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LINE-1 gene hypomethylation and p16 gene hypermethylation in HepG2 cells induced by low-dose and long-term triclosan exposure: The role of hydroxyl group

Liudan Zeng [a,b], Huimin Ma [a,b,*], Shangxia Pan [a,b,c], Jing You [a,b], Gan Zhang [a,b], Zhiqing Yu [a,b], Guoying Sheng [a,b], Jiamo Fu [a,b]

[a] State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China
[b] Guangdong Key Laboratory of Environmental Protection and Resources Utilization, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China
[c] Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

Triclosan (TCS), a frequently used antimicrobial agent in pharmaceuticals and personal care products, exerts liver tumor promoter activities in mice. Previous work showed high-dose TCS (1.25–10 μM) induced global DNA hypomethylation in HepG2 cells. However, whether or how tumor suppressor gene methylation changed in HepG2 cells after low-dose and long-term TCS exposure is still unknown. We investigate here the effects and mechanisms of DNA methylation of global DNA(GDM), repetitive genes, and liver tumor suppressor gene (p16) after exposing HepG2 cells to low-dose TCS (0.625–5 nM)for two weeks using HPLC–MS/MS, Methylight, Q-MSP, Pyrosequencing, and Massarray methods. We found that low-dose TCS exposure decreased repetitive elements LINE-1 methylation levels, but not global DNA methylation, through down-regulating DNMT1 (DNA methyltransferase 1) and MeCP2 (methylated DNA binding domain) expression, and up-regulating 8-hydroxy-2-deoxyguanosine (8-OHdG) levels. Interestingly, low-dose TCS elevated p16 gene methylation and inhibited p16 expression, which were not observed in high-dose (10 μM) group. Meanwhile, methyl-triclosan could not induce these two types of DNA methylation changes, suggesting the involvement of hydroxyl in TCS-mediated DNA methylation changes. Collectively, our results suggested low concentrations of TCS adversely affected HepG2 cells through DNA methylation dysregulation, and hydroxyl group in TCS played an important role in the effects. This study provided a better understanding on hepatotoxicity of TCS at environmentally relevant concentrations through epigenetic pathway.

1. Introduction

As a broad spectrum antibacterial agent, triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy) phenol) has been widely used in personal care, industrial, and household products at concentrations up to 0.3% (w/w) (Fiss et al., 2007). Due to its capacity to inhibit microbial growth, TCS has also been added to food coverings (Dann and Hontela, 2011). As a result of its extensive use, TCS has been detected in various environmental media in the ranges of 10 to 2300 ng/L (0.035–7.945 nM) (Sabaliunas et al., 2003) as well as in humans, e.g., urine, plasma, and breast milk (Fang et al., 2010). Additionally, increased levels of TCS were noted in the elders and people with high socioeconomic status (Singer et al., 2002). This suggests that TCS may accumulate in human body (Honkisz et al., 2012). Recent evidence showed that TCS may react with others chemicals to form carcinogen byproducts in the environment, such as chloroform (Fiss et al., 2007), 2,8-dichloriodibenzo-p-dioxin (2,8-TCDD), 2,3,7-TCDD, 1,2,8-TCDD, and 1,2,3,8-TCDD (Latch et al., 2005), which has resulted in growing awareness of TCS toxicity.

TCS was identified as a potential carcinogen by the United States Environmental Protection Agency (USEPA) in 2008. Its toxicity mainly resides in carcinogenicity and endocrine effects on mammalians, such as causing the hepatocellular adenomas and carcinomas in mice (Rodricks et al., 2010). Specially, Yueh et al. resported in Proceeings of the National Academy of sciences of United States of America(PNAS) that long-term TCS exposure (8 months) enhancing liver fibrogenesis and tumorigenesis in mice (Yueh et al., 2014). Recent cohort data showed that TCS was associated with allergy or hay fever diagnosis (Bertelsen et al., 2013). Based on its wide use in daily life and the rapidly increasing healthy toxicity database for TCS, the USEPA began new registration review progress for TCS in 2013.

Genetic studies showed that TCS had no significant genotoxicity in rodents (Klaunig et al., 2000), however, limited information is available on the causality of hepatotoxicity by TCS. Differing from genetic studies, DNA methylation describes transmission of heritable states of gene expression that do not involve sequence changes in DNA, and its

* Corresponding author.
E-mail address: mahuimin@gig.ac.cn (H. Ma).

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deregulation is an important feature of cancer development and progression (Wilson et al., 2007).For example, some hepatocarcinogens, such as polychlorinated biphenyls (PCBs), tetrachlorodibenzo-p-dioxin (TCDD), phenobarbital, hexabromocyclododecane (HBCD), estradiol, and hexachlorobenzene (HCB) exhibit abnormal epigenetics, but no genotoxicity characteristics (Aniagu et al., 2009; Vandegehuchte and Janssen, 2013).

Hypomethylation of global DNA and repetitive elements genes, and hypermethylation of tumor suppressor genes are the main types of DNA methylation biomarkers of initiation and progression of cancer (Wilson et al., 2007). Our previous work showed that TCS at high doses induced the global DNA hypomethylation in human hepatocellular HepG2 cells (Ma et al., 2013). Different from our previous study on TCS (Ma et al., 2013), repetitive elements which dispersed throughout the genome, were chosen to represent the global DNA methylation in mammalian in the current study (Baccarelli and Bollati, 2009), for they can minimize the background of normal DNA. For example, short interspersed nucleotide elements Alu and long interspersed nucleotide elements LINE gene hypomethylation were reported to be related to the initiation and progress of liver tumor (Wilson et al., 2007). Additionally, as a tumor suppressor gene, p16 (CDKN2A) gene hypermethylation was found in many kinds of tumor, such as liver tumor (Soberanes et al., 2012), and was proposed to have a prognostic value of many diseases (Krajnovic et al., 2013). Recently, p16 gene hypermethylation was reported to be a driver in cancer metastasis (Cui et al., 2015). So in the current study, the changes of p16 were evaluated to better understand the hepatotoxicity of low-dose TCS exposure. Besides, the role of hydroxyl group on TCS-induced DNA methylation was also explored.

As epigenetics factors, DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, and DNMT3b maintain and *de novo* methylated DNA, therefore they participate in DNA demethylation process. Methylated DNA binding domain protein MeCP2 indirectly inhibits global DNA hypomethylation (Ou et al., 2004, 2007). Moreover, MeCP2 has been found to maintain GDM level *in vivo* through direct binding to DNMT1 (Kimura and Shiota, 2003), so the DNMT1–MeCP2 pathway is an important way to understand the global DNA methylation changes. In addition, 8-hydroxy-2-deoxyguanosine (8-OHdG), a biomarker of oxidative damage of DNA (Valavanidis et al., 2009), negatively regulates GDM by interfering with the binding of DNMT–DNA. Indeed, in the case of high dose TCS exposure, TCS decreased GDM level in a dose-dependent manner and the decrease of GDM level was accompanied with oxidative DNA damage and inhibition of the methylated DNA binding domain (MBD2, MBD3, and MeCP2) gene expression (Ma et al., 2013).

High-dose and short-time TCS exposures (treatments at 1.25–10 μM for 24 h) are not commonly involved in human daily life. Rather, long-term exposure at low doses of environmental pollutants is more realistic, but little information is available on the hepatotoxicity after low-dose exposure to TCS. Additionally, methylation changes of repetitive elements and tumor suppressor genes induced by TCS are still unknown. The objectives of the current study are to evaluate whether the long-term and low-dose TCS exposure can affect the DNA methylation of p16 gene and repetitive elements, and to explore the role of the hydroxyl group in TCS in TCS-induced DNA methylation changes.

2. Materials and methods

2.1. Chemicals and enzymes

Methanol was purchased from Merck (Darmstadt, Germany). Formic acid, sodium acetate, and zinc butter were obtained from Dupont (Wilmington,Delaware, USA), and nuclease P1, 8-OHdG, 5mdC, snake venom phosphodiesterase I, 5-aza-deoxycytidine (AZA), and methyl-triclosan (MTCS) were purchased from Sigma (St. Louis, MO, USA). Calf intestine alkaline phosphatase and M.*Sss*I methyltransferase were obtained from New England Biolabs (Beverly, CA, USA), and 2′-deoxycytidine (dC) and 2′-deoxyguanosine (dG) were purchased from Amresco (Solon, OH, USA). All chemicals were of HPLC or analytical grade. Deionized water was prepared using a Direct-Q water purification system (Bedford, OH, USA).

2.2. Cell culture, chemical treatment, and DNA isolation

HepG2 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and were cultured in high-glucose DMEM medium (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, San Diego, CA, USA) at 37 °C in 5% CO_2 atmosphere. The cells at about 80% confluence were used for experiments.

HepG2 cells at 1×10^5 cells/ml were seeded in a cell bottle and treated in triplicate with freshly prepared TCS at - (0.625, 1.25, 2.5, 5 nM) concentrations in dimethyl sulfoxide (DMSO) for 2 weeks, and cells treated with AZA were used as positive control. The media containing different concentrations of chemicals were changed every day.

The HepG2 cells were harvested, and washed once with sterile phosphate-buffered saline (PBS), and lysed in lysis buffer containing 1 μM butylated hydroxytoluene to protect the DNA from oxidative damage. The genomic DNA of the cells was extracted using a NanoMag Reagent Genomic DNA Isolation Kit (Shannuo Scientific Company, China). The quality and quantity of the DNA samples were assessed using the DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Individual DNA samples with a ratio of A_{260}/A_{280} between 1.8 and 2.0 were aliquoted and stored at −80 °C.

2.3. HPLC–MS/MS analyzing the 5mdC and 8-OHdG

Enzymatic hydrolysis of DNA was performed as described previously (Hu et al., 2010; Ma et al., 2013). Briefly, 1 μg individual DNA samples was dissolved in deionized water, denatured in 100 °C for 3 min, and then hydrolyzed at 37 °C with 2 U of nuclease P1 for 3 h and 0.002 U of snake venom phosphodiesterase I and 0.5 U of calf intestine alkaline phosphatase overnight. The final DNA hydrolysates were filtrated through a Microcon centrifugal filter device (YM-10, MW cut-off 3000 Da, Millipore, Bedford, MA, USA) by centrifuging at 12,000 *g* at 4 °C for 30 min.

The components of individual DNA hydrolysate samples were analyzed using an Agilent 1100 series HPLC system coupled with API 4000 triple quadrupole mass spectrometer (Applied Biosystems) in electrospray ionization mode Analytical method was described previously with minor modifications (Ma et al., 2013). Optimized positive ESI-MS/MS conditions were obtained for three channels: 5mdC (m/z 241.9/126.3), dG (m/z 268.1/152.3), and 8-OHdG (m/z 284.2/152.3). The level of 5mdC was expressed as [5mdC] / [dG] × 100%, while the level of 8-OHdG was expressed as [8-OHdG] / [dG] × 10^6.

2.4. Methylight assay of Alu and LINE gene methylation

DNA was exacted, then 540 ng genomic DNA was bisulfited using Zymo bisulfited modified kit (Zymo Research, Orange, CA, USA). The methylation levels of Alu-M1 and LINE-1 were analyzed using Methylight method as described previously (Weisenberger et al., 2005; Gaudet et al., 2009). PCR was performed in the volume of 20 μl with 300 nM forward and reverse primers, 100 nM probe, 12.5 μl Thunderbird Probe qPCR Mix (Toyobo, Osaka, Japan), and bisulfited DNA (40 ng for repetitive sequence assay), using the following PCR program: 95 °C for 10 min, then 50 cycles of 95 °C for 15 s followed by 60 °C for 1 min, and the primers used are shown in Table 1. The Methylight reaction was performed on AB 7500 Real-time PCR instrument. Over methylated PBL DNA was used as the fully methylated DNA sample, whose level was considered as 100%, and Alu-C4 was used to measure the levels of input DNA to normalize the signal of repetitive element genes Methylight reaction (Gaudet et al., 2009).

Table 1
Sequence of the primers used in the current study.

Methylight	Forward primer	Reverse primer	Probe
ESR1	GGCGTTCGTTTTGGGATTG	GCCGACACGCGAACTCTAA	6FAM-CGATAAAACCGAACGACCCGAC GA-BHQ-1
LINE-1	GGACGTATTTGGAAAATCGGG	AATCTCGCGATACGCCGTT	6FAM-TCGAATATTGCGTTTTCGGATCGG TTT-BHQ-1,
Alu-M2	GCGCGGTGGTTTACGTTT,	AACCGAACTAATCTCGAACTCCTAAC	6FAM-AAATAATCCGCCCGCCTCGA CCT-BHQ-1
ALU-A4	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAG TA	ATTAACTAAACTAATCTTAAACTCCTAACCTCA,	6FAM-CCTACCTTAACCTCCC-MGB
β-actin	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	FAM-ACCACCACCCAACACACAATAACAAA CACA-TAMRA
Q-MSP	Forward primer	Reverse primer	
P16 M	GTTTTTTAAATTTTTTGGAGGGATC	AAATACCACATTCGCTAAATACTCG	
P16 U	TTTTTAAATTTTTTGGAGGGATTG	ATACCACATTCACTAAATACTCAAA	
W-β-actin	ATCTGGCACCACACCTTC	AGCCAGGTCCAGACGCA	
M-β-actin	TGGTGATGGAGGAGG TTTAGTAAGT	AACCAATAAAACCTACTCCTC CCTTAA	
Pyrosequencing	Forward primer	Reverse primer	Probe
LINE-1	TTTTGAGTTAGGTGT GGGATATA	AGTTAGGTGTGGGATATAGT	Biotin-AAAATCAAAAAATT CCCTTTC
RT-PCR	Forward primer	Reverse primer	
DNMT1	ACCTGGCTAAAGTCAAATCC	ATTCACTTCCCGGTTGTAAG	
DNMT3a	CAGCTTCCACGTTGCCTTCT	CATCTGCAAGCTGTCTCCCTTT	
DNMT3b	TTGGAATAGGGGACCTCGTGTG	AGAGACCTCGGAGAACTTGCCATC	
Mecp2	CCCCACCCTGCCTGAA	GATGTGTCGCCTACCTTTTCG	
β-actin	TGGCACCCAGCACAATGAA	TAAGTCATAGTCCGCCTAGAAGCA	
Massarray	Forward primer	Reverse primer	
p 16	aggaagagagGGTTTTTTTAGAGGACTTGAGGGAT	cagtaatacgactcactatagggagaaggctATTCCTCTTCCTTAACTTCAAAC	

A series of diluted M.*Sss*I DNA were amplified in every plate and used to generate a standard curve, and methylation levels of each genes were expressed as percent methylation ratio (PMR): PMR = 100% × (relative copy number of target gene in sample / relative copy number of control gene in sample) / (relative copy number of target gene in M.*Sss*I sample / relative copy number of control gene in M.*Sss*I sample). In general, a PMR < 4 was considered as unmethylated. Each experiment was performed in quadruplicate, and each Methylight reaction was performed in duplicate.

2.5. PCR and Pyrosequencing of LINE-1 and ESR1 gene methylation

DNA was exacted from 5 nM TCS-treated HepG2 cells after 2 weeks, then 1 μg genomic DNA was bisulfited. Methylated DNA level of LINE-1 gene was performed using a Pyrosequencing method according to a method proposed by Bollati et al. (2007). In brief, bisulfited DNA of cells was used as the template, and was amplified using TAQ enzyme kit (KAPA Biosystems, USA) according to the instructions. The PCR conditions included 95 °C for 3 mins, and 40 cycles of 90 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 7 mins. Then, the biotin-labeled products were purified and alkaline denatured to ssDNA using Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA) followed by Pyrosequencing. The Pyrosequencing reaction was performed using PyroMark Q 96 ID (QIAGEN), and the amounts of dNTP, substrates, and enzymes were according to designed results of the Pyrose Quenching soft (QIAGEN). Finally, the methylation levels of CpG sites in the sequence were calculated using the PyroQ-CpG software (QIAGEN). LINE-1 gene methylation was expressed as the methylated divided by the sum of methylated and unmethylated.

2.6. Q-MSP assay and Massarray assay of p16 gene methylation

DNA methylation state of p16 gene was detected by quantitative methylated-specific PCR (Q-MSP). In brief, 600 ng DNA were bisulfited, and DNA amplification was measured using SYBR Green qPCR by the delta–delta Ct (DDCt) method (Soberanes et al., 2012). Two sets of primers, were used to amplify the interest area of p16 gene (Chr9:21,965,166–21,965,277, NCBI36/hg18), which includes the transcription start site (see Table 1).

The Sequenom MassARRAY platform was also used to perform the quantitative methylation analysis of p16 promoter. The primers used in this system were designed using software SEQUENOM (http://www.epidesigner.com). 1 μg genomic DNA was treated with $NaHSO_4$ to transform the Cytosine to Urical while methylated cytosine remained no change. Then bisulfited DNA of cells was amplified using the designed primers which were further added the 5′-aggaagagag-3′ sequences (Table 1). The PCR was performed using the following conditions: 94 °C for 4 min, and 45 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 3 min. SAP (Shrimp alkaline phosphatase) enzyme was used to digest the unincorporated dNTPs in PCR products, followed by being treated with 95 °C for 3 min to inactivate SAP. PCR products were then transcript to RNA using T7 RNA&DNA polymerase (Epicentre, Madison, WI, USA) because Uracil base in RNA can be enzyme digested by Uracil-specific cleavage (RNase A, Sequenom, San Diego, CA, USA). The methylation data for each sample were generated by Epityper software version 1.0 (Sequenom, San Diego, CA, USA). In the end, the Uracil-ended oligonucleotides were analyzed using MALDI-TOF MS. A 512 bp fragment of p16 gene TSS area was examined in this experiment, which includes 38 CpG sits, and 15 CpG sits can be detected. In this p16 gene methylation experiments, the DNA methylation level of each CpG site was expressed as the mean value of the ratio of methylated to the sum of methylated and unmethylated, and the DNA methylation value of p16 was calculated as an average of all sites.

2.7. DNMT activity assay

Treated-HepG2 cells were harvested, and the nuclei were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (BioTeke, Beijing, China) according to the manufacturer's instruction. DNMT activity was determined using DNMT activity assay kit (Epigentek, Brooklyn, USA). HepG2 cells treated with 1 μM 5-AZA for 24 h were used as

positive control to DNMT activity kit. DNMT activity values were expressed as: DNMT Activity (OD/h/mg) = (Sample OD – Blank OD) / Protein Amount (μg) × reaction time (h) × 1000.

2.8. DNMT and MeCP2 gene expression

Gene expression assay was performed as described in our previous work (Ma et al., 2013). Briefly, the total RNA in the cells was extracted using Trizol Reagent (Invitrogen, Carlsbad, USA), and 2 μg of total RNA from each sample were reversely transcribed into cDNA using a Prime-Script™ RT reagent Kit (Takara, Dalian China). The relative levels of target gene mRNA transcripts to control β-actin were characterized by quantitative PCR using the cDNA template, specific primers and SYBR® Premix Ex Taq™ (Takara, Dalian China) on an AB 7500 Fast System (Applied Biosystems). The sequences of primers used in our experiment are shown in Table 1. The relative levels of each target mRNA transcripts to the control β-actin were analyzed by $2^{\Delta\Delta Ct}$ and expressed as fold changes.

2.9. DNMT and MeCP2 protein expression

At the end of the cell treatments, HepG2 cells were collected and centrifuged at 2000 rpm for 5 min. Proteins were extracted using the protein extraction kit of Bioteke Corporation according to the manufacturer's instruction. The cell lysates were centrifuged at 13,000 rpm and 4 °C for 15 min. 20 μg soluble proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then electrophoretically transferred to polyvinylidene fluoride membranes. Then the membranes were blocked in 5% milk/Trisbuffer saline/Tween-20. Subsequently, the membranes were incubated with rabbit anti-DNMT1(1:800, CST, 5032), anti-DNMT3a (1:1000, CST, 3598), anti-DNMT3b(1:1000, Santa, 376,043), anti-MeCP2 antibodies (1:1000, CST, 3456), anti-GAPDH (1:4000,ProteinTech Group, 60004-1), and anti-β-actin (1:4000, ProteinTech Group, 60008-1) at 4 °C overnight and washed with PBS twice. The secondary immunoglobulin conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.) was used at a 1:3000 dilution according to the manufacturer's protocol. The membranes were washed twice with PBS and the bands on the membranes were visualized using chemiluminescence method (PIERCE, USA). All Western blot analyses were performed in triplicate. Bio-rad Quantity Ones software was used to analyze the gray value of the protein expression in each group. All amounts of the proteins was relatively quantified based on the normalization to GAPDH protein, and 0.01% (v/v) DMSO was used as the control.

2.10. Statistical analysis

Statistically analyses were performed using SPSS software 9 version 13.0, (SPSS Inc., Chicago, IL, USA). The difference among groups was analyzed using an ANOVA/Dunnett's post-hoc multiple comparison test; the difference between the two groups was analyzed using Student's *t*-test. A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Effects of low-dose TCS on methylation levels of GDM and repetitive elements after a two-week exposure

The GDM level in normal HepG2 cells was 3.37 ± 0.05% compared with the dG levels. The exposure of the cells to 3 μM AZA for 24 h down-regulated GDM to 75.5% of the control group, which validated our HPLC–MS/MS method that was used to investigate 5mdC level in HepG2 cells. To assess the effects of TCS on GDM, HepG2 cells were treated with TCS at environmentally relevant doses (from 0.625 to 5.0 nM) for two weeks. The exposure to TCS at test levels did not change the relative GDM levels to the control group (Fig. 1.A, $p > 0.05$).

Methylation levels of repetitive elements LINE-1-M1 and Alu-M2 genes were analyzed by the Methylight method. In this method, a reproducible and sensitive CpG methylation independent Alu control, Alu-C4 gene, was chosen to measure the amount of input DNA (Weisenberger et al., 2005) and AZA was used as the positive control (Fig. 1B). PMR values of Alu-M2 and LINE-1-M1 genes in the cells treated with 5 nM TCS were 47.3 ± 8.04 and 34.2 ± 6.60, respectively, compared with over methylated PBL DNA. As expected, LINE genes showed a significant hypomethylation in TCS treated group, and the relative methylation levels reduced to 82.8 ± 6.8% ($p < 0.05$). The inter assay coefficients based on triplicate PMR for Alu-M2 and LINE-1-M1 were 5.34 and 13.48, respectively.

For further confirmation of the TCS-induced hypomethylation, the Pyrosequencing method was used to investigate the LINE-1 gene methylation. As shown in Fig. 1C LINE-1 gene methylation levels in DMSO and TCS group of HepG2 cells were 37.5 ± 0.87% and 34.3 ± 2.70%, respectively. As expected, TCS induced 8.5 ± 2.7% decreases in LINE-1 gene methylation levels compared with the control group.

3.1. Effect of Hydroxyl group of TCS on LINE-1 methylation changes

The changes of GDM levels and LINE-1 gene methylation in MTCS-exposed cells were compared with the TCS-exposed cells to investigate the contribution of hydroxyl group in TCS to hypomethylation of GDM and repetitive elements. As shown in Fig. 1C and D, incubation with 5 nM MTCS for 2 weeks did not change the gene methylation levels of GDM (Fig. 1E) and LINE-1 (Fig. 1D and Fig. 1E). After replacing the hydroxyl group in TCS with methoxy group in MTCS (Fig. 1E), the observed LINE-1 gene hypomethylation disappearenced, suggesting that hydroxyl group in TCS may play an important role on the methylation in HepG2 cells.

3.1. Effects of low-dose TCS on DNMT activity and DNMT1 expression after exposure for two weeks

DNMT activity, gene expression, and protein expression were investigated in TCS-treated HepG2 cells to reveal the mechanisms of LINE-1 hypomethylation. Low-dose TCS treatments at 0.625, 1.25, 2.5, or 5.0 nM significantly decreased DNMT activity (Fig. 2A) and DNMT1 gene expression (Fig. 2B) ($p < 0.05$).The latter was further validated by protein expression (Fig. 2C-D). The activity of DNMTs data showed that 3 μM AZA, the positive control, reduced DNMT activity to 17% after 24-h treatment (Fig. 2A), which validated the results of low-dose TCS treatments. Comparatively, treatment with 5 nM MTCS for 2 weeks did not significantly change DNMT1 gene expression in HepG2 cells (104.6 ± 8.4%, $p > 0.05$).

3.2. Effects of low-dose of TCS on MeCP2 expression after exposure for two weeks

Our previous data showed that TCS at high concentrations induced changes of the MBD gene expression. Therefore, the changes of MeCP2 gene transcription in HepG2 cells after low-dose TCS exposure were assessed by real-time PCR. As shown in Fig. 3A, TCS at low doses significantly inhibited the MeCP2 gene transcription ($p < 0.05$), which was confirmed by the western blotting assay (Fig. 3 B-C). Conversely, the changes of MeCP2 gene expression were not observed in MTCS-exposed HepG2 cells (the relative gene expression of MeCP2 was 100.6 ± 5.5% ($p > 0.05$) after a 2-week exposure to 5 nM MTCS).

3.3. Effects of low-dose TCS on 8-OHdG levels after exposure for two weeks

To further elucidate the mechanism by which low-concentration TCS promoted LINE-1 gene hypomethylation, HepG2 cells were treated with TCS at different doses for two weeks, and the contents of 8-OHdG in the genomic DNA were determined by HPLC–MS/MS. The background

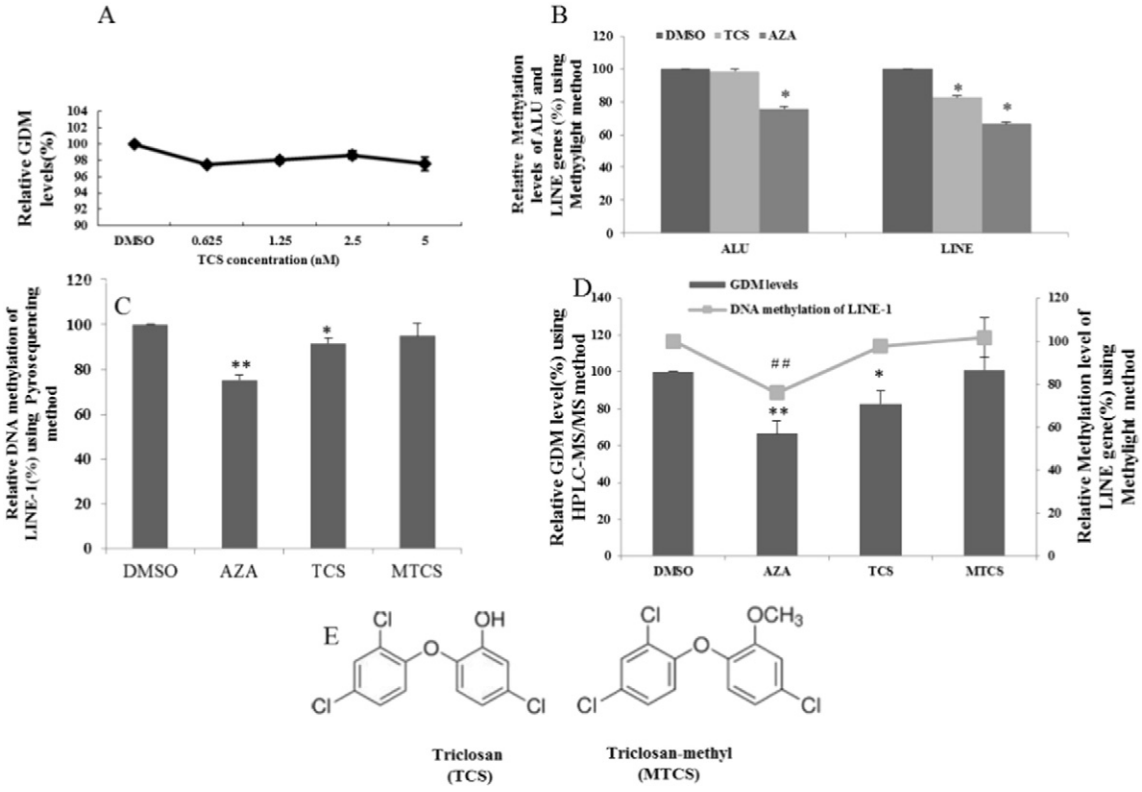

Fig. 1. Effects of low-dose TCS on methylation levels of GDM and repetitive elements in HepG2 cells. A) Relative GDM levels in the cells after being treated with TCS at various concentrations for two weeks, and relative GDM levels were analyzed using HPLC–MS/MS; B) Alu and LINE-1 gene methylation levels which were measured using Methylight method in cells treated with 5 nM TCS and 10 μM AZA for two weeks and 48 h, respectively. C) LINE-1 gene methylation in HepG2 cells which were treated with 10 μM AZA (24 h), 5 nM TCS (2 weeks), and 5 nM MTCS (2 weeks), and LINE-1 gene methylation was analyzed by pyrosequencing method. D) The GDM (bar) and LINE-1 gene methylation (line) in HepG2 cells which were treated with 10 μM AZA, 5 nM TCS, 5 nM and MTCS for 24 h, 2 weeks, and 2 weeks respectively, and the GDM and LINE-1 gene methylation were investigated using HPLC–MS/MS and Methylight method, respectively. E) The structures of TCS and MTCS. Control cells were treated with 0.1% DMSO (v/v) in the medium. Data are presented as mean ± standard deviation values of four replicates. Significant difference between the treatments and the control was indicated by * ($p < 0.05$), ** ($p < 0.01$), and ## ($p < 0.01$).

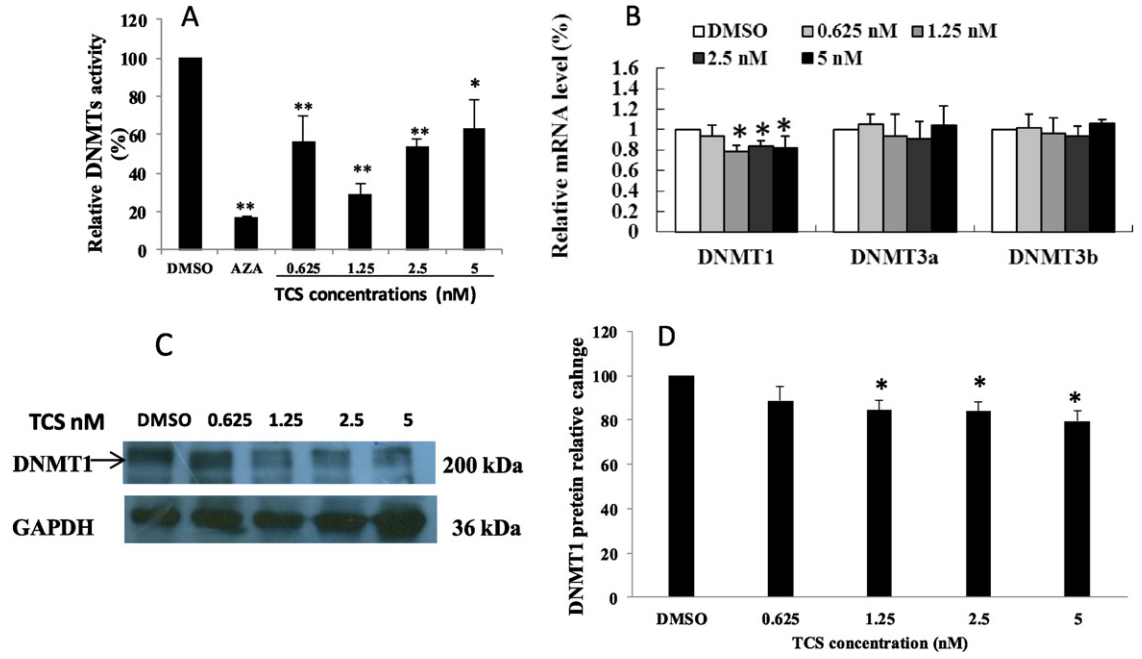

Fig. 2. Effects of low-dose TCS treatment on DNMT activity, gene and protein expression. HepG2 cells were treated with 0.625 to 5.0 nM TCS for two weeks. A) Effects of TCS treatment on DNMT activity in HepG2 cells. The treatment with 3 μM AZA for 24 h was used as a positive control. B) Relative levels of DNMT1, DNMT3a, and DNMT3b mRNA transcription to the control β-actin which were determined by RT-PCR. The levels of target gene mRNA transcription were designated as 1 in control group. Data are expressed as the mean ± SD ($n = 3$). C) DNMT1 protein expression was down-regulated by TCS. D) DNMT1 protein expression in HepG2 cells after TCS treatments. The gray values of each band of DNMT1 protein were analyzed by Biorad Quantity Ones software. GAPDH protein was used as a loading control. Significant difference from the control group was indicated by * ($p < 0.05$) and ** ($p < 0.01$).

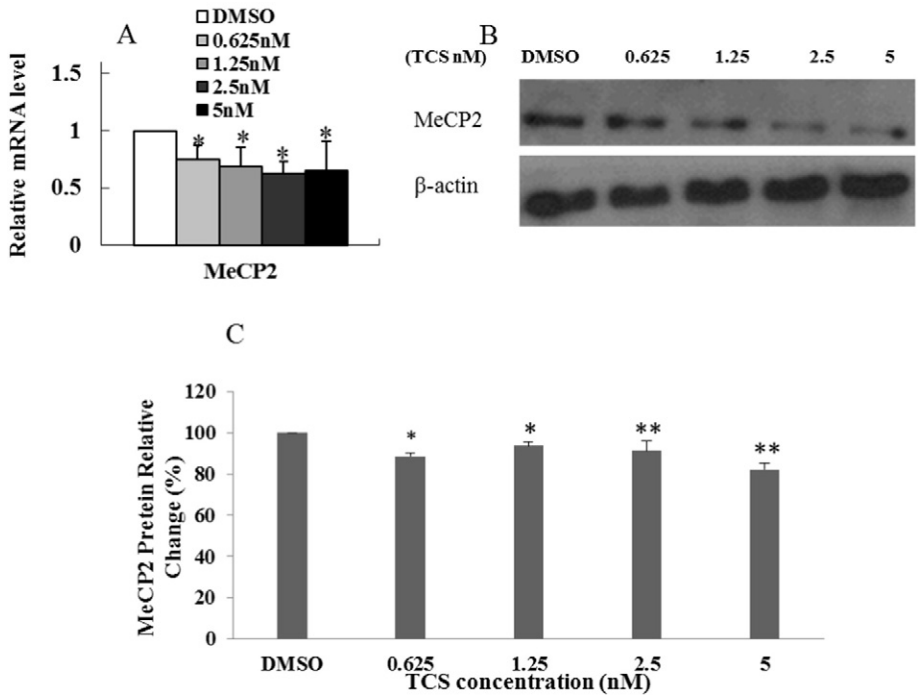

Fig. 3. Effects of low-doses of TCS exposure on MeCP2 gene and protein expression. HepG2 cell were treated with the indicated concentrations of TCS for two weeks. A) Relative levels of MeCP2 mRNA transcription to the control β-actin which were determined by RT-PCR. The levels of target gene mRNA transcription were designated as 1 in control group. Data are expressed as the mean ± SD (n = 3). B) MeCP2 protein expression was down-regulated by TCS. C) MeCP2 protein expression in HepG2 cells after TCS treatments. The gray values of each band of MeCP2 protein were analyzed by Bio-rad Quantity Ones software. GAPDH protein was used as a loading control. Significant difference from the control group was indicated by * ($p < 0.05$) and ** ($p < 0.01$).

level of 8-OHdG levels in HepG2 cells was $2.3/10^6$ dG, which was similar to the reported data by Yuan et al. (2009). After treated with low-dose TCS, 8-OHdG levels significantly increased ($p < 0.01$) compared with the control group (Fig. 4A).

To assess the acceleration effects of 8-OHdG on LINE-1 hypomethylation, 8-OHdG inhibitor NAC was used to check if NAC can impair the TCS-mediated hypomethylation. Firstly, the inhibition effect of NAC on LINE-1 gene hypomethylation was checked at high concentration of TCS (10 μM) because more significant DNA hypomethylation could be induced at high dose of TCS compared with low-dose TCS exposure. As shown in Fig. 4 B, DNA methylation levels of TCS + NAC group were 77.7 ± 9.6%, which was higher than that of the TCS group (67.0 ± 13.1%) but lower than that of the NAC group (84.4 ± 3.0%). This result conformed our hypothesis that 8-OHdG may contribute to LINE-1 gene hypomethylation by steric hindrance.

3.1. Effects of low-dose TCS exposure on p16 gene hypermethylation

The tumor suppressor gene p16 was chosen to evaluate the hepatotoxicity of low-dose exposure to TCS (Fig. 5). After treatment with 5 nM TCS, p16 gene methylation was measured using the Q-MSP method and it increased 39.7 ± 12.6% compared with the control (Fig. 5A), and the gene expression decreased to 31.58 ± 9.8% (Fig. 5C).In addition, DNA methylation inhibitor AZA group was used as a positive control, which down-regulated p16 gene methylation (Fig. 5A) and up-regulated mRNA expression (Fig. 5C).

Massarray assay method was used to confirm p16 gene hypermethylation (Fig. 5B). In this method, the 829 bp DNA sequence of p16 gene promoter area was detected, which included the target sequences of Q-MSP method. After treating the cells with 10 nM TCS for 2 weeks, p16 gene hypermethylation was induced while gene expression

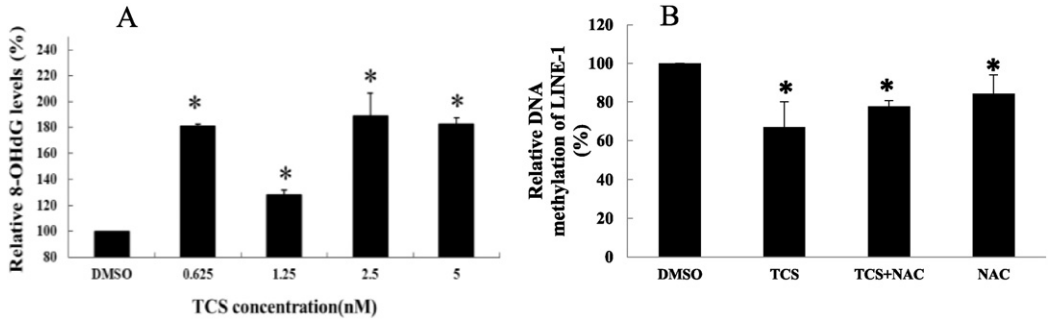

Fig. 4. Effects of TCS on 8-OHdG levels in HepG2 cells. A) HepG2 cells were treated with TCS at 0.625 to 5.0 nM for two weeks and the levels of 8-OHdG were determined using HPLC–MS/MS; B) after HepG2 cells were exposed to TCS (10 μM), NAC (100 μM), and TCS (10 μM) + NAC (100 μM), respectively, for 24 h, DNA methylation of LINE-1 gene was examined using Methylight method. Data are expressed as mean ± SD (n = 4) and cells treated with 0.1% DMSO (v/v) was considered as the control (100%). Significant difference from the control was indicated by * ($p < 0.01$).

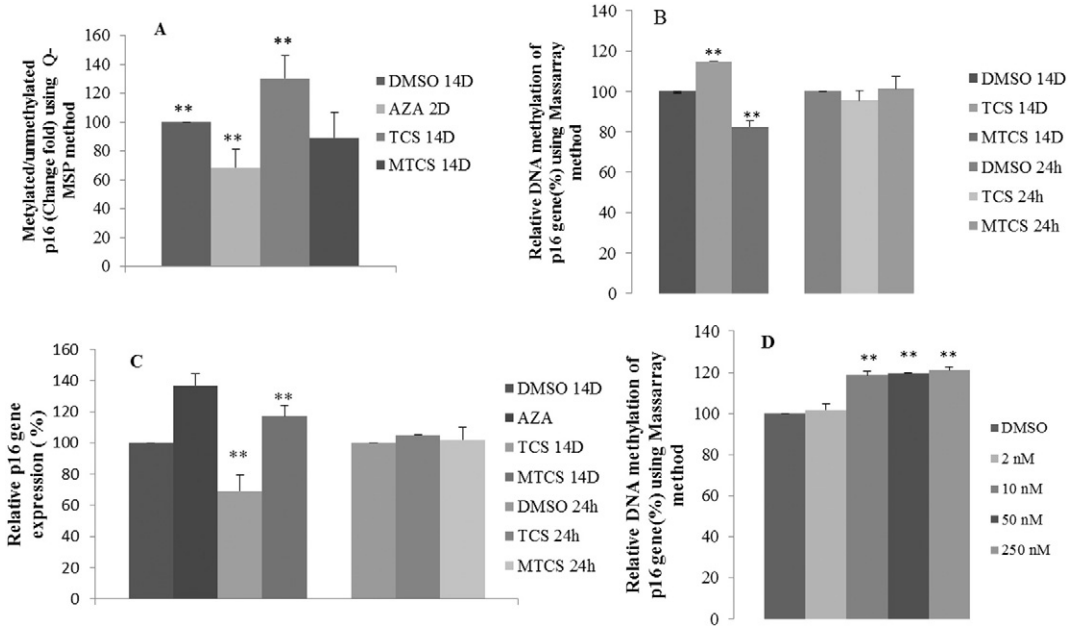

Fig. 5. Effects of low-dose TCS exposure on p16 gene methylation. HepG2 cells were treated with 5 nM TCS or MTCS for 2 weeks, and treated with 10 μM TCS or MTCS for 24 h. A) Changes of p16 gene methylation were investigated using Q-MSP method; B) changes of p16 gene methylation were investigated using Massarray method; (C) RT-PCR method was used to analyze the gene expression changes of p16 gene. Data are expressed as the mean ± SD (n = 4); Cells treated with 0.1% DMSO (v/v) was considered as the control (100%). D) Changes of p16 gene methylation were investigated in different concentrations TCS exposed HepG2 cells using Massarray method after two weeks exposure. Significant difference from the control was indicated by * ($p < 0.05$) and ** ($p < 0.01$).

decreased (Fig. 5C). Reverse results were noted in the MTCS group (Fig. 5B-C) Moreover, the changes of DNA methylation and gene expression were not detected in high-dose short-term exposure groups (10 μM TCS or MTCS for 24 h) (Fig. 5B and C).

3.2. Effects of Low-dose TCS exposure on ESR1 gene hypermethylation

To check if low-dose TCS could induce hypermethylation of other tumor suppressor genes in low-dose exposed HepG2 cells, ESR1 gene was chosen. Using the Pyrosequencing method, the DNA methylation of ESR1 promoter area was not changed (100.1 ± 0.8%, compared to DMSO group).

3.3. Dose-dependent effects of low-dose TCS exposure on DNA methylation of p16 gene

The dose-dependent effect of low-dose TCS exposure on DNA methylation of p16 gene was also explored in HepG2 cells after two weeks of exposure. And the results (Fig. 5D) showed that p16 gene methylation level was increased with the increase of exposed TCS concentrations (from 2 nM to 250 nM).

4. Discussion

In order to determine whether low-dose TCS dysregulate DNA methylation of global DNA, repetitive elements, and tumor suppressor genes in HepG2 cells, we investigated the effects of TCS exposure at low concentrations on GDM, ALU-1, LINE-1, and p16 gene methylation levels. Meanwhile, DNMTs, MeCP2, and 8-OHdG factors were also studied to explore the possible mechanisms. We found that low-dose TCS induced hypomethylation in repetitive element LINE-1 gene and hypermethylation in p16 genes in HepG2 cells. In mechanism, LINE-1 gene hypomethylation may directly or indirectly resulted from the down regulation of DNMT activity, DNMT1 and MeCP2 gene and protein expressions, and the accumulation of 8-OHdG. Furthermore, hydroxyl group of TCS may contribute to these TCS-mediated methylation changes.

As a DNA methylation biomarker of initiation and progression of cancer, changes of GDM levels were firstly investigated. The HPLC–MS/MS measured GDM level in normal HepG2 cells was similar to that in previous reports (Aniagu et al., 2009), and the positive control AZA significantly decreased the GDM level, which validated our experimental method. Yet, we found that low-dose TCS cannot induce GDM changes, which was different from our previous result of TCS exposure at high dose TCS (Ma et al., 2013).

Because of the high presentation throughout the genome, the Alu and LINE have been widely used in environmental epigenetics investigation, such as air pollution, benzene and PM10 exposure, which induced Alu and LINE gene hypomethylation in human tissues (Baccarelli and Bollati, 2009). LINE-1 comprises about 20% of human gene, and includes 6000 base pairs. There are approximately half million copies in human gene, and most of them are mutations or truncated; only approximately 200 copies are able to be transcribed. LINE-1 gene is reported to associate with human development, tissue differentiation, and gene expression. Hypomethylation may induce active transcriptions of LINE-1, then it can insert near or within genes (includes Alu genes), continues to dysregulate gene expression and cell functions. All of these actions may lie in the fact that LINE-1 gene code for an enzyme with both endonuclease and reverse transcriptase properties (Wright et al., 2010). In this study, LINE gene methylation assay showed that the hypomethylation was induced by TCS at low-dose. Compared with the GDM data, repetitive gene methylation seems more sensitive, because repetitive genes are considered as non-functional genes and would be less regulated by relative DNA methylation repair enzymes than other genes, while GDM data respond to not only repetitive genes but also to other functional genes (Wright et al., 2010).

On the contrary, the extent of decreased hypomethylation assessed by Alu gene is not significant ($p > 0.05$), suggesting that Alu gene may not be the target gene of low-dose TCS exposure. LINE gene was more sensitive than Alu gene in the evaluation of the impact of environmental pollutants on DNA methylation in human tissues. In fact, it is LINE but not Alu gene methylation in the blood samples of shortly exposed subjects decreased after exposure to black carbon and PM 2.5 in the air (Baccarelli et al., 2009). Furthermore, a cancer study data also showed

that more types of cancers presented LINE than Alu gene hypomethylation (Wilson et al., 2007). The extent of decreased LINE gene is more significant than that of the global DNA methylation measured by HPLC–MS/MS method at 5 nM TCS exposure (97.58 ± 0.82%), suggesting that hypermethylation may occur in other genomic areas, such as the promoter areas of cancer suppressor genes. Overall, methylation of repetitive elements only contributes to about half of global DNA methylation (Wright et al., 2010; Hou et al., 2010).

Two competing theories on how hypomethylation might occur are widely accepted: 1) the passive DNA demethylation (e.g. impairment of DNMTs) and 2) the active DNA demethylation (e.g. mis-regulation of demethylation enzymes or proteins) (Wild and Flanagan, 2010). To our best knowledge, the maintenance and *de novo* DNA methylation function of DNMTs were investigated for the first time by the observation of gene and protein expression and activity change of DNMTs. The results of that investigation showed that DNMT activity was significantly down-regulated. Then DNMT1 gene and protein expression was also found to be decreased. The global DNA hypomethylation was also accompanied by the down-regulation of DNMT1 expression in (−)-epigallocatechin-3-gallate, a green tea catechin (Nandakumar et al., 2011) and ultraviolet B (Wu et al., 2013) treated human skin cancer cells and $CD4^+$ T cells, respectively. Moreover, knockout of DNMT1 may lead to genomic hypomethylation (Yamada et al., 2005).The DNMT activity assay in this study shows that low-dose TCS caused DNMT activity and DNMT1 expression significantly decreased, supporting that the TCS-induced dysregulation of GDM may be through the DNMT1 pathway. But the gene and protein expressions in 0.625 nM TCS exposed group cannot be used to explain why the DNMT activity completely decreased at this concentration because the expression was not significantly inhibited. This also suggests that DNMT1 may not be the only key to induce DNMT activity down-regulation, other proteins or enzymes, such as MeCP2, may also contribute to the changes of DNMT activity.

As the first reported MBP protein, MeCP2 protein may play an important role in maintaining DNA methylation (Ou et al., 2004). In Ou's study, they reported that in human embryonic kidney cells, which were transiently transfected with methylated GFP reporter gene under CMV promoter and that over-expression of MeCP2 inhibited demethylation of the GFP reporter gene, while antisense knock down of MeCP2 promoted demethylation under the same promoter. We found that in the HepG2 cells which were exposed to low-dose TCS, MeCP2 gene and protein expression were significantly down-regulated, implying that down-regulated MeCP2 promoted demethylation. Indeed, our results were supported by Kimura's report that MeCP2 interacted directly with DNMT1 to maintain GDM *in vivo* (Kimura and Shiota, 2003). Therefore, we considered that the repetitive element hypomethylation induced by low-dose TCS resulted from the down-regulated DNMT1–MeCP2 pathway. Besides, the decrease of MeCP2 by low-dose TCS is an important finding for down-regulation of MeCP2 which may lead to Rett syndrome, a neurodevelopmental disease, as MeCP2 plays a key role in developing Rett syndrome (Chahrour et al., 2008). Moreover, the decreased MeCP2 expression may enhance the global DNA hypomethylation-mediated gene transcription as MeCP2 usually uses transcriptional co-repressor molecules to silence gene transcription (Bakker et al., 2002). Currently, we do not know whether the decrease of MeCP2 expression is the cause or the consequence of global DNA hypomethylation.

Another passive methylation factor we expected is the increased 8-OHdG level. It is known that 8-OHdG may lead to hypomethylation by inhibiting the binding of DNMTs to DNA by a steric block and also inhibiting the binding of methylated-DNA to MBDs, both of which are important to maintain DNA methylation (Valinluck et al., 2004). Our previous data had shown that 8-OHdG inhibitor NAC mitigated TCS-mediated decrease of GDM in HepG2 cells (Ma et al., 2013). In this study we found that 8-OHdG increased in low-dose TCS exposed HepG2 cells, suggesting that the low-dose TCS induced oxidative stress (Tamura et al., 2012), also the methylation levels of LINE-1 gene data supported that 8-OHdG decrease may impair global DNA hypomethylation. In summary, we believe that the increased 8-OHdG levels played an indirect role in inducing genomic hypomethylation in TCS exposed HepG2 cells.

Tumor suppressor gene hypermethylation is another DNA methylation biomarker of initiation and progression of cancer. As the tumor suppressor gene, p16 gene hypermethylation is the potential biomarker of liver tumor (Ueberham et al., 2015). So p16 gene DNA methylation changes were studied to explore the mechanism of TCS induced hepatotoxicity. Firstly, p16 gene hypermethylation were detected in low-dose TCS exposed group using Q-MSP method, then Massarray method was used to confirm the DNA methylation changes in the similar sequence, for Massarry and Pyrosequencing methods are the most two reproducible and accurate methods in all fragment DNA methylation methods. Massarry method can explore DNA methylation of less than 1000 bp DNA, and Pyrosequencing works on less than 70 bp DNA. It is clear that TCS may inhibit p16 gene expression through an increased DNA methylation level in area including TSS sites. The most interesting thing is that p16 methylation is not increased in higher dose TCS treated group, but in long-term and low-dose TCS treatment group, which suggests that low-dose and long-term TCS exposure may induce an adverse effect on human, and this gene methylation changes may contribute to the hepatotoxicity of TCS. And the data of low-dose TCS exposure on ESR1 gene methylation levels suggests that the p16 gene hypermethylation may be the gene-special change in TCS exposed HepG2 cell. Actually, some environmental chemicals can induce p16 gene hypermethylation, such as particulate matter 2.5 (Soberanes et al., 2012), arsenic (Chanda et al., 2006; Zhang et al., 2007), nickel (Govindarajan et al., 2002), HCB, TCDD (Ozden et al., 2015), and chromium (Kondo et al., 2006).

Besides, DNA hypomethylation of p16 gene was decreased in MTCS 2 week treated group, and the relative gene expression was up-regulated. This suggests that MTCS may induce a benign effect on human, which also leads us to realize the toxicity of low-concentrations and long-term TCS exposure to human health. And the result also means that more TCS change to MTCS, less toxicity of TCS polluted environment. To our knowledge, this is the first time to report that environmental pollutant TCS decreases p16 gene expression through hypermethylation at low-dose and long-term exposure. Further study is warranted on how much TCS or MTCS transfers in cells after long-term exposure to induce p16 gene hypermethylation, and on extending this result to animals and human.

Next, we focused on which group in TCS structure may play an important role in DNA methylation dysregulation. MTCS, the main environmental metabolite of TCS, which only has the difference on hydroxyl group with TCS, was chosen to study the role of hydroxyl group in TCS (Fig. 1C). As expected, in MTCS exposed cells, methylation levels of repetitive element have no significant difference compared with the control group, and as for p16 gene, the adverse effect transformed to benign effects after the hydroxyl group was replaced in TCS, which suggests that the methylation changes may be contributed by the hydroxyl group. A similar phenomenon has reported that the hydroxyl group is more responsive to metabolic pathways in *Escherichia coli* than the methoxy group (Su et al., 2012).The reason is not clear, maybe the higher activity and bioavailability of hydroxyl and larger steric block contribute to this. More work was needed to investigate the hydroxyl group role in environmental pollutions, which have similar structure with TCS, such as hydroxylated polybrominated diphenyl ethers (OH-PBDEs), on DNA methylation changes.

5. Conclusion

We have demonstrated that low-dose and long-term TCS exposure caused repetitive elements hypomethylation (especially LINE-1 gene) and tumor suppressor gene, p16 gene hypermethylation. The increase

in 8-OHdG levels, decrease in DNMT activity, DNMT1 and MeCP2 gene and protein expressions may directly or indirectly contribute to the LINE-1 hypomethylation. Comparing the effects of TCS and MTCS on DNA methylation level in HepG2 cells, the hydroxyl group may play an important role than the methoxy group. All these DNA methylation changes may ultimately result in hepatocellular cytotoxicity. The two DNA methylation changes suggess that low concentration and long term exposure TCS has adverse health outcomes, and also these findings will open new avenues for better understanding of TCS hepatotoxicity associated to epigenetics disorders. However, HepG2 cells used in our paper are carcinoma cells, have different characters from normal cells, though they are widely used as the liver model cell line. And also, further studies were needed using more cell lines and animals to conform the DNA methylation changes induced by low-dose and long-term TCS exposure.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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