*Original Article*

**SAGE Open Medicine**

SAGE Open Medicine
3: 2050312115578317

DOI: 10.1177/2050312115578317
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# Cigarette smoke condensate and individual constituents modulate DNA methyltransferase expression in human liver cells

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## Abstract

**Objectives:** Previous studies found higher expression levels of DNA methyltransferase 1 in liver samples from smokers compared to those from non-smokers. In contrast, expression levels of DNA methyltransferase 3a and DNA methyltransferase 3b were similar in smokers and non-smokers. This study extends these studies to establish a causal linkage to cigarette smoke exposure by examining whether DNA methyltransferase expression is modulated by cigarette smoke condensate.

**Methods:** These experiments were conducted in an in vitro system using HepG2 human liver cells. The dose range of cigarette smoke condensate was 0.1–120 µg/mL. The duration of exposure was up to 72 h.

**Results:** In a 24-h exposure, DNA methyltransferase 1 expression was found to increase significantly in a dose-dependent manner (greater than threefold at 100 µg/mL cigarette smoke condensate). Expression levels of DNA methyltransferase 3a and DNA methyltransferase 3b were, however, not affected under these conditions. The effect of two cigarette constituents, nicotine and cotinine, on DNA methyltransferase 1 expression was also examined. Nicotine exposure significantly increased DNA methyltransferase 1 expression in a dose-dependent manner (greater than twofold at 50 µM). However, under these conditions, cotinine did not increase DNA methyltransferase 1 expression.

**Conclusion:** These results clearly provide additional support of the modulating effect of cigarette smoke on DNA methyltransferase 1 expression. Given the potential of alterations in DNA methyltransferase expression to affect cellular function, this pathway may play a critical role in cigarette smoke-induced toxicity.

## Keywords

Cigarette smoke condensate, DNA methyltransferase, human liver cells, nicotine

Date received: 19 November 2014; accepted: 3 February 2015

## Introduction

Cigarette smoking is a major established environmental risk factor for numerous diseases, including cancer and diseases of the cardiovascular and respiratory systems.[1] Several modes of action are involved in the toxicology of cigarette smoke, including epigenetic mechanisms.[2] Epigenetic mechanisms resulting in changes in gene expression can lead to disruption in cellular function and cause a variety of diseases. Cytosine methylation is a major epigenetic modification of human DNA and has been shown to influence a number of cellular processes.[3] Aberrant DNA methylation is causally implicated in human cancer,[4,5] and there is increasing evidence of its involvement in diseases of the respiratory and cardiovascular systems, various autoimmune diseases, and multiple neuronal disorders.[6,7] A global loss of DNA methylation (hypomethylation) and a gene-specific gain of DNA methylation (hypermethylation) are two distinct hallmarks of carcinogenesis.[8,9] Numerous studies associate cigarette smoking with gene promoter hypermethylation in tobacco-related cancers.[10–12] In

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two recent epigenome-wide association studies comparing smokers and never smokers, a number of the CpG sites identified as differentially methylated were, however, found to be hypomethylated in smokers.[13,14] Several studies have also examined the association between cigarette smoke exposure and global DNA methylation.[15,16]

DNA methylation is the result of the activity of a family of DNA methyltransferase (DNMT) enzymes, including DNMT1, DNMT3a, and DNMT3b, that catalyze the transfer of a methyl group from the ubiquitous methyl donor S-adenosyl methionine to the 5-position of cytosines residing in the dinucleotide sequence cytosine-guanine.[17] DNMTs 1, 3a, and 3b have been implicated to different extents in initiating gene silencing through de novo methylation and recruitment of chromatin remodeling proteins.[18–20] DNMT1 has both maintenance and de novo methyltransferase activity, associates with chromatin, and is responsible for ~90% of methyltransferase activity in mammalian cells. DNMT3a and DNMT3b are ubiquitously expressed and can be detected in most adult tissues. DNMT3a and 3b are thought to function as de novo DNMTs. These enzymes were shown to have equal preferences in vitro for unmethylated and hemimethylated DNA. Studies have shown that DNMTs function in cooperation with each other to facilitate DNA methylation in both human and mouse systems.[21]

Elevated levels of DNMTs have been found in many cancer cells in vitro and in several types of human tumors in vivo, including hepatocellular carcinoma.[20,22–25] In addition, increased DNMT levels are required to maintain the phenotype of fibroblasts transformed with *ras* and with the *fos* oncogene.[26] Induced DNMT1 over-expression in cultured cell lines gradually induces CpG hypermethylation and cell transformation.[27] Altered DNMT expression is related to changes in genome-wide DNA methylation patterns that can have potent effects on expression of the large number of genes that are controlled by promoter methylation.

Previous studies in our laboratory examined the potential role of cigarette smoking in influencing DNMT expression in human tissue by comparing expression levels in liver samples from smokers and non-smokers.[28,29] Cigarette smoking is causally associated with liver cancer.[30] Higher expression levels of DNMT1 were found in smokers compared to non-smokers. In contrast, expression levels of DNMT3a and DNMT3b were similar in smokers and non-smokers. This study extends these studies to establish a causal linkage to cigarette smoke exposure by examining whether DNMT expression is modulated by cigarette smoke condensate (CSC) in an in vitro system using HepG2 human liver cells. The effects of nicotine and cotinine, as constituents of cigarette smoke, were also investigated.

## Material and methods

### *Cell lines and treatment conditions*

The human liver cell line, HepG2, was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were grown in William's E medium, supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 15,000 U penicillin, 15,000 U streptomycin, 2 mM L-glutamine (Life Technologies, Carlsbad, CA). Cells were routinely maintained at 37°C in a humidified 5% $CO_2$ atmosphere. CSC was purchased from Murty Pharmaceuticals (Lexington, KY) and was prepared using a smoking machine designed for Federal Trade Commission testing. The particulate matter from Kentucky standard cigarettes (1R3F; University of Kentucky, Lexington, KY) was collected on Cambridge glass fiber filters and the amount of CSC obtained was determined by weight increase on the filter. CSC was prepared by dissolving the collected smoke particulates in dimethyl sulfoxide (DMSO) to yield a 4% solution (w/v). The CSC was diluted into DMSO and aliquots were stored at −80°C. For smoke condensate exposure experiments, cells (400,000 cells per plate) were cultured in 100 mm dishes in appropriate media with or without CSC (0.1, 1.0, 10, 100, 120 µg/mL; in DMSO; final vol., 0.1%) for 24, 48, or 72 h. Nicotine and cotinine were purchased from Sigma-Aldrich (St. Louis, MO). Cells were treated with or without nicotine or cotinine (0.1, 1.0, 10.0, 20.0, 50.0 µM) for 24, 48, or 72 h. Cell proliferation was assessed by MTT techniques using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI).

### *RNA isolation and quantitative real-time polymerase chain reaction analysis*

Qiagen RNeasy isolation kit (Valencia, CA) was used to isolate total RNA. A Clontech cDNA synthesis kit (Mountain View, CA) was used for cDNA synthesis. Quantitative real-time polymerase chain reaction (QRT-PCR) was performed to determine expression levels of the DNMTs. The primers for real-time polymerase chain reaction (PCR) (synthesized by Sigma-Genosys; Woodlands, TX) were *DNMT1* (forward: GCACAAACTGACCTGCTTCA and reverse: GCC TTTTCACCTCCATCAAA), *DNMT3a* (forward: 5′CGTCT CCGAACCACATGAC and reverse: 5′-CGTCTCCGAAC CACATGAC), *DNMT3b* (forward: 5′-CCAGCTGAAG CCCATGTT and reverse: 5′-ATTTGTCTTGAACGCTTG), *GAPDH* (forward: 5′GAAGGTGA AGGTCGGAGTC and reverse: 5′-GAAGATGGTGATGGG ATTTC) . QRT-PCR reactions were performed in a volume of 50 µL containing 50 ng cDNA, 400 nM of each primers, deionized water, and 25 µL SYBR Green Master Mix (Stratagene, Cedar Creek, TX) using the following conditions: 50°C for 2 min and denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Optical data were collected during the 60°C step. PCRs were carried out in 96-well thin-wall PCR plates covered with optically clear sealing film (Applied Biosystems, Forest City, CA). Amplification, detection, and data analysis were performed using the ABI PRISM 7000 Sequence Detector system (Applied Biosystems). Results were expressed using the comparative threshold method after

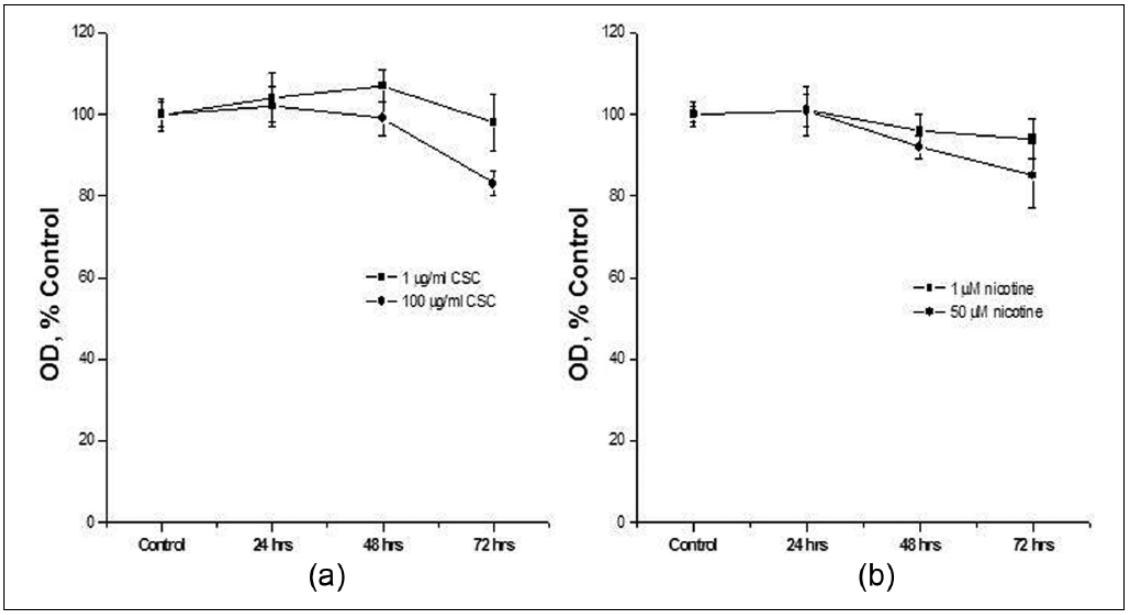


**Figure 1.** Effect of CSC and nicotine on cell proliferation. HepG2 cells were treated with (a) 1 or 100 µg/mL CSC for 24, 48, or 72 h or (b) 1 or 50 µM nicotine for 24, 48, or 72 h and assayed by MTT techniques. Data are presented as mean ± SD of at least three determinations.

validation, following the recommendations of the manufacturer (Applied Biosystems). The threshold cycle number (CT) value for DNMT was normalized against GAPDH and calculated as $\Delta CT = CT_{DNMT} - CT_{GAPDH}$. Thereafter, the relative mRNA levels of these genes after treatment were calculated using the $\Delta\Delta C_T$ method: $\Delta C_T$ (treatment) $- \Delta C_T$ (vehicle) $= \Delta\Delta C_T$ (treatment). The fold changes of mRNA levels were expressed as $2^{-\Delta\Delta CT}$. All PCR reactions were performed in triplicate in three independent experiments.

### *Statistical analysis*

Prism IV software (GraphPAD Software, Inc., La Jolla, CA) was used for graphical analyses. Data were analyzed for statistical significance using Student's *t* test. Differences with *p* values of $<0.05$ were considered statistically significant.

## Results

The effect of CSC and nicotine on cell growth was assessed. In these experiments, the dose of CSC was 1 µg/mL or 100 µg/mL. The duration of exposure was up to 72 h. Under these conditions, there was a modest inhibition of cell growth that did not exceed 20% (Figure 1(a)). Similar results were observed with nicotine (1 and 50 µM; Figure 1(b)).

The effect of CSC on expression of DNMT1, DNMT3a, and DNMT3b was assessed in HepG2 cells. In these experiments, the dose range of CSC was 0.1–120 µg/mL. The duration of exposure was up to 72 h. In a 24-h exposure, DNMT1 expression was found to significantly increase (up to 3.4-fold) in a dose-dependent manner (1–100 µg/mL), as shown in Figure 2. Induction of DNMT1 expression was greatest at 24-h exposure compared to exposure durations of 48 or 72 h (Figure 3). Cells in these experiments were exposed to 100 µg/mL CSC. Under the conditions included in this study, expression levels of DNMT3a and DNMT3b were, however, not affected (Figures 2 and 3).

The effect of two cigarette constituents, nicotine and cotinine, on DNMT1 expression was also examined in this in vitro system. Cotinine is also a metabolite of nicotine. Nicotine exposure significantly increased DNMT1 expression (up to 2.1-fold) in a dose-dependent manner (1–50 µM; Figure 4). Cotinine was, however, not shown to increase DNMT1 expression under the conditions employed in this study. Cells were exposed for 48 h because the highest increase in DNMT1 expression was shown at this exposure duration at a dose level of 50 µM (Figure 5).

## Discussion

In this study, an in vitro model system was employed to provide direct evidence that cigarette smoke induces the expression of DNMT1 in human liver cells. No effect was demonstrated with DNMT3a or DNMT3b. These results are consistent with our previous observations in human liver tissue samples in which higher levels of DNMT1 were found in smokers while levels of DNMT3a and DNMT3b were similar in smokers and non-smokers.[28,29] Reports of other in vitro studies of the effects of CSC on DNMT expression are limited, and the results have been varied. Similar to the findings in the current study, CSC exposure in T-24 bladder cancer cells induced a significant time-dependent increase in the

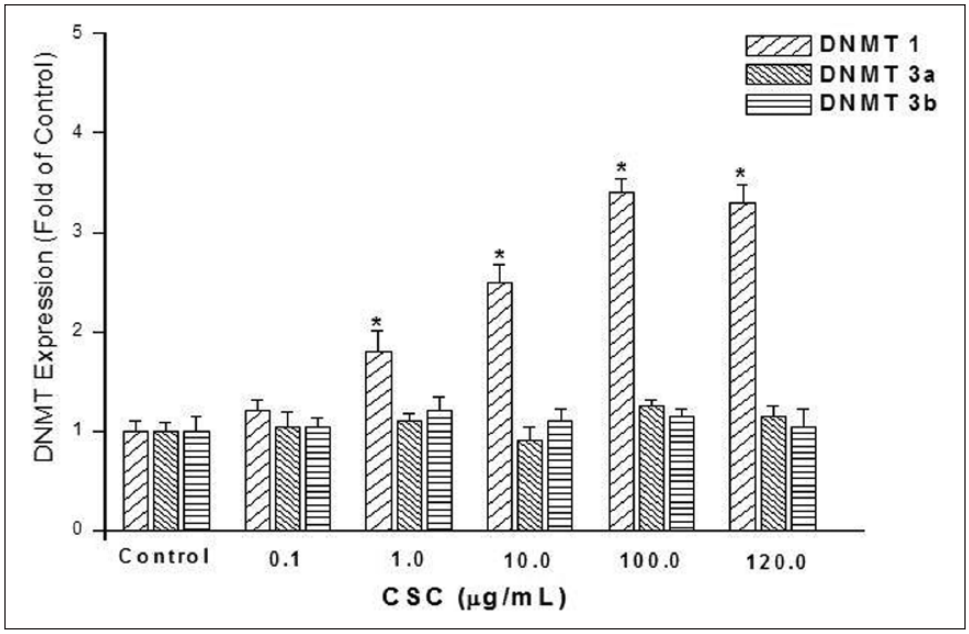


**Figure 2.** Effect of CSC dose on expression levels of DNMTs 1, 3a, and 3b. HepG2 cells were exposed to 0.1, 1.0, 10, 100, or 120 µg/mL CSC for 24 h. Expression levels were determined by quantitative real-time RT-PCR. Data are presented as mean ± SD of at least three determinations. The symbol "*" Denotes a significant difference ($p < 0.05$) compared to controls.

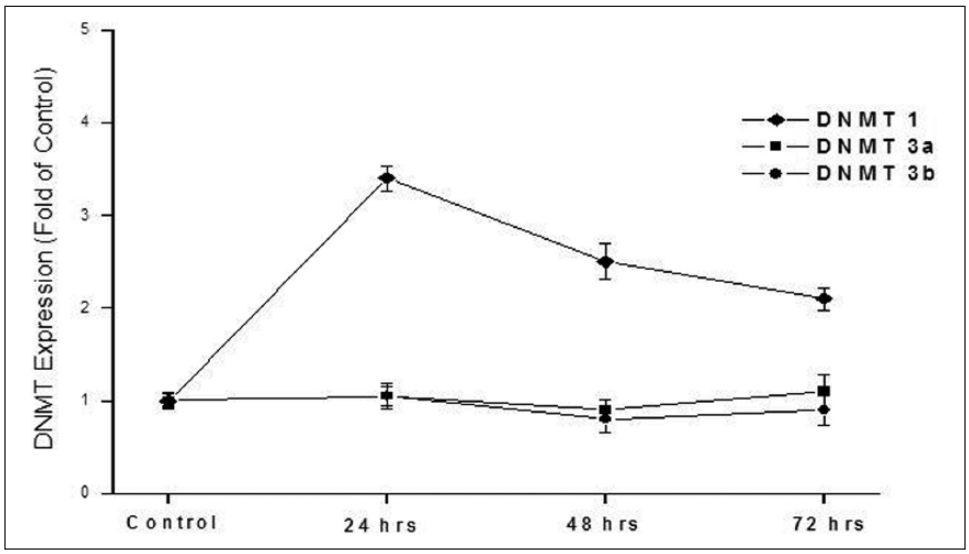


**Figure 3.** Effect of CSC exposure duration on expression levels of DNMTs 1, 3a, and 3b. HepG2 cells were exposed to 100 µg/mL CSC for 24, 48, or 72 h. Expression levels were determined by quantitative real-time RT-PCR. Data are presented as mean ± SD of at least three determinations.

level of DNMT1, but had no effect on DNMT3a and DNMT3b.[31] The time dependence did, however, differ with the current study given the decrease in DNMT1 expression after 24 h that was shown in this study. In another study, increased DNMT1 expression was observed in B2B cells treated with 1% CSC for 12 weeks.[32] However, in an examination of expression levels of DNMTs 1, 3a, and 3b in A549 lung cancer cells, a time-dependent (12–72 h) decrease in the levels of DNMT3b by CSC exposure was detected.[33] There was no effect on DNMT3a expression. In human bronchial epithelial cells *(*HBEC) exposed to CSC, after 5 months, decreased DNMT1 and increased DNMT3B expression were observed.[34] Exposures of shorter durations were found not to affect the expression of either DNMT. Reasons for these variations in results are unknown but may reflect different cell types, doses, or durations of exposure.

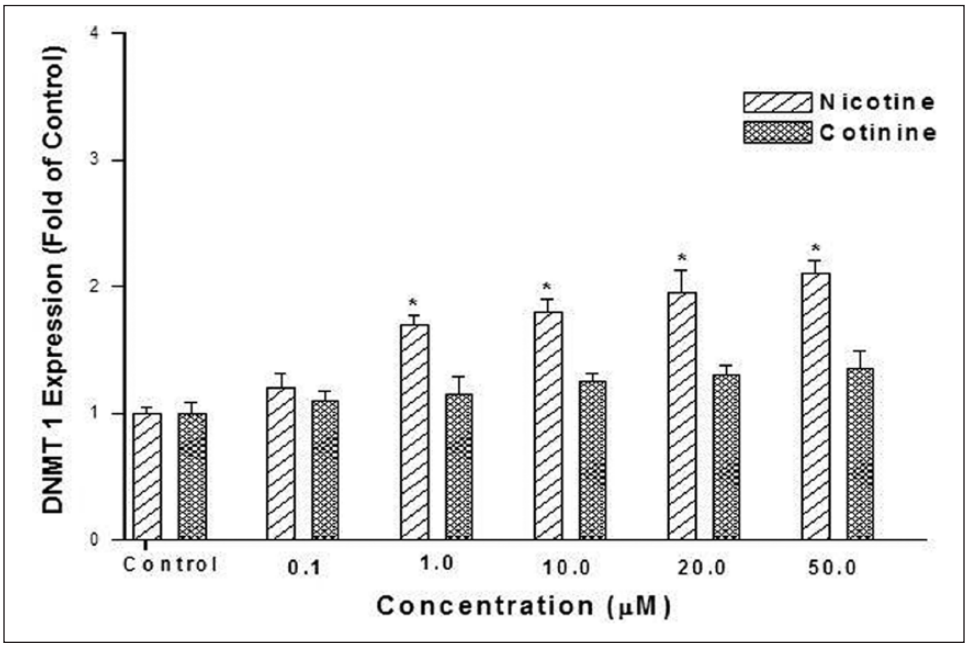


**Figure 4.** Effect of nicotine and cotinine dose on expression levels of DNMT1. HepG2 cells were exposed to 0.1, 1.0, 10.0, 20.0, and 50.0 μM nicotine or cotinine for 48 h. Expression levels were determined by quantitative real-time RT-PCR. Data are presented as mean ± SD of at least three determinations. The symbol "*" denotes a significant difference ($p < 0.05$) compared to controls.

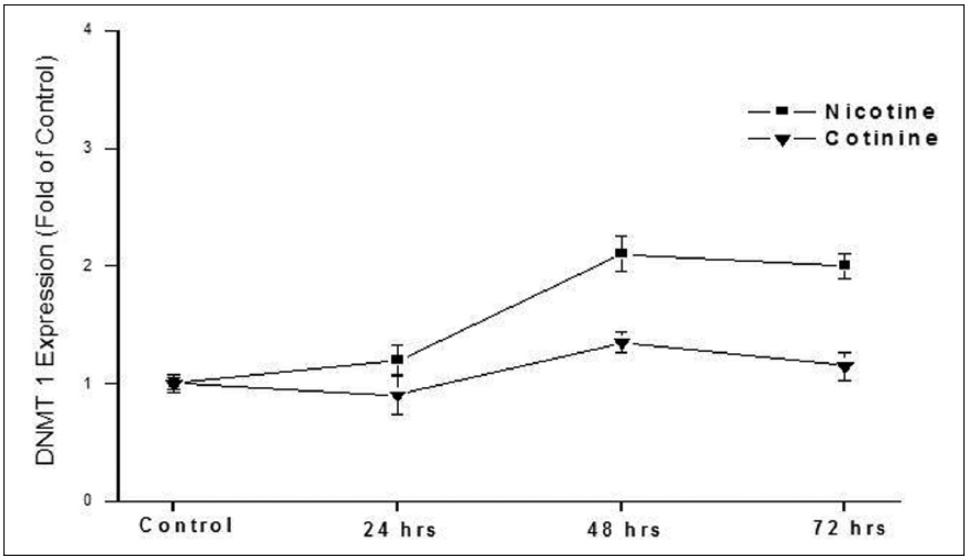


**Figure 5.** Effect of nicotine and cotinine exposure duration on expression of DNMT1. HepG2 cells were exposed to 50.0 μM nicotine or cotinine for 24, 48, or 72 h. Expression levels were determined by quantitative real-time RT-PCR. Data are presented as mean ± SD of at least three determinations.

Only limited data appear to be available on the effects of cigarette smoke constituents on DNMT expression. Our examination of the effect of nicotine and cotinine on DNMT1 expression explored the contribution of these constituents. At least one other report describes the effect of nicotine on DNMT1. Results were, however, in contrast to those found in this study. Down-regulation of DNMT1 expression was demonstrated in the frontal cortex of mice injected with nicotine.[35] The effect of other cigarette smoke constituents has also been investigated. Exposure of HBEC lung cells to methylnitrosourea and benzo(a)pyrene-diolepoxide increased significantly levels of DNMT1.[36] Additional research will be needed to develop this area of study.

The underlying mechanism(s) for the observed influence of CSC on DNMT expression remain to be completely established. Earlier studies suggest that one of the mechanisms

involved in increased DNMT1 in smokers may be the *ras* pathway since smoking has been found to be associated with increased *ras* expression.[37–39] The JUN/FOS transcription factors have been shown to activate murine DNMT1 transcription through the AP-1 target motifs.[40] Furthermore, oncogenic RAS-stimulated signal transduction pathways increase murine DNMT1 expression[40] and expression in human T cells[41] and alter DNA methylation patterns.[42] More recent studies have identified several other signaling pathways involved in the regulation of DNMT expression that may have a role in CSC-induced expression of DNMT1. These include transforming growth factor-β (TGF-β),[43] glioma-associated oncogene family zinc finger 1 (GLI1),[44] a transcriptional factor in Hedgehog signaling pathway, and Sp1/NF-κB.[45] Levels of each of these transcriptional factors have been shown to respond to cigarette smoke.[46–48] DNMT1 can also be regulated by microRNAs, as demonstrated with miR-148a and miR-152.[49,50] Although shown for other microRNAs, whether cigarette smoke affects these microRNAs has, however, not been reported.

A limitation of this study is noted. CSC is widely used in model systems to study in vitro effects of tobacco smoke.[31–34,51–55] A disadvantage is that cell culture models often do not exhibit all the differentiated and functional characteristics of the corresponding native epithelium or the entire organ. A concern can also be the inherent instability, especially on long-term culture. Treatment with CSC may not exactly replicate in vivo responses to smoke exposures. However, in in vitro research, cellular and subcellular functions can be studied with more ease in a simplified, direct biological model system using guidelines for good cell culture practice, which allows the prediction of mechanisms that may be relevant to in vivo situations. HepG2 are widely used and are useful as a model system in vitro for human hepatic cells. These cells are highly differentiated and display many of the genotypic features of normal liver cells.[56] This cell line is, however, known to have low metabolic capacities.[57] Despite the potential limitation, our experiments yielded several interesting and potentially relevant findings.

In summary, a full understanding of the harmful effects of cigarette smoke requires consideration of its epigenetic action. Results from this study clearly provide additional support of the modulating effect of cigarette smoke on DNMT1 expression. Although the underlying mechanisms are not yet known, given the potential of alterations in DNMT expression to affect cellular function, this pathway may play a critical role in cigarette smoke-induced diseases.

## Declaration of conflicting interests

The views presented in this article do not necessarily reflect those of the Food and Drug Administration.

## Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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