Toxicology 274 (2010) 57–64

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Alterations in tumor biomarker GSTP gene methylation patterns induced by prenatal exposure to PFOS

Yan-jian Wan [1], Yuan-yuan Li [1], Wei Xia, Jun Chen, Zi-quan Lv, Huai-cai Zeng, Ling Zhang, Wen-jie Yang, Tian Chen, Yi Lin, Jie Wei, Shun-qing Xu *

Ministry of Education Key Laboratory of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, Wuhan 430030, China

ARTICLE INFO

Article history:
Received 18 March 2010
Received in revised form 19 May 2010
Accepted 19 May 2010
Available online 26 May 2010

Keywords:
Perfluorooctane sulfonate
Rat
GSTP
DNA methylation
Prenatal

ABSTRACT

The adverse environmental exposure in early life may have adverse effects on animals through epigenetic aspects. The current study examined the possibility of early epigenetic alteration in PFOS-exposed rat liver. Pregnant Sprague–Dawley (SD) rats were exposed to perfluorooctane sulfonate (PFOS) at doses of 0.1, 0.6 and 2.0 mg/kg/d and 0.05% Tween 80 as control by gavage from gestation days 2 to 21. The dams were allowed to give birth and liver samples from weaned (postnatal day 21) offspring rats were analyzed for PFOS content, relative liver weight, global DNA methylation, methylation of LINE-1 regulatory region, tumor suppressor gene glutathione S-transferase pi (GSTP) and p16 promoter methylation level, as well as related genes expression level. In PFOS-exposed weaned rats, compared to the control, global DNA methylation and methylation of LINE-1 regulatory region decreased significantly only in the 2.0 mg/kg/d group. Up to 30% of critical CpG sites (+79, 81 and 84) in GSTP promoter region were methylated in the livers of PFOS-treated rats, while p16 promoter methylation was not affected. In addition, the up-regulated expression of GSTP was observed and this increase was associated with its main pathway of transcription regulation: Keap1–Nrf2/MafK. Thus, early-induced changes in critical cytosines within the GSTP gene promoter region may be a biomarker of hepatic PFOS burden, though their direct role in PFOS-induced hepatotoxicity, including its potential carcinogenic action, needs further research.

1. Introduction

Perfluorooctane sulfonate (PFOS), a widespread environmental pollutant, is a breakdown product of related perfluorooctanesulfonamides, which were used in many industrial and commercial applications, such as repellant coatings for carpets, textiles, leather, paper, and food packing materials, or as surfactants in diverse cosmetics, and fire-fighting foams (OECD, 2002). Due to the extremely stable and accumulative nature of PFOS (Giesy and Kannan, 2001; Smithwick et al., 2005), it has been considered as a persistent organic pollutant and has been found in high concentrations in serum and liver in wildlife and humans (Kannan et al., 2001; Taniyasu et al., 2003; Smithwick et al., 2005).

Increased incidence of liver carcinogenesis accompanied by proliferation peroxisomal bodies and cytoplasmic vacuolation, subsequent hepatocellular hypertrophy, hepatomegaly (Seacat et al., 2002, 2003; Lau et al., 2007; Midasch et al., 2007) have been reported to be the toxicity and hazard profile of PFOS. However, the mechanism underlying hepatic effects observed in the PFOS-treated mammalians was not well known (Lau et al., 2007). Notably, it is proven that PFOS can cross the placental barrier and cause toxicity in developmental mammalians (Lau et al., 2003, 2004; Thibodeaux et al., 2003; Midasch et al., 2007). As we known, early life stage exposure to toxicants would increase the risk of adverse effects. In the past decade, increasing evidence has been reported to support the associations between exposures during the intrauterine period and health outcomes later in life (Hales and Barker, 2001; Jirtle and Skinner, 2007). So it is possible to find a predictive biomarker for increased incidence of liver toxicity induced by PFOS.

Based on the research of gene/environment interactions and epigenetics, many research efforts have been launched for studying epigenetic changes in targets correlated with environment factors. Particularly, aberrant promoter methylation as biomarkers for early diagnosis has been reviewed in various environment associated diseases (Chouliaras et al., 2010; Phe et al., 2010; Zhang et al., 2007; Anglim et al., 2008; Brooks et al., 2009). Research on earlier stages of life could provide more insight into its underlying pathogenesis.

Although it is still not fully clear whether the observed epigenetic changes actually represent a cause or a consequence of body

* Corresponding author. Tel.: +86 27 83657705; fax: +86 27 83657781.
E-mail addresses: xuscience@hotmail.com, shunqing@mails.tjmu.edu.cn (S.-q. Xu).
[1] These authors contributed equally to this work.

0300-483X/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2010.05.006

disorders, the changes may provide a unique opportunity for early detection of environmental contaminants-induced late on-set toxicity. For example, the recent studies showed the altered cellular epigenetic status may be associated with early pyrazinamide- or inorganic arsenic-induced hepatotoxicity (Kovalenko et al., 2007; Xie et al., 2007). Importantly, epigenetic changes, especially alterations in global DNA methylation, as well as promoter methylation status of particular genes, have been suggested to be more sensitive indicators of liver toxicity than classic parameters in toxic potential assessment, thus may be useful predictive biomarkers for potential toxicities (Verma and Srivastava, 2002; Watson et al., 2004; Kovalenko et al., 2007). Conclusively, methylation change appears to be an early event, and it may give an advantage for timely detection of toxic potential compared to the currently used indicators, such as histopathological observation or biochemical indicators.

PFOS is a non-genetic toxicant (Luebker et al., 2005). Thus, it was hypothesized in this study that PFOS exposure during early development stage in life would induce unfavourable epigenetic changes in rats. The early epigenetic changes emerging from adverse effects might be used as sensitive biomarkers for biological monitoring. Tumor suppressor gene glutathione S-transferase pi (GSTP) and p16 both belong to tumor suppressor genes. GSTP gene encodes an enzyme protecting cells against oxidants and electrophilic carcinogens by conjugating them with glutathione, and plays a role in susceptibility to cancer (Daniel, 1993). p16 is a gene that regulates cyclin-dependent kinases, known as CDKN2A (cyclin-dependent kinase inhibitor 2A). It plays an important role in regulating the cell cycle, and genetic or epigenetic changes in p16 correlate with the risk of developing a variety of cancers (Nobori et al., 1994; Verma and Srivastava, 2002). Alterations in promoter methylation of GSTP and p16 are two of the most frequently sensitive biomarkers observed in hepatocarcinogenesis, either in human or in toxicant-induced animal models (Esteller et al., 1998; Steinmetz et al., 1998; Tchou et al., 2000; Yang et al., 2003; Kostka et al., 2007; Kovalenko et al., 2007; Harder et al., 2008). Based on these considerations, the present study was undertaken to determine whether the PFOS exposure *in utero* is associated with alterations of global DNA methylation and the promoter methylation status of GSTP and p16 in livers of weaned rats.

2. Materials and methods

2.1. Chemicals

PFOS (purity >98%) was purchased from Fluka Chemical (Buchs, Switzerland); sodium bisulfite (purity >99%) and hydroquinone (purity >99%) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Animals and treatment

SD rats (Vital Rivers, Peking, China) were housed in polypropylene plastic cages in a room with *ad libitum* access to food and water, with an ambient temperature of 22–25 °C and a 12/12-h light/dark cycle. Cohabitation and confirmation of mating were performed. Upon confirmed mating (gestation day 0, GD 0) through sperm positive, females were removed and housed individually in nesting boxes containing nesting material throughout the remainder of the study. Pregnant rats were assigned to groups that received the vehicle (0.05% Tween 80) or 0.1, 0.6 or 2.0 mg/kg/d of PFOS from GD 2 to GD 21 by gavage. Dosages were adjusted daily for body-weight changes (1 mL/kg). Dams were allowed for spontaneous delivery (10 dams per group), and at PND 4, six of the rat offsprings per litter were randomly selected and maintained to PND 21. On PND 21, rat offsprings were sacrificed, blood was collected for serum preparation and livers were removed, weighed, and then stored at −80 °C until use.

To collect liver tissues of PND 0 rats, four groups of rats was treated in the same way as described above. Six pups from each litter were sacrificed by decapitation on PND 0. Livers were collected, and then stored at −80 °C until use.

2.3. Histological examinations

Paraformaldehyde fixed liver tissues were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin.

2.4. Determination of PFOS content in serum and livers

The procedures of sample pretreatments of liver tissues and serum samples for determining PFOS content by high-performance liquid chromatography with an electrospray tandem mass spectrometer (LC/MS/MS) were in accordance with previous reports with some minor modifications (Kannan et al., 2001; Olivero-Verbel et al., 2006). Briefly, homogenate of liver tissues or serum samples was extracted by 1 mL of 0.5 M tetrabutylammonium hydrogen sulfate solution and 2 mL of 0.25 M sodium carbonate buffer in a 15-mL polypropylene tube for extraction. After being thoroughly mixed, 5 mL of methyl tert-butyl ether (MTBE) was added to the solution, and the mixture was shaken for 20 min. After being separated by centrifugation, an exact volume of MTBE (4 mL) was removed from the solution, and the process was repeated twice. The solvent was allowed to evaporate under nitrogen before being reconstituted in 1 mL of methanol. The sample was vortexed and passed through a 0.2 μm nylon filter into an autosampler vial. Analysis was then performed using LC/MS/MS (Agilent 1100 LC-MSD-trap-XCT, Agilent 1100 Series LC/MS Ion-Trap, Agilent, Palo Alto, CA) with a sample volume of 5 μL. Separation was achieved on an Inertsil ODS-3 column (2.1 mm× 150 mm, 5 μm), being carried out using a mobile phase of 1.0 mM ammonium acetate/methanol (v/v) at a flow rate of 0.2 mL/min. The gradient profile was as follows: linear increase from 40% to 75% methanol solution for 5–12 min, then hold at 75% for 3 min. PFOS was quantitatively analyzed by single mass mode using characteristic ions at m/z 498.9 (Martin et al., 2004; Tseng et al., 2006).

2.5. Global DNA methylation determination

DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Global DNA methylation was assessed with Methylamp™ Global DNA Methylation Quantification kit (Epigentek, Brooklyn, NY, USA) according to the manufacturer's instructions.

2.6. Bisulfite treatment

The bisulfite reaction was carried out under conditions described by Clark et al. (1994) with minor modifications. Briefly, 2 μg of DNA was denatured with freshly prepared NaOH (final concentration 0.3 M). After 15 min denaturation at 37 °C, the DNA was treated with freshly prepared solutions of sodium bisulfite (final concentration 3.1 M, purity >99%, Sigma–Aldrich, Inc., St. Louis, MO, USA) and hydroquinone (final concentration 0.5 mM). This mixture was overlaid with mineral oil and incubated in the dark at 55 °C for 16 h. The samples were purified using Wizard DNA Clean-Up System desalting columns (Promega, Madison, WI, USA), eluted in 50 μL of H_2O and incubated within 0.3 M NaOH for 15 min at 37 °C. The solutions were neutralized by addition of NH_4OAc to 3 M and the DNA in each tube was ethanol precipitated, dried, resuspended in 20 μL TE [10 mM Tris–HCl (pH 8), 0.1 mM EDTA] and stored at −20 °C.

2.7. Methylation analysis of LINE-1 repetitive elements

Methylation status of long interspersed nucleotide elements (LINE-1) was determined by the combined bisulfite restriction analysis (COBRA) as described previously (Xiong and Laird, 1997; Eads and Laird, 2002), including bisulfite modified genomic DNA, PCR amplification, and digestion of PCR products with restriction enzymes *BstU*I or *Rsa*I (Tryndyak et al., 2007). The primer sequences which correspond to the nucleotides in the regulatory region of LINE-1 sequence (product size, 163 bp, GenBank: U87600) were: forward, 5′-TTT GGT GAG TTT GGG ATA-3′; reverse, 5′-CTC AAA AAT ACC CAC CTA AC-3′. The PCR conditions were 40 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 30 s and finally at 72 °C for 7 min (Tryndyak et al., 2007). The undigested or digested PCR products were separated on 3% agarose gels and the band intensity was analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.8. Determination of the GSTP and p16 promoter methylation status

The converted DNA was amplified by polymerase chain reaction (PCR) using GoTaq® Hot Start Green Master Mix (Promega, Madison, WI, USA) with GSTP (product size, 403 bp) forward primer: 5′-AAA TAG GGA TGG GTA GAA GGT AGA-3′ and reverse primer: 5′-AAC CCA AAC CCC AAA AAC TAC-3′. PCR amplifications were performed in 30 μL reaction mixtures containing pooled 2 μL of bisulfite-treated genomic DNA, under the following reaction conditions: 3 min at 94 °C, 40 cycles for 30 s at 94 °C, 30 s at 59 °C and 30 s at 72 °C, and finally at 72 °C for 7 min. For p16 (product size, 236 bp), the forward primer was 5′-AGG GTT TTA TTG GTT ATA TTA TTG G-3′, the reverse primer was 5′-AAA TAA AAC ATT CCT TAC CTA CCT ATA TC-3′, and the annealing was performed at 56 °C. The PCR products were separated on 2% agarose gels and purified by gel extraction using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), then cloned into pCRII vector using TA Cloning kit Dual Promoter (Invitrogen, Grand Island, NY). Plasmids DNA from 10 colonies were prepared using TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China) and sequenced.

Table 1
PCR primer list for determination of genes expression.

Gene	Forward primer	Reverse primer	Product size (bp)	Accession
β-Actin	CAACGGCTCCGGCATGTGC	CTCTTGCTCTGGGCCTCG	153	NM_031144.2
GSTP	CTATGTGGCTCGCCTCAGTG	GGAGTTCCTGTCCCTTCGTC	119	NM_012577.2
Nrf2	GGGCTGTGATCTGTCCCTGTG	GCGGTGGGTCTCCGTAAATG	158	NM_031789.1
MafK	CACAGAGCCAGCAAACAGCC	GCAACAACACACACACGCCA	124	NM_145673.2
Keap1	GCAGAAGAGGCAGCAGAACAAG	TCCAGGGGCTATGACAGAAGG	121	NM_057152.1
DNMT1	CCAGATACCTACCGGTTATTCG	TCCTTTAACTGCAGCTGAGGC	153	NM_053354.3
DNMT3a	CTGAAATGGAAAGGGTGTTTGGC	CCATGTCCCTTACACACAAGC	164	NM_001003957.1
DNMT3b	AGGAAGGATGGGTGGAGTGG	ATTGGGGTCAGGGAGAGGGA	95	NM_001003959.1

2.9. RNA extraction, reverse transcription and real-time PCR

Total RNA was isolated using SV Total RNA Isolation System (Promega, Madison, WI, USA). Reverse transcription for cDNA synthesis was performed with 2 μg total RNA using RevertAid™ First Strand cDNA Synthesis Kits (Fermentas, Hanover, MD, USA) with random primers. Real-time PCR was carried out in a 10 μL final volume and performed in triplicates using Power SYBR Green PCR Master Mix reagents (Applied Biosystems, Framingham, MA, USA) in an ABI PRISM 7900 sequence detection system (Applied Biosystems, Framingham, MA, USA) according to the manufacturer's protocol. Primer sets used for amplification of β-actin, GSTP, Nrf2, MafK, Keap1, DNMT1, DNMT3a, and DNMT3b genes, and sizes of PCR products were shown in Table 1. The conditions for real-time PCR were as follows: 95 °C for 10 min followed by 40 cycles at 94 °C for 10 s, and 60 °C for 1 min. Differences in gene expression between groups were calculated using cycle time (Ct) values, which were normalized against β-actin and expressed as relative values (means ± S.D.) compared to the control. S.D. represents standard deviation.

2.10. Western blot analysis

Liver tissue was thawed on ice, washed with PBS and suspended at 4 °C in RIPA lysis buffer (Beyotime, Shanghai, China). Tissues was disrupted by sonication (3× for 10 s) and centrifuged for 30 min at 10,000 × *g* (4 °C). Protein concentrations in the supernatant were determined by Bradford Protein Assay Kit (Beyotime, Shanghai, China). Proteins were electrophoretically resolved on 10% SDS-polyacrylamide gels (25 μg per lane), and then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Non-specific binding was reduced by incubating the membrane in blocking buffer (Beyotime, Shanghai, China) for 1 h. Membranes were incubated with specific primary antibodies (1:200 diluted, Santa Cruz, CA, USA) overnight at 4 °C, washed 3× with PBS for 15 min, and incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000; Beyotime, Shanghai, China) for 1 h. The membranes were washed 4× and developed with BeyoECL Plus (Beyotime, Shanghai, China). β-Actin was used as the internal control.

2.11. Statistical analysis

For global DNA methylation, genes expression and relative liver weight, the data were expressed as the relative values compared to the control (means ± S.D.). Methylation status of LINE-1 was expressed by ratio between *BstU*I-cut (methylated) and uncut (unmethylated) (means ± S.D.). The two-way analysis of variance (ANOVA) was employed to calculate the statistical significance between control and treated groups. Rank sum test was used to assess the significance of the promoter methylation frequency. Significance was accepted for p values <0.05.

3. Results

3.1. Animal data

Six offsprings per litter were allowed to survive to PND 21 after PND 4. A statistically significant difference in postnatal mortality in 3 days, body weight or mean relative liver weight on postnatal day 21 was only observed in the 2.0 mg/kg/d treated group. There were no statistically significant differences in these indicators among control and treated rats in the other two groups (Table 2). The mean PFOS content in weaned rat serum and livers was 0.37–4.26 μg/mL and 1.43–20.52 μg/g, respectively (Table 2). The growth rate of dams and pups was shown in supplemental Table 1.

Additionally, supplemental Fig. 1 shows no significant pathological changes in weaned rats treated with PFOS compared to control. Histopathological analysis of livers of weaned rats treated with PFOS revealed no morphological changes characterized of cytoplasmic vacuolation, or hypertrophy of hepatocytes.

3.2. Effect of PFOS on global DNA methylation and DNMTs expression in livers of weaned rats

The extent of DNA methylation in the weaned rat livers, in the group 2.0 mg/kg/d, became slightly hypomethylated (90.8 ± 4.7%) compared to the control (100 ± 3.2%). However, global DNA methylation in rats livers of group 0.6 mg/kg/d (97.0 ± 4.1%) or 0.1 mg/kg/d (96.8 ± 3.3%), did not differ significantly from control (Fig. 1A).

The global extent of DNA methylation is regulated by DNMT. While DNMT1 functions as a maintenance methylase, DNMT3a and DNMT3b mainly function as de novo methyltransferases (Bogdanovic and Veenstra, 2009). In this study, DNMT3a expression was up-regulated significantly (2.02 ± 0.14) in the 2.0 mg/kg/d treated group compared to the control, but not significantly in other two groups (Fig. 1B and C). However, DNMT1 and DNMT3b did not change significantly (Fig. 1B).

3.3. Effect of PFOS on LINE-1 methylation in liver

Changes in DNA methylation induced by xenobiotics exposure may not be revealed by global DNA methylation change in the initial stage, due to the existence of repetitive DNA sequences like LINE-1 (Xie et al., 2007). In mammalians, more than 90% of all 5-methylcytosines lie within the CpG islands in the transposons, including long/short interspersed nucleotide elements (LINE and SINE). LINE-1 methylation status has been shown to be a good indicator of genome-wide methylation (Yang et al., 2004). In the present study, the amount of 5-methylcytosine/cytosine in the LINE-1 repetitive sequence in livers of weaned rats was evaluated using COBRA method (Fig. 2A and B). A statistically significant dif-

Table 2
Effect of *in utero* PFOS exposure on weaned rat: postnatal mortality within PND 3, weight gain, relative liver weight and PFOS content in serum and livers.

Treatment	Delivered pups	Mortality (%)	Body weight (g)	Liver weight (g)	Relative liver weight	PFOS in serum (μg/mL)	PFOS in liver (μg/g)
Control	13.5 ± 1.3	3.6 ± 0.1	52.8 ± 3.4	2.13 ± 0.19	0.040 ± 0.002	ND	ND
0.1 mg/kg/d	13.6 ± 2.3	3.2 ± 0.1	53.5 ± 3.7	2.18 ± 0.18	0.040 ± 0.002	0.37 ± 0.12	1.43 ± 0.59
0.6 mg/kg/d	12.7 ± 2.1	3.5 ± 0.1	50.4 ± 3.4	2.10 ± 0.18	0.041 ± 0.003	1.86 ± 0.35	7.68 ± 1.62
2.0 mg/kg/d	11.0 ± 2.5*	22.9 ± 0.1*	45.3 ± 3.8*	2.12 ± 0.18	0.046 ± 0.001*	4.26 ± 1.73	20.52 ± 4.59

Each data represents means ± S.D. of 10 litters for mortality and body weight, and means ± S.D. (n = 6) for other values, ND represents the value lower than the limit of detection.

* Statistically significant difference (p < 0.05) from corresponding control.

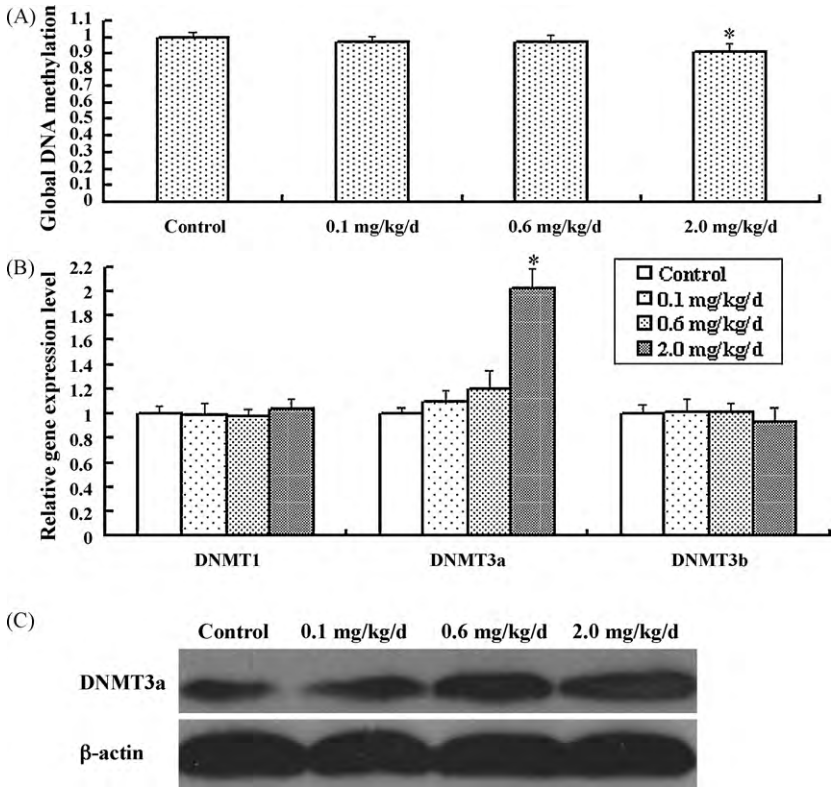

Fig. 1. Global DNA methylation assay and DNMTs relative expression level assay. (A) Global DNA methylation in weaned rat livers treated with differential dose of PFOS. (B) Effects of PFOS on DNMTs expression in weaned rat livers. Asterisks (*) indicate statistically significant difference ($p < 0.05$) from corresponding control ($n = 6$). Error bars represent the S.D. (C) Representative photograph of Western blot for DNMT3a.

ference was only observed between livers of 2.0 mg/kg/d group (methylated/unmethylated = 1.15 ± 0.04) and the control (methylated/unmethylated = 1.28 ± 0.03) (Fig. 2C).

3.4. Effect of PFOS on promoter methylation of GSTP and p16 genes

Xenobiotics exposure may not only affect global DNA methylation, but also affect genes (e.g., GSTP or P16) that play an important role in the regulation of cellular metabolic pathways or cell cycle through epigenetic alteration.

Methylation was up to 30% in the promoter region of GSTP in weaned rats treated with PFOS compared to the control (0%). However, no methylation in the promoter region of the GSTP gene was observed in neonatal (PND 0) rat livers, either in dosage groups or control (supplemental Fig. 2A). More importantly, the methylation occurred in the critical sites (+79, +81 and +84) within the promoter (Fig. 3A and B), which was in accordance with reports of Steinmetz et al. (Steinmetz et al., 1998; Kovalenko et al., 2007). 20% and 30% hypermethylation in +79 and +84, respectively, were observed in weaned rats treated with 2.0 mg/kg/d PFOS. And 10% in +81, 30% in +84 were observed in livers of weaned rats treated with 0.6 mg/kg/d PFOS. However, no methylation change occurred in any of the three sites in either livers of weaned rats treated with 0.1 mg/kg/d PFOS or control. This indicated the alteration was dose-dependent to some extent. In addition, there was no significant difference between average methylation ratio of male and female offspring.

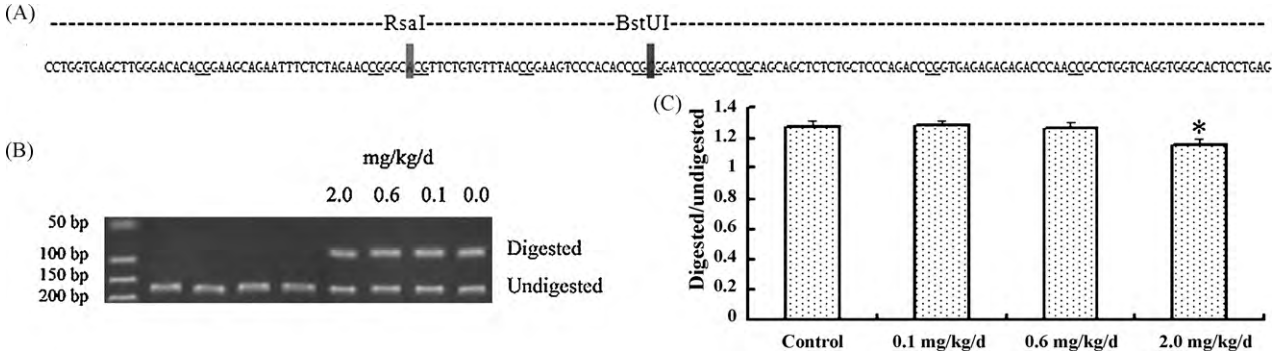

Fig. 2. Methylation status of LINE-1 in the liver of control and PFOS-treated rats as detected by COBRA assay. (A) Diagram of the PCR product of rat LINE-1 regulatory region. The location of *Rsa*I and *BstUI* sites analyzed for methylation status by COBRA is shown. (B) Representative photograph of the COBRA assay with restriction by *BstUI*: the left four lanes following the marker were PCR products (163 bp) of rat LINE-1 regulatory region; the right four lanes were digested PCR products of *BstUI*-cut (80–83 bp) and uncut (163 bp). (C) Ratio between *BstUI*-cut (methylated) and uncut (unmethylated) PCR products ($n = 6$, means ± S.D.). Asterisks (*) indicate statistically significant difference ($p < 0.05$) from corresponding control.

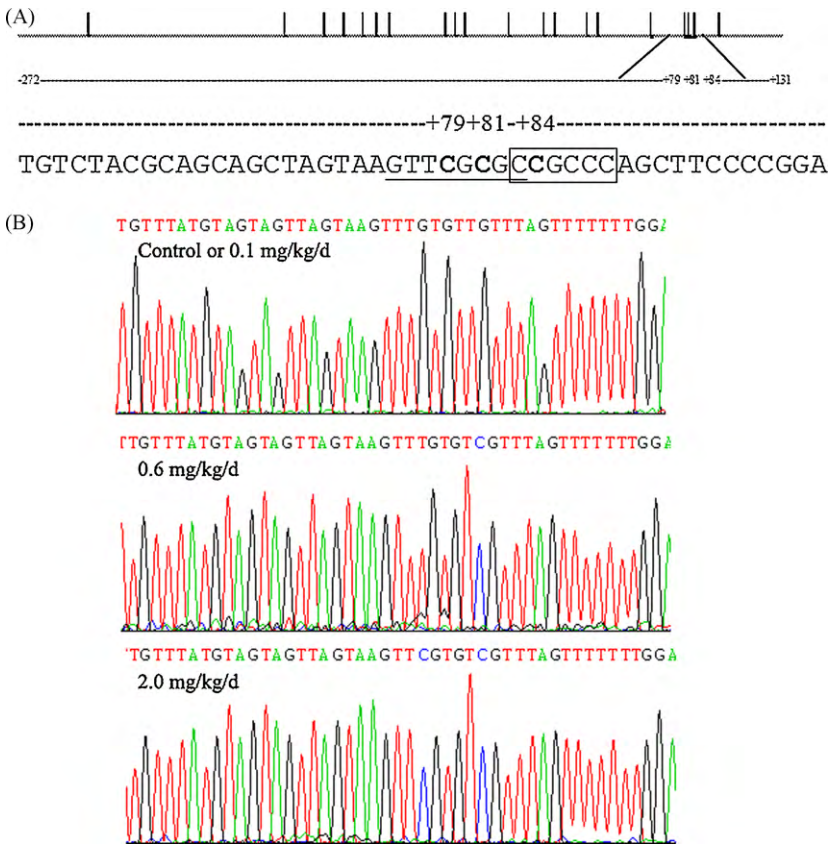

Fig. 3. Graphic representation of critical CpG site locations in the rat GSTP 5′ regulatory region, and effects of PFOS on critical CpG site of GSTP promoter in weaned rat livers. (A) CpG sites +79 and +81 are located within an E2F sequence (GTTCGCGC, underline), and CpG site +84 is located within a degenerate Sp1 sequence (GGGCGG, hollow rectangle) adjacent to the E2F sequence. (B) Effects of PFOS on critical CpG site of GSTP promoter in postnatal day 21 rat livers; black part indicates percentage of methylation and white indicates unmethylated. (C) Representative chromatogram for differential group.

No statistically significant difference was obtained from p16 gene promoter, since there were no methylation occurred in any sites in the CpG islands either in the three treated groups or the control (supplemental Fig. 3).

3.5. *Expression of GSTP was up-regulated in rat liver*

GSTP expression can be induced by exposure to xenobiotics, and this process involves the mediation of Nrf2/MafK heterodimer interacting with potent GSTP enhancer I after activation of the transcriptional trans-activator Nrf2 via release from Keap1 complexes (Dinkova-Kostova et al., 2002; Sakai and Muramatsu, 2007). GSTP mRNA expression in liver tissues significantly increased (1.30 ± 0.07) in groups treated with 2.0 mg/kg/d PFOS compared to the control (Fig. 4A and B). Although the Nrf2 and MafK expression did not increase significantly in any dosed group, the expression of Keap1 (the inhibitor of Nrf2) was negatively related with the increased expression of GSTP at 2.0 mg/kg/d (Fig. 4A and B). This change was considered as the compensatory stress reaction of phase II metabolic enzyme induced by xenobiotics.

4. Discussion

The number of born pups, the mortality within PND 3, and the body weight, the liver weight, the relative liver weight, as well as the mean PFOS content in weaned rat serum and livers were consistent with values reported in similar studies (Lau et al., 2003, 2007). It is noteworthy that PFOS content in human serum were up to 2.440 and 0.056 μg/mL (maximal mean concentration) in occupationally exposed workers or non-occupationally populations, respectively (Fromme et al., 2009), which is similar to or several orders of magnitudes lower than the levels observed in this study. Taken into consideration of lower elimination rate in human than rat, human may be in danger of PFOS exposure (Lau et al., 2007).

The extent of global DNA methylation decrease may be related to the carcinogenic potential of a xenobiotic (Pogribny et al., 2008). Hypomethylation of LINE-1 may contribute to the decrease of global DNA methylation in the livers of rats treated with 2.0 mg/kg/d PFOS. Meanwhile, the increased DNMT3a expression observed in this study may be a feedback regulation of decreased global DNA methylation. DNMT3a and DNMT3b are essential for de novo methylation and mammalian development, and they are able to methylate previously unmethylated CpG sequences, while DNMT1 functions as a maintenance methylase, copying the preexisting methylation marks onto the new strand during replication (Okano et al., 1999; Jeltsch, 2006; Bogdanovic and Veenstra, 2009). Therefore, the increased DNMT3a expression in this study may be considered as a compensatory reaction for the decreased global methylation.

GSTP is a member of glutathione S-transferases (GSTs). GSTs are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. They are categorized into four main classes: alpha, mu, pi, and theta. The GSTP is thought to function in xenobiotics metabolism and play a role in susceptibility to cancer, and other diseases (Daniel, 1993; Esteller et al., 1998; Krajinovic et al., 2001; Nakayama et al., 2004; Wang et al., 2006; Harder et al., 2008).

Additionally, it is well established that GSTP gene promoter hypermethylation often occurs in hepatocellular carcinoma and even at preneoplastic stages (Esteller et al., 1998; Tchou et al.,

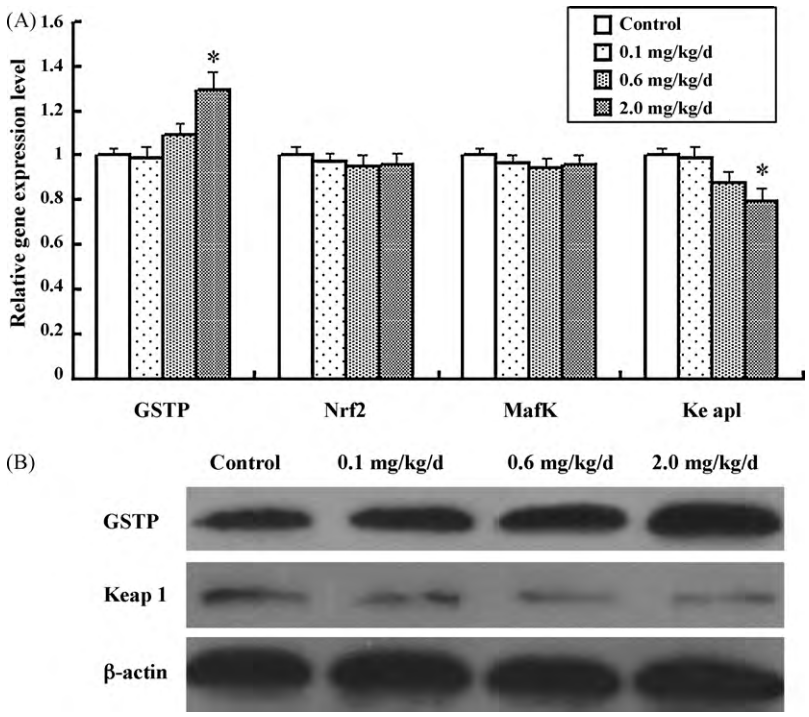

Fig. 4. Comparison of GSTP expression among rats exposed to different dose of PFOS and the expression of main factors in GSTP transcription regulation pathway. (A) Quantitative PCR was performed on four genes, GSTP was shown as increased in the 2.0 mg/kg/d PFOS-treated group, one was shown as decreased in the 2.0 mg/kg/d group (Keap1, the inhibitor of Nrf2), and Nrf2 (transcription factor of GSTP) and MafK (the heterodimer of Nrf2) was shown as unaltered as compared to control. Asterisks (*) indicate statistically significant difference ($p < 0.05$) from corresponding control ($n = 6$). Error bars represent the S.D. (B) Representative photograph of Western blot for GSTP and Keap1.

2000; Yang et al., 2003; Kovalenko et al., 2007). To our knowledge, the change of GSTP gene promoter methylation in rat liver tissue often occurs at several critical CpG sites (Steinmetz et al., 1998; Kovalenko et al., 2007). The change observed in this study was consistent with the critical sites (+79, +81 and +84) reported by Steinmetz et al. Furthermore, this alteration is dose-dependent in this experiment, and may be associated with exposure level of PFOS in rat development.

According to the report of Steinmetz et al., CpG sites +79 and +81 are located within an E2F consensus sequence (GTTCGCGC), and +84 within a degenerate Sp1 sequence (GGGCGG) adjacent to the E2F sequence (Steinmetz et al., 1998). Previous studies have shown that CpG methylation status within these consensus sequences are important for protein binding (Kovesdi et al., 1987; Macleod et al., 1994), and for the interaction of E2F with Sp1 (Karlseder et al., 1996; Lin et al., 1996). Sp1 binding is believed to 'protect' CpG sites from methylation during development (Brandeis et al., 1994).

Other CpG sites of GSTP promoter region determined in this experiment were all unmethylated (supplemental Fig. 2B) in rat livers from either PFOS-treated groups or control.

In control rat liver tissues, the methylation status of critical sites (+79, +81 and +84) within GSTP promoter observed in weaned rat was unmethylated. However, weaned rat of 2.0 and 0.6 mg/kg/d PFOS exposure revealed a different status in critical CpG sites in GSTP promoter. This indicated that weaned rat treated with PFOS, different from the control, fell into a status which was unfavourable for detoxification or cancer prevention via GSTP.

Interestingly, in the 2.0 mg/kg/d group, the methylation change of two CpG sites often occurred in one plasmid clone, corresponding to the primary one copy of the genomic DNA. And this combined methylation was only observed in the highest dose treated group but not others. This implied that there may be more potent interaction between MBD (5-methyl-CpG-binding domain) contained transcription repressors and the DNA sequence (Bakker et al., 2002; Nakayama et al., 2004), than the effect induced by the separate methylated CpG site in different copies of the genomic DNA in 0.6 mg/kg/d group.

Although GSTP promoter hypermethylation should be followed by the decrease in GSTP transcripts or expression (Esteller et al., 1998; Tchou et al., 2000), our real-time PCR assessment did not show the decreasing tendency. Previously, the similar discrepancy was reported (Lin et al., 2001).

Exposure to xenobiotics can induce GSTP expression, a process involving increased GSTP gene transcription, mediated by activation of the transcriptional trans-activator Nrf2 released from Keap1 complexes (Dinkova-Kostova et al., 2002; Nakayama et al., 2004). Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation analyses (ChIP) demonstrate that the Nrf2/MafK heterodimer regulates GSTP expression through the strong enhancer element-GSTP enhancer I (−2.5 kb), which is an important cis-element for activation of GSTP gene (Matsumoto et al., 1999; Sakai and Muramatsu, 2007). The GSTP up-regulation observed in this experiment was consistent with the reported change induced by xenobiotics (Sakai and Muramatsu, 2007; Xie et al., 2007) and it negatively related with the down-regulation of Keap1 (inhibitor of GSTP via Nrf2). This induction likely prevents or attenuates the development of cancer upon exposure to carcinogens (Ramos-Gomez et al., 2001; Nakayama et al., 2004). It can be considered as a protection initiated by cells.

When certain carcinogenic xenobiotics entered into organisms, the protective stress response would be launched. A balance between adverse effects and self-protection response may be formed during early exposure period, thus toxicity of xenobiotics would escape from normal detection; but finally the self-protection factors can hardly impede carcinogenesis. The evidence pointing towards an involvement of epigenetic regulation in PFOS induced hepatocarcinogenesis may provide an earlier insight into the adverse aspects. Since it is a two-year period for observation

of long-term effect of PFOS like potential hepatocarcinogenesis, on liver of rat offspring, it was not demonstrated in this study.

In conclusion, this study showed for the first time that prenatal PFOS exposure is associated with GSTP gene promoter methylation in PND 21 rats in 0.6 and 2.0 mg/kg/d group, while global DNA methylation did not alter significantly in 0.6 mg/kg/d group. Therefore, GSTP promoter methylation change was more sensitive than that of global DNA, LINE-1 regulatory region or p16 promoter in this experiment.

Conflict of interest statement

None.

Acknowledgements

This work was supported by the National High Technology Research and Development Program of China (grant number 2006AA06Z401), the National Basic Research Program of China (grant number 2008CB418206), the National Natural Science Foundation of China (grant numbers 20677018 and 20807017), the Key Projects in the National Science and Technology Pillar Program (grant number 2007BAC16B07) and the R&D Special Fund for Public Welfare Industry (Environment) (grant number 200709052).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2010.05.006.

References

Anglim, P.P., Alonzo, T.A., Laird-Offringa, I.A., 2008. DNA methylation-based biomarkers for early detection of non-small cell lung cancer: an update. Mol. Cancer 7, 81.

Bakker, J., Lin, X., Nelson, W.G., 2002. Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells. J. Biol. Chem. 277, 22573–22580.

Bogdanovic, O., Veenstra, G.J., 2009. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. Chromosoma 118, 549–565.

Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., Cedar, H., 1994. Sp1 elements protect a CpG island from de novo methylation. Nature 371, 435–438.

Brooks, J., Cairns, P., Zeleniuch-Jacquotte, A., 2009. Promoter methylation and the detection of breast cancer. Cancer Causes Control 20, 1539–1550.

Chouliaras, L., Rutten, B.P., Kenis, G., Peerbooms, O., Visser, P.J., Verhey, F., van Os, J., Steinbusch, H.W., van den Hove, D.L., 2010. Epigenetic regulation in the pathophysiology of Alzheimer's disease. Prog. Neurobiol. 90, 498–510.

Clark, S.J., Harrison, J., Paul, C.L., Frommer, M., 1994. High sensitivity mapping of methylated cytosines. Nucleic Acids Res. 22, 2990–2997.

Daniel, V., 1993. Glutathione S-transferases: gene structure and regulation of expression. Crit. Rev. Biochem. Mol. Biol. 28, 173–207.

Dinkova-Kostova, A.T., Holtzclaw, W.D., Cole, R.N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., Talalay, P., 2002. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc. Natl. Acad. Sci. U.S.A. 99, 11908–11913.

Eads, C.A., Laird, P.W., 2002. Combined bisulfite restriction analysis (COBRA). Methods Mol. Biol. 200, 71–85.

Esteller, M., Corn, P.G., Urena, J.M., Gabrielson, E., Baylin, S.B., Herman, J.G., 1998. Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. Cancer Res. 58, 4515–4518.

Fromme, H., Tittlemier, S.A., Volkel, W., Wilhelm, M., Twardella, D., 2009. Perfluorinated compounds – exposure assessment for the general population in Western countries. Int. J. Hyg. Environ. Health 212, 239–270.

Giesy, J.P., Kannan, K., 2001. Global distribution of perfluorooctane sulfonate in wildlife. Environ. Sci. Technol. 35, 1339–1342.

Hales, C.N., Barker, D.J., 2001. The thrifty phenotype hypothesis. Br. Med. Bull. 60, 5–20.

Harder, J., Opitz, O.G., Brabender, J., Olschewski, M., Blum, H.E., Nomoto, S., Usadel, H., 2008. Quantitative promoter methylation analysis of hepatocellular carcinoma, cirrhotic and normal liver. Int. J. Cancer 122, 2800–2804.

Jeltsch, A., 2006. Molecular enzymology of mammalian DNA methyltransferases. Curr. Top. Microbiol. Immunol. 301, 203–225.

Jirtle, R.L., Skinner, M.K., 2007. Environmental epigenomics and disease susceptibility. Nat. Rev. Genet. 8, 253–262.

Kannan, K., Franson, J.C., Bowerman, W.W., Hansen, K.J., Jones, P.D., Giesy, J.P., 2001. Perfluorooctane sulfonate in fish-eating water birds including bald eagles and albatrosses. Environ. Sci. Technol. 35, 3065–3070.

Karlseder, J., Rotheneder, H., Wintersberger, E., 1996. Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. Mol. Cell. Biol. 16, 1659–1667.

Kostka, G., Urbanek, K., Ludwicki, J.K., 2007. The effect of phenobarbital on the methylation level of the p16 promoter region in rat liver. Toxicology 239, 127–135.

Kovalenko, V.M., Bagnyukova, T.V., Sergienko, O.V., Bondarenko, L.B., Shayakhmetova, G.M., Matvienko, A.V., Pogribny, I.P., 2007. Epigenetic changes in the rat livers induced by pyrazinamide treatment. Toxicol. Appl. Pharmacol. 225, 293–299.

Kovesdi, I., Reichel, R., Nevins, J.R., 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. Proc. Natl. Acad. Sci. U.S.A. 84, 2180–2184.

Krajinovic, M., Ghadirian, P., Richer, C., Sinnett, H., Gandini, S., Perret, C., Lacroix, A., Labuda, D., Sinnett, D., 2001. Genetic susceptibility to breast cancer in French-Canadians: role of carcinogen-metabolizing enzymes and gene-environment interactions. Int. J. Cancer 92, 220–225.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., Seed, J., 2007. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol. Sci. 99, 366–394.

Lau, C., Butenhoff, J.L., Rogers, J.M., 2004. The developmental toxicity of perfluoroalkyl acids and their derivatives. Toxicol. Appl. Pharmacol. 198, 231–241.

Lau, C., Thibodeaux, J.R., Hanson, R.G., Rogers, J.M., Grey, B.E., Stanton, M.E., Butenhoff, J.L., Stevenson, L.A., 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II. Postnatal evaluation. Toxicol. Sci. 74, 382–392.

Lin, C.H., Hsieh, S.Y., Sheen, I.S., Lee, W.C., Chen, T.C., Shyu, W.C., Liaw, Y.F., 2001. Genome-wide hypomethylation in hepatocellular carcinogenesis. Cancer Res. 61, 4238–4243.

Lin, S.Y., Black, A.R., Kostic, D., Pajovic, S., Hoover, C.N., Azizkhan, J.C., 1996. Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. Mol. Cell. Biol. 16, 1668–1675.

Luebker, D.J., Case, M.T., York, R.G., Moore, J.A., Hansen, K.J., Butenhoff, J.L., 2005. Two-generation reproduction and cross-foster studies of perfluorooctanesulfonate (PFOS) in rats. Toxicology 215, 126–148.

Macleod, D., Charlton, J., Mullins, J., Bird, A.P., 1994. Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev. 8, 2282–2292.

Martin, J.W., Kannan, K., Berger, U., de Voogt, P., Field, J., Franklin, J., Giesy, J.P., Harner, T., Muir, D.C., Scott, B., Kaiser, M., Jarnberg, U., Jones, K.C., Mabury, S.A., Schroeder, H., Simcik, M., Sottani, C., van Bavel, B., Karrman, A., Lindstrom, G., van Leeuwen, S., 2004. Analytical challenges hamper perfluoroalkyl research. Environ. Sci. Technol. 38, 248A–255A.

Matsumoto, M., Imagawa, M., Aoki, Y., 1999. Identification of an enhancer element of class pi glutathione S-transferase gene required for expression by a co-planar polychlorinated biphenyl. Biochem. J. 338 (Pt 3), 599–605.

Midasch, O., Drexler, H., Hart, N., Beckmann, M.W., Angerer, J., 2007. Transplacental exposure of neonates to perfluorooctanesulfonate and perfluorooctanoate: a pilot study. Int. Arch. Occup. Environ. Health 80, 643–648.

Nakayama, M., Gonzalgo, M.L., Yegnasubramanian, S., Lin, X., De Marzo, A.M., Nelson, W.G., 2004. GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. J. Cell. Biochem. 91, 540–552.

Nobori, T., Miura, K., Wu, D.J., Lois, A., Takabayashi, K., Carson, D.A., 1994. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368, 753–756.

OECD (Organization for Economic Co-operation and Development), 2002. Co-operation on existing chemicals. Hazard assessment of perfluorooctane sulfonate (PFOS) and its salts, ENV/JM/RD(2002)17/FINAL, Paris.

Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247–257.

Olivero-Verbel, J., Tao, L., Johnson-Restrepo, B., Guette-Fernandez, J., Baldiris-Avila, R., O'Byrne-Hoyos, I., Kannan, K., 2006. Perfluorooctanesulfonate and related fluorochemicals in biological samples from the north coast of Colombia. Environ. Pollut. 142, 367–372.

Phe, V., Cussenot, O., Roupret, M., 2010. Methylated genes as potential biomarkers in prostate cancer. BJU Int..

Pogribny, I.P., Tryndyak, V.P., Boureiko, A., Melnyk, S., Bagnyukova, T.V., Montgomery, B., Rusyn, I., 2008. Mechanisms of peroxisome proliferator-induced DNA hypomethylation in rat liver. Mutat. Res. 644, 17–23.

Ramos-Gomez, M., Kwak, M.K., Dolan, P.M., Itoh, K., Yamamoto, M., Talalay, P., Kensler, T.W., 2001. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 98, 3410–3415.

Sakai, M., Muramatsu, M., 2007. Regulation of glutathione transferase P: a tumor marker of hepatocarcinogenesis. Biochem. Biophys. Res. Commun. 357, 575–578.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Clemen, L.A., Eldridge, S.R., Elcombe, C.R., Butenhoff, J.L., 2003. Sub-chronic dietary toxicity of potassium perfluorooctanesulfonate in rats. Toxicology 183, 117–131.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Olsen, G.W., Case, M.T., Butenhoff, J.L., 2002. Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in cynomolgus monkeys. Toxicol. Sci. 68, 249–264.

Smithwick, M., Muir, D.C., Mabury, S.A., Solomon, K.R., Martin, J.W., Sonne, C., Born, E.W., Letcher, R.J., Dietz, R., 2005. Perflouroalkyl contaminants in liver tissue from East Greenland polar bears (Ursus maritimus). Environ. Toxicol. Chem. 24, 981–986.

Steinmetz, K.L., Pogribny, I.P., James, S.J., Pitot, H.C., 1998. Hypomethylation of the rat glutathione S-transferase pi (GSTP) promoter region isolated from methyl-deficient livers and GSTP-positive liver neoplasms. Carcinogenesis 19, 1487–1494.

Taniyasu, S., Kannan, K., Horii, Y., Hanari, N., Yamashita, N., 2003. A survey of perfluorooctane sulfonate and related perfluorinated organic compounds in water, fish, birds, and humans from Japan. Environ. Sci. Technol. 37, 2634–2639.

Tchou, J.C., Lin, X., Freije, D., Isaacs, W.B., Brooks, J.D., Rashid, A., De Marzo, A.M., Kanai, Y., Hirohashi, S., Nelson, W.G., 2000. GSTP1 CpG island DNA hypermethylation in hepatocellular carcinomas. Int. J. Oncol. 16, 663–676.

Thibodeaux, J.R., Hanson, R.G., Rogers, J.M., Grey, B.E., Barbee, B.D., Richards, J.H., Butenhoff, J.L., Stevenson, L.A., Lau, C., 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I. Maternal and prenatal evaluations. Toxicol. Sci. 74, 369–381.

Tryndyak, V.P., Kovalchuk, O., Muskhelishvili, L., Montgomery, B., Rodriguez-Juarez, R., Melnyk, S., Ross, S.A., Beland, F.A., Pogribny, I.P., 2007. Epigenetic reprogramming of liver cells in tamoxifen-induced rat hepatocarcinogenesis. Mol. Carcinogen. 46, 187–197.

Tseng, C.L., Liu, L.L., Chen, C.M., Ding, W.H., 2006. Analysis of perfluorooctanesulfonate and related fluorochemicals in water and biological tissue samples by liquid chromatography-ion trap mass spectrometry. J. Chromatogr. A 1105, 119–126.

Verma, M., Srivastava, S., 2002. Epigenetics in cancer: implications for early detection and prevention. Lancet Oncol. 3, 755–763.

Wang, J., Qin, Y., Li, B., Sun, Z., Yang, B., 2006. Detection of aberrant promoter methylation of GSTP1 in the tumor and serum of Chinese human primary hepatocellular carcinoma patients. Clin. Biochem. 39, 344–348.

Watson, R.E., McKim, J.M., Cockerell, G.L., Goodman, J.I., 2004. The value of DNA methylation analysis in basic, initial toxicity assessments. Toxicol. Sci. 79, 178–188.

Xie, Y., Liu, J., Benbrahim-Tallaa, L., Ward, J.M., Logsdon, D., Diwan, B.A., Waalkes, M.P., 2007. Aberrant DNA methylation and gene expression in livers of newborn mice transplacentally exposed to a hepatocarcinogenic dose of inorganic arsenic. Toxicology 236, 7–15.

Xiong, Z., Laird, P.W., 1997. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 25, 2532–2534.

Yang, A.S., Estecio, M.R., Doshi, K., Kondo, Y., Tajara, E.H., Issa, J.P., 2004. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res. 32, e38.

Yang, B., Guo, M., Herman, J.G., Clark, D.P., 2003. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. Am. J. Pathol. 163, 1101–1107.

Zhang, Y.J., Wu, H.C., Shen, J., Ahsan, H., Tsai, W.Y., Yang, H.I., Wang, L.Y., Chen, S.Y., Chen, C.J., Santella, R.M., 2007. Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. Clin. Cancer Res. 13, 2378–2384.