Bioanalyzer and Agilent RNA6000 Nano kit (Agilent Technologies). Microarray analysis (Clariom™ S Assay) was conducted by Filgen, Inc. (Aichi, Japan). All systems used for the microarray analysis were purchased from Thermo Fisher Scientific. Briefly, fragmented and labeled cDNA samples were prepared using the GeneChip™ WT PLUS Reagent Kit for whole-transcriptome expression analysis. Hybridization was carried out using GeneChip™ Hybridization Oven 645, and the array was incubated at 45°C for 16 h (60 rpm). GeneChip™ Fluidics Station 450 was used to wash the array, and GeneChip™ Scanner 3000 7G was used to scan the array according to the GeneChip™ Command Console 4.0 User Manual. Data were analyzed using Transcriptome Analysis Console v4.0. Kyoto encyclopedia of genes and genomes (KEGG) pathways were determined using DAVID Bioinformatics Resources (v103.1), and gene set enrichment analysis (GSEA) was performed using GSEA v4.3.2 (Broad Institute, Cambridge, MA, USA) with the microarray datasets.

Statistical analysis

Data are presented as means ± standard deviation (SD). Significant differences between the groups were analyzed using Dunnett's test or Tukey's test following one-way analysis of variance (ANOVA) with GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

HTP aerosol extracts were not as cytotoxic as the RC extract, but affected cellular metabolic activity

The effects of RC, HTP1, and HTP2 extracts on the metabolic activity of A549 cells were evaluated using the WST-8 assay. RC markedly decreased cell metabolic activity at concentrations higher than 0.1 mg TPM/mL (Fig. 1A). Contrarily, cell metabolic activity was increased by HTP1 (0.1 to 0.4 mg TPM/mL) and HTP2 (0.125 to 2 mg TPM/mL); however, it was decreased by HTP1 at a dose of 2 mg TPM/mL (Fig. 1B, C). The release of LDH into the medium was simultaneously detected to evaluate cytotoxicity. RC significantly increased LDH release at concentrations higher than 0.15 mg TPM/mL (Fig. 1D). In contrast, HTP1 and HTP2 slightly decreased LDH release, even at high doses (Fig. 1E, F).

RC, but not HTPs, decreased global 5-mC and 5-hmC levels

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The effects of RC, HTP1, and HTP2 extracts on global 5-mC and 5-hmC levels in the DNA of A549 cells were evaluated using DNA dot blot analysis. RC significantly decreased global 5-mC and 5-hmC levels, whereas HTP1 and HTP2 did not alter their levels (Fig. 2A, B).

HTP1 increased TET1 mRNA expression as much as RC

The effects of RC and HTP1 extracts on the mRNA expression of *TET* and *DNMT* family enzymes, which are related to DNA methylation, in A549 cells were evaluated using RT-qPCR. RC increased *TET1*, *TET3*, and *DNMT3A* mRNA expression, but did not alter *TET2*, *DNMT1*, and *DNMT3B* mRNA expression (Fig. 3A, B). Similarly, HTP1 increased *TET1* mRNA expression, but did not alter *TET2*, *TET3*, *DNMT1*, *DNMT3A*, and *DNMT3B* mRNA expression (Fig. 3A, B).

HTP1 altered the pattern of CpG methylation

The effects of RC (0.1 mg TPM/mL) and HTP1 (2 mg TPM/mL) extracts on CpG methylation in A549 cells were evaluated using RRBS. HTP1 decreased CpG methylation levels compared with the control and RC (Fig. 4A). The decrease in CpG methylation by HTP1 was observed in each chromosome and is indicated as the mean value (Fig. 4B). In contrast, the number of DMC sites (vs. control) was higher in the RC group than in the HTP1 group for both hypo- and hypermethylation (Fig. 4C). The DMC/DMR heatmap indicated that HTP1 altered CpG methylation (Fig. 4D). Methylation levels in the CpG island (CGI) and promoter regions are particularly important for gene

transcription regulation. The distribution of DMR changes in CpG regions showed that the alteration rates in CGI were low in both RC and HTP1 groups (Fig. 4E). Furthermore, the genomic distribution of DMR changes showed that the alteration rates in the promoter regions were low in both RC and HTP1 groups (Fig. 4F).

GO enrichment of DMRs showed a similar pattern in the RC and HTP1 groups

The top ten significantly enriched GO terms overlapped in the RC and HTP1 groups regardless of hypo- or hypermethylation (Fig. 5). A comparison of *P* values showed that DMR changes were mainly enriched in genes involved in developmental processes.

HTP1 affected gene expression, but not as much as RC

DNA microarray analysis was conducted to profile the gene expression changes induced by RC and HTP1 extracts. HTP1 affected a smaller number of genes compared with RC (Fig. 6A). In both groups, the number of upregulated genes was higher than that of downregulated genes. Hierarchical clustering showed that 72 genes were differentially expressed (≥ 2-fold) by HTP1, indicating a similarity between HTP1 and RC extracts (Fig. 6B). GSEA revealed that both RC and HTP1 negatively affected the DNA methylation gene set (Fig. 6C, D). The enriched KEGG pathways ($P < 0.05$) are shown in Figure S1. RC affected a large number of pathways, such as DNA replication, whereas HTP1 affected only a small number of pathways (Fig. S1). RT-qPCR was performed to

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validate DNA microarray results and to examine TPM dose dependency. RC significantly increased *VEGFA*, *CDKN1A*, *GDF15*, and *HMOX1* mRNA expression in a dose-dependent manner (Fig. 6E). In the HTP1 group, high TPM concentration (2 mg TPM/mL) increased *VEGFA*, *GDF15*, and *HMOX1* mRNA expression, whereas low TPM concentration (0.2 mg TPM/mL) did not (Fig. 6E). Furthermore, RC significantly decreased *DKK1*, *IL6*, *TGFB2*, and *RASSF9* mRNA expression in a dose-dependent manner (Fig. 6F). Similarly, in the HTP1 group, *DKK1* and *RASSF9* mRNA expression was decreased; however, *IL6* and *TGFB2* mRNA expression was not affected (Fig. 6F).

HTP1 particularly affected CYP1A1 mRNA expression and promoter methylation

CYP1A1 mRNA expression was analyzed using RT-qPCR because the gene was most notably affected by the HTP1 extract based on DNA microarray results. A high TPM concentration of HTP1 increased *CYP1A1* mRNA expression by more than six times compared to the control. A low TPM concentration of HTP1 also increased the expression of *CYP1A1* (Fig. 7A). However, RC significantly increased *CYP1A1* mRNA expression (Fig. 7A). In addition, both RC and HTP1 affected methylation levels in the *CYP1A1* promoter region (Fig. 7B).

Discussion

In this study, we evaluated the effects of HTP aerosol extracts on cytotoxicity, DNAm, and gene transcription in human lung epithelial cells and compared them with those of the RC smoke

extract. Aromatic amines, carbonyls, and phenolic compounds are known to be involved in the mutagenicity, genotoxicity, and cytotoxicity of tobacco smoke [21, 22]. These harmful components are less abundant in HTPs than in RC, but HTPs are characterized by higher contents of glycerol and propylene glycol [20, 21]. In this study, the RC smoke extract exhibited cytotoxicity against A549 cells in a dose-dependent manner, whereas HTP aerosol extracts did not. However, a high concentration of HTP1 decreased cell metabolic activity. The components contained in the aerosols of HTP1, a high-temperature (200°C) heating product, and HTP2, a low-temperature (40°C) heating product, are different even at the same TPM concentration [20]. HTP1 contains more toxic substances, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN), than HTP2 [20]. Previously, it was shown that exposure to the HTP2 aerosol extract for three days did not inhibit the proliferation of A549 cells [18]. Furthermore, significant toxicity against A549 cells treated with aerosol extracts of another type of HTP (heating temperature > 300°C) was reported, albeit to a lesser extent than the RC extract [23]. Consistently, our study also demonstrated that HTP extracts exhibit lower cytotoxicity than RC. However, high concentrations of the HTP1 extract can impair cell growth.

Furthermore, we examined whether high concentrations of the HTP extracts altered DNAm. Although CS is a well-known cause of DNAm abnormalities [2], the effects of HTPs on DNAm in lung epithelial cells have not yet been elucidated. In this study, we analyzed global DNAm alterations using dot blot analysis. RC significantly decreased 5-mC and 5-hmC levels, whereas

HTPs did not. The RC-induced decrease in 5-hmC levels are thought to be reflected a decrease in 5-mC levels. Previous studies also showed a decrease in 5-mC levels upon exposure to a different CS extract [24], indicating that CS induces proteasome-mediated degradation of DNMTs [25]. Furthermore, a recent study indicated significantly lower global 5-hmC levels in peripheral blood leukocytes of healthy smokers compared to those of nonsmokers [26]. A reduction in global 5-mC and 5-hmC levels is a characteristic of cancer, and smoking may be a triggering factor [27]. Our study showed for the first time that the effects of HTPs on global 5-mC and 5-hmC levels in lung epithelial cells may be smaller than those of RC.

Moreover, HTP1 was found to have a smaller effect on DNAm-related gene expression than RC. However, HTP1 increased *TET1* expression. A previous study reported that exposure to a different CS extract for 24 h also increased *TET1* expression in lung epithelial cells, whereas *TET2* and *TET3* expression remained unaltered [24]. These results suggest that *TET1* may be susceptible to tobacco components.

In addition, we performed RRBS analysis to determine DNAm alterations at CpG sites. HTP1 was found to markedly reduce CpG methylation. Additionally, no distinctive changes were observed when CpG methylation alterations were analyzed by chromosomes, indicating that the effects of HTP1 on DNAm are not chromosome-specific. In contrast, the number of DMC sites was lower in the HTP1 group than in the RC group. Therefore, the decrease in CpG methylation, indicated as the mean value, by HTP1 may be attributed to the larger ratio of hypomethylation to hypermethylation

events. Changes in DMRs in the CGI and promoter regions, which are known to play critical roles in gene transcription, were not markedly different between the RC and HTP1 groups. However, a recent study demonstrated that CpG methylation in other regions, such as exons, is also involved in the regulation of gene transcription [28]. Therefore, we cannot exclude the possibility that RC and HTP1 affect gene transcription by altering CpG methylation.

Interestingly, GO enrichment analysis of DMRs in RC and HTP1 groups showed a similar pattern, and genes involved in the top enriched terms, such as developmental processes, may be susceptible to CpG methylation in A549 cells treated with tobacco extracts. These results suggest that both RC and HTP1 induce DNAm changes at CpG sites.

Furthermore, we performed DNA microarray analysis to profile gene expression alterations. The number of genes affected by HTP1 was lower than that affected by RC, although a high TPM concentration of HTP1 was used. Importantly, the DNAm gene set was enriched in both RC and HTP1 groups. These results suggest that HTP1 decreased CpG methylation levels via transcriptional downregulation of the DNAm gene set, although detailed analyses are required. Furthermore, pathway analysis showed that signaling pathways involved in lung pathogenesis were significantly enriched in the RC group, but not in the HTP1 group. Additionally, we performed RT-qPCR to analyze the mRNA expression of genes associated with lung cancer to investigate dose dependencies. The mRNA expression of *VEGFA*, *GDF15*, and *HMOX1* was upregulated and that of *DKK1* and *RASSF9* was downregulated by HTP1 as well as RC. In contrast, RC decreased *TGFB2* and *IL6*

expression, but HTP1 did not. Significant changes in gene expression have been linked to cancer exacerbation [29]. HTPs have been reported to increase *HMOX1* expression and induce an oxidative stress response at a potential similar to that of conventional cigarette products in alveolar epithelial cells [30]. Furthermore, a previous study that examined the effects of another HTP on a 3D airway tissue indicated that the HTP had a lower impact on gene expression than RC [16]. Our results suggested that HTP1 affected genes that were primarily affected by RC, with the magnitude of the effect of HTP1 being smaller than that of RC.

In this study, *CYP1A1* was identified as one of the genes with the most significantly altered expression. A high concentration of the HTP1 extract increased *CYP1A1* mRNA expression in A549 cells by more than six times compared to the control. Similarly, a low concentration of the HTP1 extract also increased *CYP1A1* expression compared to the control group, although the increase was less than that of the RC group. HTP1 and RC also affected methylation levels in the *CYP1A1* promoter region. Notably, *CYP1A1* methylation has been reported to be altered in lung cancer tissues from individuals with a history of smoking compared to those without a smoking history [6]. CYP1A1 is involved in the metabolic activation of polycyclic aromatic hydrocarbons into mutagenic and carcinogenic derivatives that bind to DNA and subsequently induce neoplastic transformations [7, 8]. Benzopyrene, a polycyclic aromatic hydrocarbon, is present in HTP aerosols [31]. This study revealed that exposure to HTP1 aerosols can increase *CYP1A1* expression with altering promoter methylation. A previous study reported that *CYP1A1* expression is regulated through the aryl

hydrocarbon receptor (AhR), which mediates the recruitment of specificity protein 1 (Sp1) to demethylated DNA [32]. Moreover, several transcription factors are known to be activated by CS, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and hypoxia-inducible factor (HIF) [33]. These transcription factors may also affect *CYP1A1* expression [34, 35].

A limitation of this study is that we did not examine protein levels because we aimed to focus on the relationship between epigenetics and gene transcription. Furthermore, whether DNAm changes increase or decrease gene transcription is dependent on the cell type [27]. Recently, it was reported that environmental PM2.5, which is associated with lung cancer risk, promotes lung cancer development by acting on cells that harbor pre-existing oncogenic mutations in healthy lung tissues [36]. Therefore, the effects of HTPs can vary depending on the initial or original cellular conditions. In addition, repeated exposure to PM may cause a more notable degree of DNAm changes than a single exposure [10]. Moreover, HTPs may alter gene expression not only via DNAm changes, but also via histone modifications and other factors [37].

Previous studies have suggested that HTPs are associated with significant reductions in exposure to harmful and potentially harmful constituents compared to CS [38]. In a cohort study, HTP users displayed abnormal DNAm and transcriptome profiles in peripheral blood mononuclear cells, albeit to a lesser extent than CS users [39]. CS-induced DNAm alterations can be reduced by ceasing CS exposure or switching to HTPs [40]. However, no tobacco product has been proven to be safe and risk-free [12, 41, 42]. Taken together, these results suggest that HTP users are at risk of

DNAm changes. Further investigations are warranted to better understand the health effects associated with the use of HTPs.

Conclusion

HTP aerosol extracts had lower impacts on cytotoxicity and global 5-mC and 5-hmC levels in A549 cells than the RC smoke extract. However, HTP extract altered the CpG methylation patterns and expression of cancer-related genes, albeit to a lesser extent than the RC extract. In particular, HTP extract affected the mRNA expression and promoter methylation of *CYP1A1*, which is associated with carcinogenic risk. This study suggests that HTPs as well as combustible cigarettes cause CpG methylation and gene transcription alterations in lung epithelial cells.

CRediT authorship contribution statement: Ayami Sato: Investigation, Formal analysis, Writing - original draft. Akihito Ishigami: Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest: The authors declare no conflict of interest.

Data availability: The data in the current study are available from the first author upon reasonable request.

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Fig. 1. HTP aerosol extracts were not as cytotoxic as the RC extract, but affected cellular metabolic activity.

The extracts were diluted with DMSO to the indicated total particulate matter (TPM) concentration and added to the culture medium at 2% (v/v). A549 cells were incubated in a medium containing different concentrations of RC, HTP1, and HTP2 extracts for 24 or 48 h. (A-C) Cell metabolic activity was evaluated using the WST-8 assay. (D-F) Release of LDH, a marker of cytotoxicity, was detected in the cell supernatant. Data are presented as means ± SD (n = 5). Statistical analysis was performed using Dunnett's test following one-way ANOVA (*$P < 0.05$, vs. 0 μM).

Fig. 2. RC, but not HTPs, decreased global 5-mC and 5-hmC levels.

A549 cells were incubated in a medium containing different concentrations of RC, HTP1, and HTP2 extracts for 12 h. Global (A) 5-mC and (B) 5-hmC levels, and a representative image of dot blot analysis of control, RC, HTP1, and HTP2 groups. Box plots represent the minimum and maximum values of each sample (n = 5–6). Statistical analysis was performed using Dunnett's test following one-way ANOVA, and the *P* values ($P < 0.05$, vs. control) are shown.

Fig. 3. HTP1 and RC increased *TET1* mRNA expression.

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A549 cells were incubated in a medium containing different concentrations of RC and HTP1 aerosol extracts for 12 h. (A and B) *TET1*, *TET2*, *TET3*, *DNMT1*, *DNMT3A*, and *DNMT3B* mRNA expression was analyzed by RT-qPCR. Gene expression was normalized to that of 18S rRNA. Data are presented as means ± SD (n = 5). Statistical analysis was performed using Dunnett's test following one-way ANOVA, and the *P* values ($P < 0.05$, vs. control) are shown.

Fig. 4. HTP1 altered the CpG methylation pattern.

A549 cells were incubated in a medium containing 0.1 mg TPM/mL RC and 2 mg TPM/mL HTP1 extracts for 12 h. Subsequently, RRBS was performed. (A) CpG methylation levels at different loci were plotted (n = 4,815,335). Statistical analysis was performed using Tukey's test following one-way ANOVA, and the *P* values are shown. (B) Changes in CpG methylation levels on each chromosome in RC vs. control and HTP1 vs. control. (C) The number of differentially methylated cytosine (DMC) sites in RC vs. control and HTP1 vs. control was counted from the RRBS data. A difference in the methylation rate of 25% or greater was included in the counts for hypomethylation (decrease) and hypermethylation (increase). (D) Heatmap illustrating DMC/differentially methylated region (DMR) of 32,000 randomly chosen CpG sites. Blue indicates low methylation rates and yellow indicates high methylation rates. (E) Distribution of DMR changes in CpG regions (CpG island [CGI], CpG shore, CpG shelf, and inter CGI) in RC vs. control and HTP1 vs. control. (F)

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Genomic distribution of DMR changes (Promoter, Intron-exonboundary, Intron, Intergenic, Exon, 5'UTR, 3'UTR, and 1 to 5kb) in RC vs. control and HTP1 vs. control.

Fig. 5. GO enrichment of DMRs in RC and HTP1 groups showed a similar pattern.

The top ten significantly enriched biological processes based on GO enrichment analysis of DMRs (methylation difference ≥ 25% and $q < 0.001$) from the RRBS data. Hypo- and hypermethylation in RC vs. control and HTP1 vs. control are indicated as *P* values.

Fig. 6. HTP1 affected gene expression, but not as much as RC.

A549 cells were incubated in a medium containing 0.1 mg TPM/mL RC or 2 mg TPM/mL HTP1 extracts for 12 h. Subsequently, DNA microarray was performed. (A) Venn diagram showing the number of genes affected by RC and HTP1 (≥ 2-fold). (B) Hierarchical clustering showing 72 genes that were differentially expressed (≥ 2-fold) in HTP1 vs. control. The enrichment of the DNA methylation gene set was determined using gene set enrichment analysis (GSEA) in both (C) RC vs. control and (D) HTP1 vs. control. (E, F) The mRNA expression of genes identified using DNA microarray (*VEGFA*, vascular endothelial growth factor A; *CDKN1A*, cyclin-dependent kinase inhibitor 1A; *GDF15*, growth differentiation factor 15; *HMOX1*, heme oxygenase 1; *DKK1*, dickkopf WNT signaling pathway inhibitor 1; *IL-6*, interleukin 6; *TGFB2*, transforming growth factor beta 2; *RASSF9*, Ras association (RalGDS/AF-6) domain family (N-terminal) member 9) was analyzed

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using RT-qPCR. Gene expression was normalized to that of *18S rRNA*. Data are presented as means ± SD (n = 6). Statistical analysis was performed using Dunnett's test following one-way ANOVA, and the *P* values ($P < 0.05$, vs. control) are shown.

Fig. 7. HTP1 markedly affected *CYP1A1* mRNA expression and promoter methylation.

(A) The mRNA expression of cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*) was analyzed using RT-qPCR. Gene expression was normalized to that of 18S rRNA. Data are presented as means ± SD (n = 6). Statistical analysis was performed using Dunnett's test following one-way ANOVA, and the *P* values ($P < 0.05$, vs. control) are shown. (B) Methylation changes in *CYP1A1* promoter regions (n = 86) in RC vs. control and HTP1 vs. control were analyzed from the RRBS data.

Fig. S1. KEGG pathway enrichment of differentially expressed genes.

Enrichment of the signaling pathways affected by RC and HTP1 was analyzed using DAVID ($P < 0.05$ vs. control, ≥ 1.5-fold). The number of genes counted in each pathway is shown in the upper column.

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Figure 1.

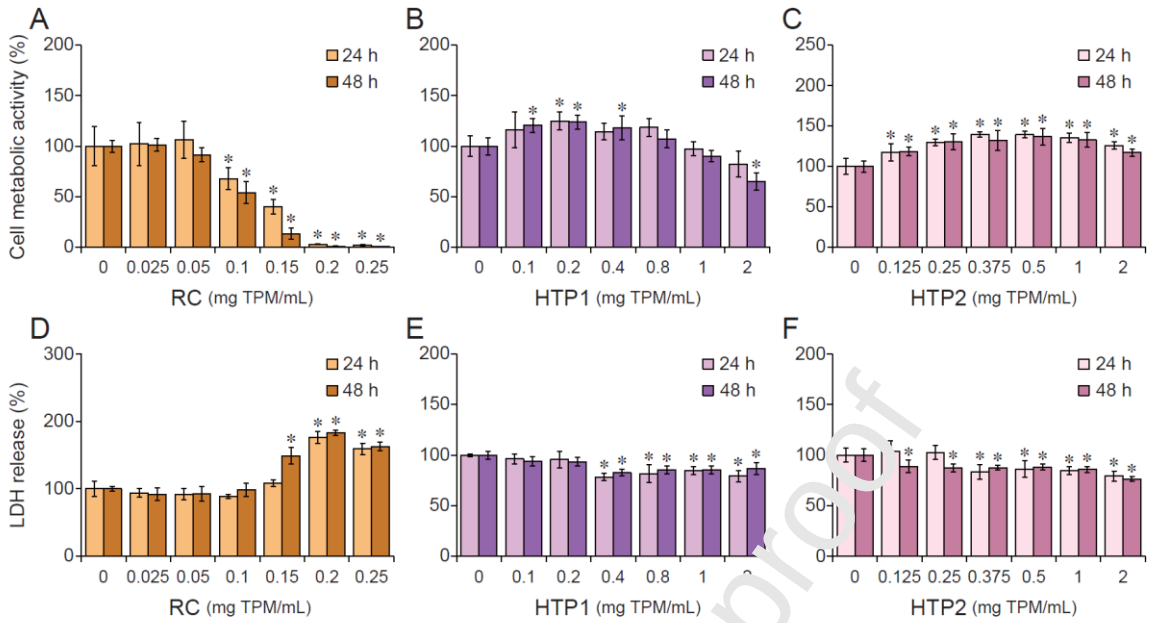

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Figure 2.

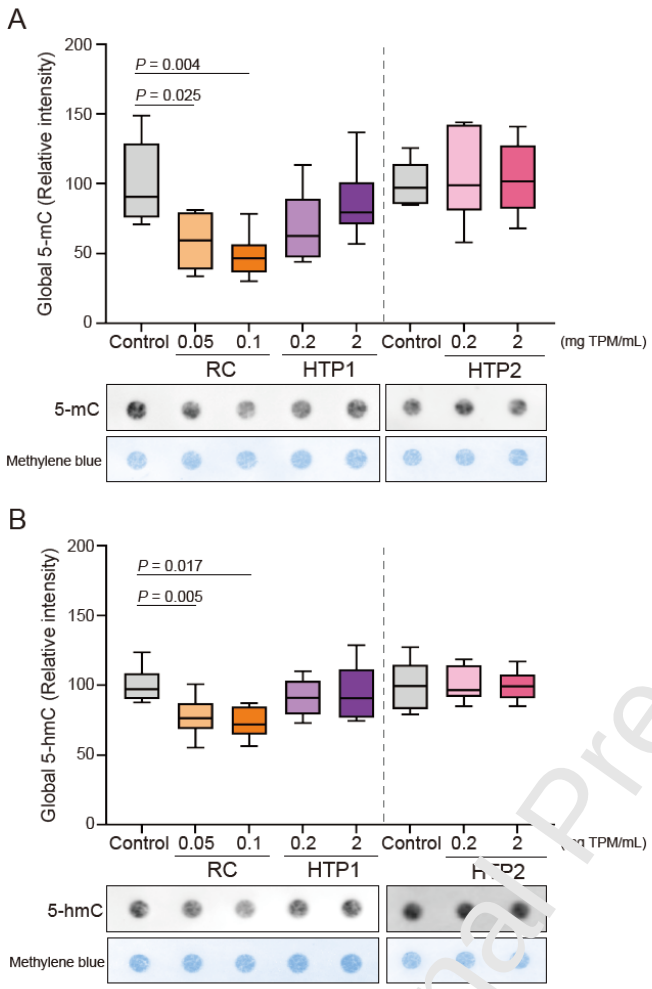

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Figure 3.

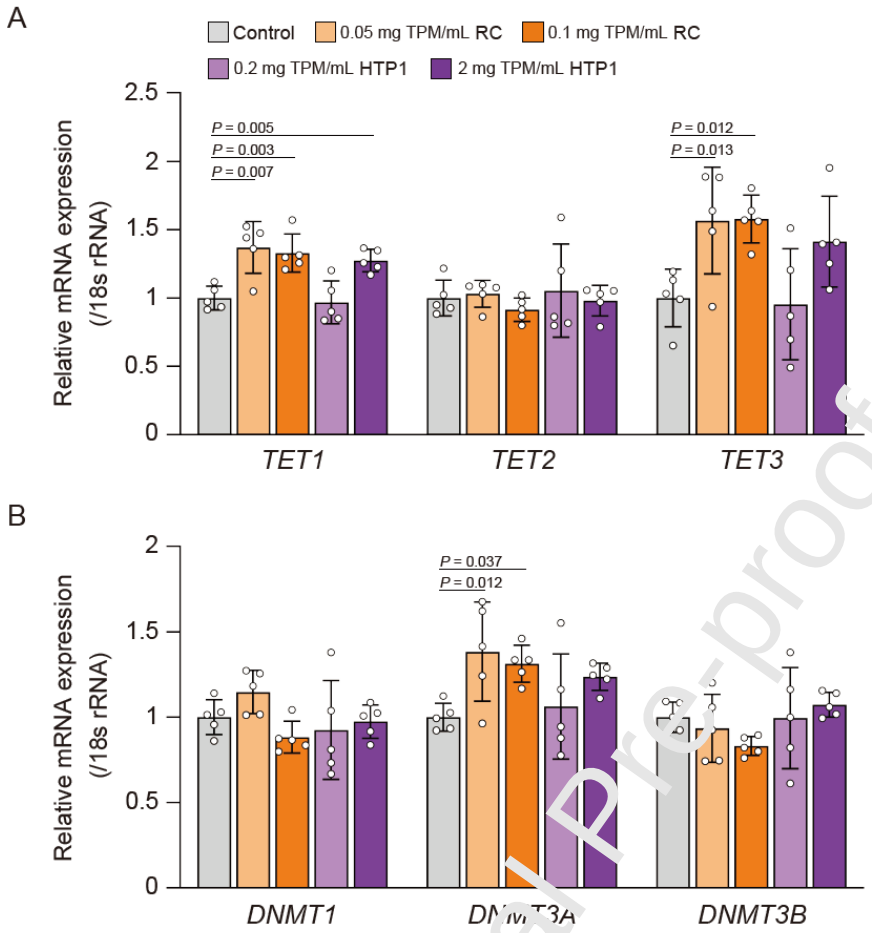

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Figure 4.

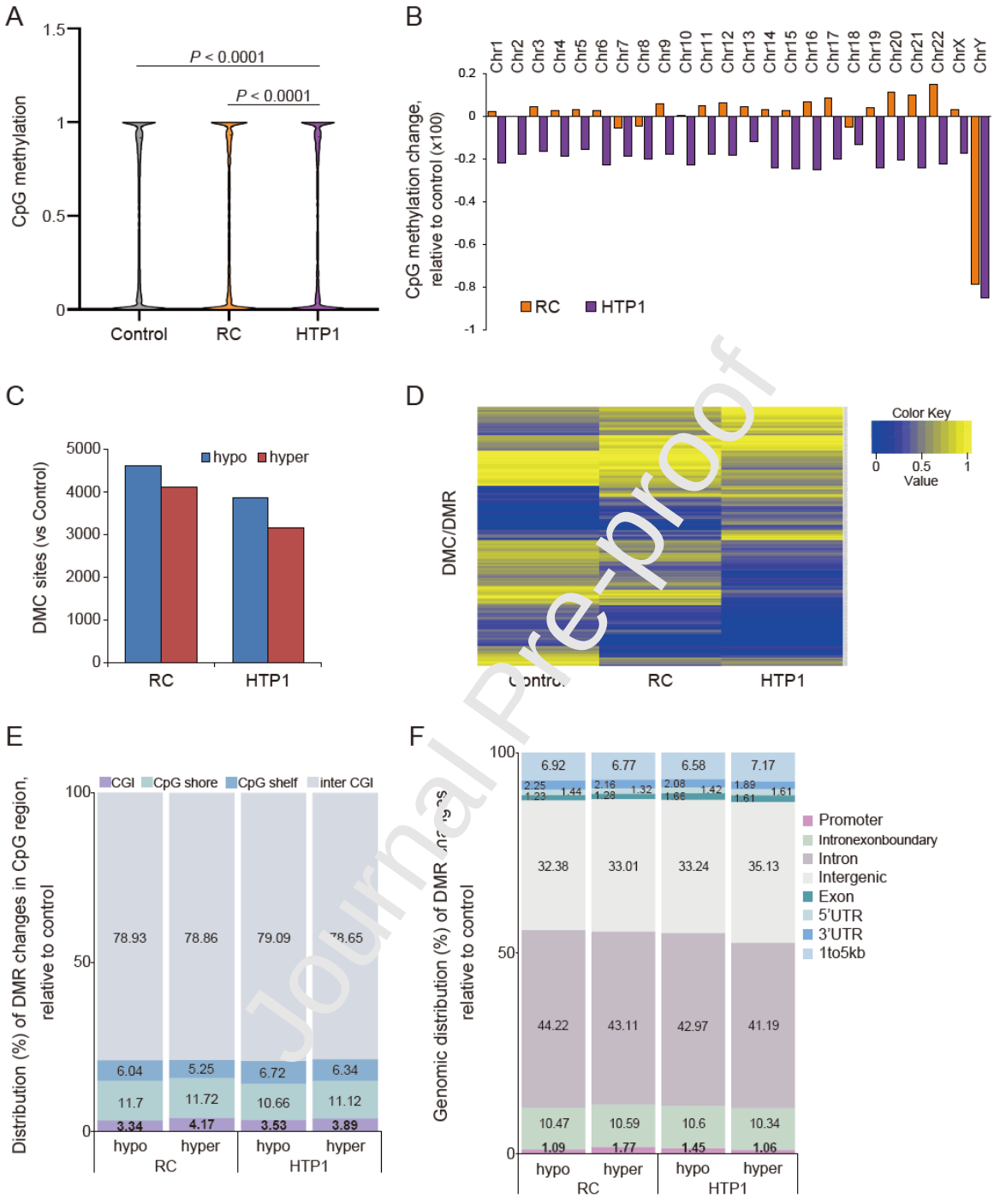

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Figure 5.

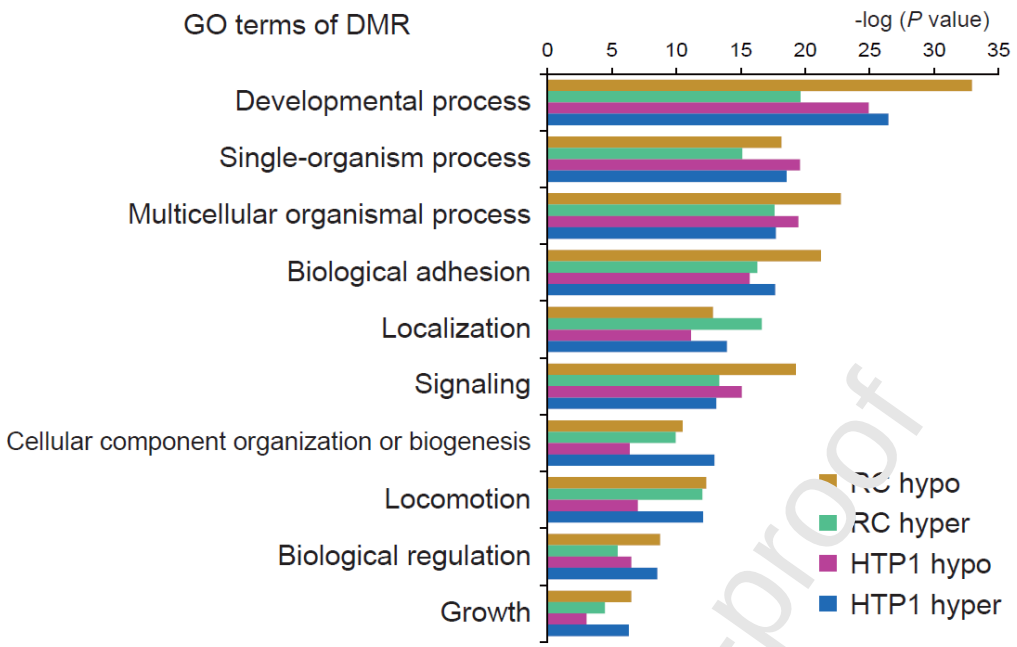

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Figure 6.

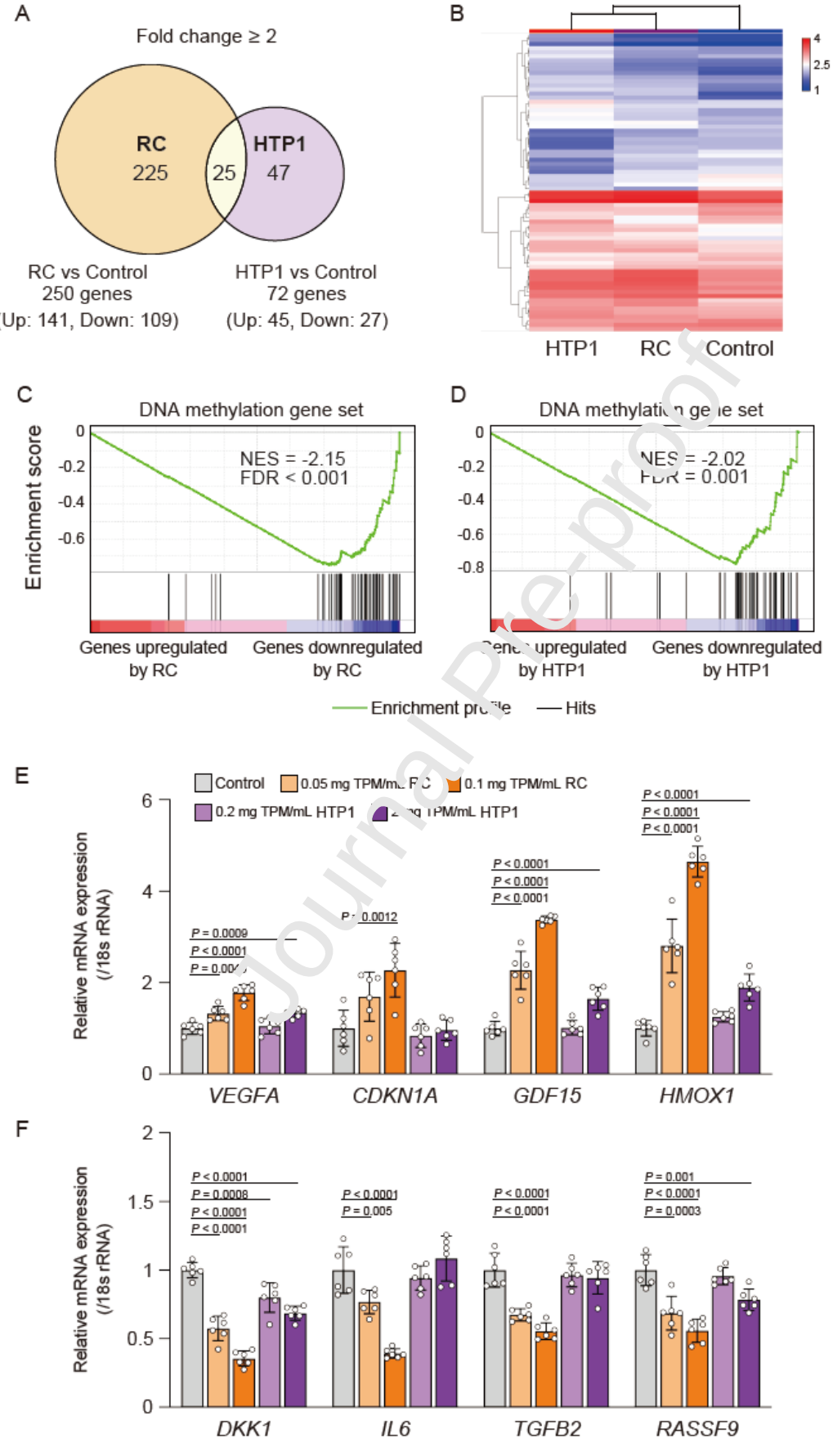
A
Fold change ≥ 2
RC
225
25
HTP1
47
RC vs Control
250 genes
(Up: 141, Down: 109)
HTP1 vs Control
72 genes
(Up: 45, Down: 27)
B
4
2.5
1
HTP1
RC
Control
C
DNA methylation gene set
Enrichment score
NES = -2.15
FDR < 0.001
Genes upregulated by RC
Genes downregulated by RC
D
DNA methylation gene set
NES = -2.02
FDR = 0.001
Genes upregulated by HTP1
Genes downregulated by HTP1
Enrichment profile
Hits
E
Control
0.05 mg TPM/mL RC
0.1 mg TPM/mL RC
0.2 mg TPM/mL HTP1
Relative mRNA expression (/18s rRNA)
VEGFA
CDKN1A
GDF15
HMOX1
F
Relative mRNA expression (/18s rRNA)
DKK1
IL6
TGFB2
RASSF9

Figure 7.

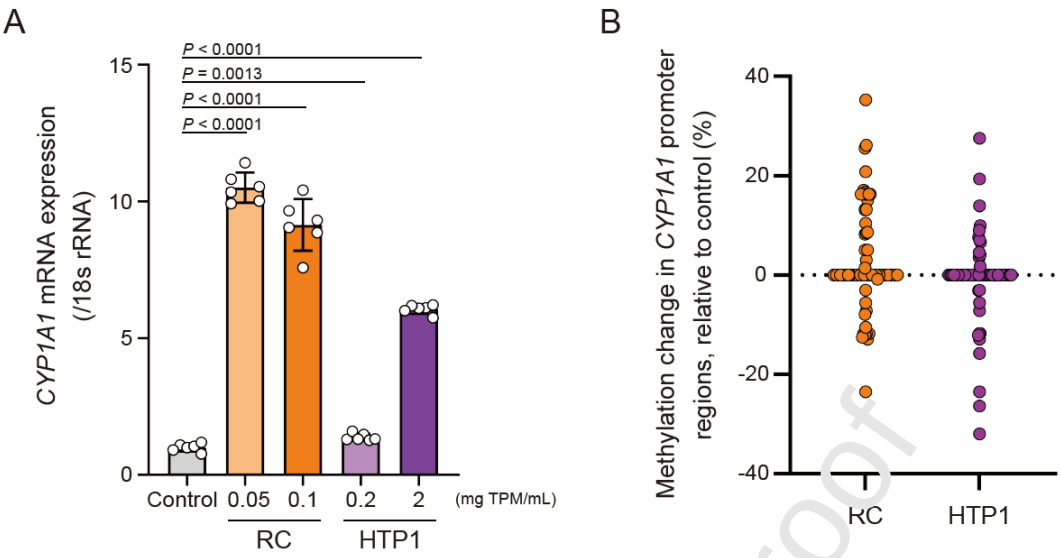

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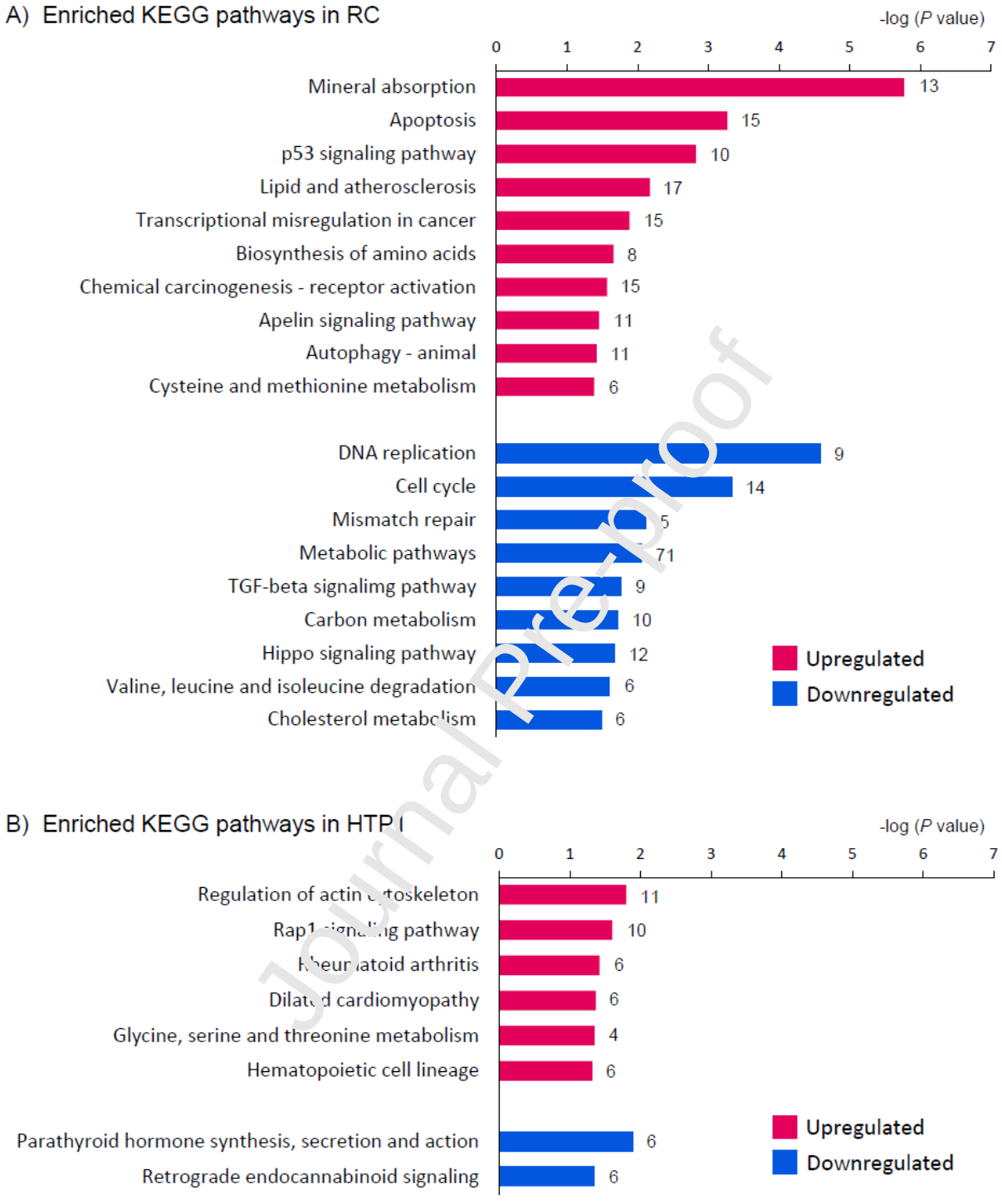
Figure S1.



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CRediT authorship contribution statement: Ayami Sato: Investigation, Formal analysis, Writing - original draft. Akihito Ishigami: Supervision, Funding acquisition, Conceptualization.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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- Heated tobacco products (HTPs) can alter CpG methylation in lung epithelial cells.
- HTP affected gene expression, but not as much as combustible reference cigarette.
- HTP particularly affected *CYP1A1* mRNA expression and promoter methylation.

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