Title: Association of environmental benzo[a]pyrene exposure and DNA methylation alterations in Hepatocellular Carcinoma: a Chinese case-control study

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Abstract: Epidemiological studies implicate environmental risk factors and epigenetic alterations in the multistage process of hepatocellular carcinoma (HCC) development. However, associations between environmental factors and DNA methylation of tumour suppressor genes (TSGs) in HCC development remain ambiguous. Understanding how possible interactions influence risk may provide insights into the complexity of hepatocarcinogenesis. For this study, blood samples were collected from HCC patients (n=90) and healthy volunteers (n=99) from Xiamen (China) and data for selected environmental risk factors [e.g., benzo[a]pyrene (B[a]P), hepatitis B or C virus (HBV or HCV) infection, smoking and alcohol consumption] were recorded; factors identified as significantly higher (P <0.05) amongst case subjects compared to controls were identified. In order to assess associations for epigenetic alterations and HCC risk factors, serum DNA methylation of TSGs was quantified using high-resolution melting (HRM) analysis. Our results clearly indicate elevated methylation patterns for detoxification gene [glutathione-S-transferase Pi (GSTP)] promoter regions in cases compared to control subjects. Additionally, GSTP promoter hypermethylation and B[a]P diol epoxide-albumin (BPDE-Alb) were positively correlated with HCC incidence. Our epidemiological and in vitro cell model studies indicated that GSTP promoter DNA methylation regulates this gene's expression. Moreover, GSTP also plays an important role in B[a]P detoxification and potential protective role against B[a]P-induced liver cell toxicity and hepatocarcinogenesis.

Response to Reviewers: Reviewers/Editor comments:
The submission still suffers from spelling, grammar, and flow issues around language. Please have it reviewed and revised by a native English speaker.

Response: We really appreciate the comments and suggestion from editor and reviewers. We have invited Prof. Frank Martin reviewed and revised
the manuscript carefully and made the related revision in the revised manuscript (highlighted in yellow).
Association of environmental benzo[a]pyrene exposure and DNA methylation alterations in hepatocellular carcinoma: a Chinese case-control study

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Abstract

Epidemiological studies implicate environmental risk factors and epigenetic alterations in the multistage process of hepatocellular carcinoma (HCC) development. However, associations between environmental factors and DNA methylation of tumour suppressor genes (TSGs) in HCC development remain ambiguous. Understanding how possible interactions influence risk may provide insights into the complexity of hepato-carcinogenesis. For this study, blood samples were collected from HCC patients (n=90) and healthy volunteers (n=99) from Xiamen (China) and data for selected environmental risk factors [e.g., benzo[a]pyrene (B[a]P), hepatitis B or C virus (HBV or HCV) infection, smoking and alcohol consumption] were recorded; factors identified as significantly higher (P <0.05) amongst case subjects compared to controls were identified. In order to assess associations for epigenetic alterations and HCC risk factors, serum DNA methylation of TSGs was quantified using high-resolution melting (HRM) analysis. Our results clearly indicate elevated methylation patterns for detoxification gene [glutathione-S-transferase Pi (GSTP)] promoter regions in cases compared to control subjects. Additionally, GSTP promoter hypermethylation and B[a]P diol epoxide-albumin (BPDE-Alb) were positively correlated with HCC incidence. Our epidemiological and in vitro cell model studies indicated that GSTP promoter DNA methylation regulates this gene’s expression. Moreover, GSTP also plays an important role in B[a]P detoxification and potential protective role against B[a]P-induced liver cell toxicity and hepato-carcinogenesis.

Keywords: B[a]P; Case control study; DNA methylation; Epigenetic; GSTP; Hepatocellular carcinoma
1. Introduction

Hepatocellular carcinoma (HCC) is the 4th most common cancer worldwide and a major cause of cancer-related deaths, especially in sub-Saharan countries, Southeast Asia and China. Incidence rates of HCC in China have increased to 30/100,000; this equates to approximately 55% of total worldwide cases (Chen et al., 2005, and Parkin et al., 2002). Epidemiological studies suggest that both environmental risk factors and genetic alterations are associated with HCC development (Chen et al., 2002, Johnson et al., 2010 and Lambert et al., 2011), but the precise underlying mechanism(s) leading to hepato-carcinogenesis remain unclear.

HCC is considered as mostly an environmental-related cancer, with both viral and chemical carcinogen components implicated in its multistage process (Zhao et al., 2010). Hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is considered a major risk factor (Lambert et al., 2011). Apart from infection, environmental pollution or unhealthy lifestyle may also provide a direct exposure route to many toxins that destabilize genomic integrity and/or deregulate epigenetic markings. Among many pollutants, benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH), has been reported to occur in the environment as result of incomplete combustion of organic materials. B[a]P is a pro-carcinogen and, systemically in humans, can be converted into reactive metabolites [e.g., B[a]P diol epoxide (BPDE)] by cytochrome P4501A1 (CYP1A1); such reactive metabolites covalently bind to DNA, potentially causing genomic alterations (Ko et al., 2004). Covalent interaction between BPDE and nucleophilic centres in DNA is thought to be a critical event in the initiation of B[a]P-induced tumorigenesis. Hence, B[a]P and its cellular metabolite BPDE may
instigate genomic and epigenomic alterations (Feng et al., 2002 and Tang et al.,
2012).

Aberrant DNA methylation is widely documented as an important epigenomic
event in the development and progression of many cancers, including HCC (Herceg
2007, Huang et al., 2011, Lambert et al., 2011 and Zhang et al., 2007). Recent studies
demonstrate that aberrant DNA methylation of specific TSG promoters (e.g., APC,
GSTP, P15, SOCS-1 and RASSF1A) and global genomes have been frequently
observed in HCC (Esteller et al., 2001, Lambert et al., 2011 and Yang et al., 2003).

Hypermethylation of TSG CpG islands inhibit transcriptional initiation and silence
expression of downstream genes; particularly, if detoxification genes are silenced, by
allowing intracellular accumulation of toxins, this may facilitate the carcinogenesis
process. However, the association between environment pollutants, epigenetic
modifications and HCC risk remains unknown. Herein, we investigated a
hospital-based case-control cohort resident in the middle of the South East coastline
of China, a high HCC incidence belt (Xu et al., 2003). In addition, an in-vitro cell
model was employed to assess the effects of B[a]P exposure and its association with
detoxification gene promoter DNA methylation modifications and HCC development.

2. Materials and methods

2.1 Samples and clinical characteristics of participants

Peripheral blood samples from newly diagnosed HCC patients (n=90) before
treatment and healthy volunteers (n=99) were obtained from Zhongshan Hospital of
Xiamen University, Xiamen Hospital of Traditional Chinese Medicine and the 174th hospital of the People’s Liberation Army. All the HCC cases were clinic patients who were determined by clinical and radiological evaluation not to suffer any other cancer types. Written informed consent was obtained from all participants. A structured questionnaire and survey was conducted, as previously described (Zhao et al., 2010). The structured questionnaire included participant demographics (e.g., sex, age, height, body weight, income, employment status and occupational history); additional information including history of cigarette smoking, alcohol consumption, disease, diabetes mellitus, physical activity and viral infection (HBV, HCV) were collected from the participants or clinical documents with the permission of related stakeholders (Niu et al., 2010 and Zhao et al., 2012).

2.2 Measurement of the serum benzo[a]pyrene diol epoxide-albumin (BPDE-Alb)

Serum BPDE-Alb adducts, a widely used biomarker for B[a]P exposure, were measured using reverse-phase high performance liquid chromatography, as previously described (Islam et al., 1999). In brief, 4 mL blood from participants were collected into Na-heparin tubes, and plasma separated by centrifugation at 1200×g for 10 min. Plasma was obtained and precipitated with one volume of saturated ammonium sulfate overnight, followed by 15 min centrifugation at 1200×g. To 1 mL of supernatant, 10 μL of concentrated acetic acid was added. The solution was left at room temperature overnight followed by 15 min centrifugation at 1200×g. The precipitated albumin, was washed with 4 mL acetone:ethylacetate (1:1) to remove unbound B[a]P metabolites. The precipitate was air-dried at room temperature and
solubilized in 900 μL of 10 mM Tris-HCl/1.0 mM EDTA (pH 8.0); the protein concentration was then determined using the protein quantitative kit (Bio-Rad Laboratories, USA). To 900 μL albumin was added 100 μl of 1 M HCl, and this solution was incubated at 90°C for 3 h. Water and methanol were added to a final volume of 5 mL, resulting in a 10% methanol solution. This solution was applied to pre-conditioned (5 ml methanol followed by 10 mL water) Sep-Pak C18 cartridges (Millipore, Milford, MA) followed by 10 ml washing with water and elution with 5 ml of methanol. The eluent was evaporated at 45°C under a nitrogen stream and re-solubilized in 500 μL of 10% methanol. The samples were stored at -20°C until analysis. Post-hydrolysis, the samples were analyzed with HPLC. For each sample 200 μL was injected. The separation was performed on a Nova-Pak C18 3.9×150 mm column (Waters, Milford, USA) with a flow rate of 1.0 mL/min within a Waters LC system (Waters, Milford, USA), equipped with a LC fluorescence detector. The excitation wavelength was 341 nm and the emission was measured at 381 nm. B[a]P-tetrahydrotetrol was separated by a linear gradient of methanol and water, 30% methanol to 100% methanol in 17 min, 8 min at 100% methanol and then 10 min at 30% methanol before the next injection.

2.3 Treatment of cell lines with B[a]P

To investigate the association between GSTP promoter region methylation status and GSTP expression, normal immortal human liver (L02) and lung adenocarcinoma (A549) cell lines were selected as in vitro models. Both L02 and A549 cell lines were obtained from the Cell Bank of the Chinese Academy of
Sciences (Shanghai, China) where they were characterized by mycoplasma detection, immunoperoxidase staining, DNA profiling and cell viability tests. Cell lines were immediately expanded and frozen so they could be restarted every 4 to 5 months from a frozen vial of the same batch of cells. Both cell lines were cultured in DMEM high-glucose medium (Hyclone, USA). Medium were supplemented with 10% FBS (Hyclone, USA), 100 unit/mL penicillin and 100 unit/mL streptomycin. L02 and A549 cells were collected for GSTP promoter methylation status analysis and GSTP mRNA expression. A549 cells were employed as a methylation analysis control. Toward B[a]P exposure experiments, L02 cells were exposed to 0.1, 1 or 10 nM B[a]P (Sigma-Aldrich, St. Louis, Mo) or DMSO alone (vehicle control). Meanwhile, in order to explore the GSTP detoxification in B[a]P exposure, L02-cell GSTP promoter regions were de-methylated and gene expression restored with 5 μM 5-aza-2′-deoxycytidine (DAC) for 2 days treatment prior to exposure to 0.1, 1 or 10 nM B[a]P or DMSO alone (vehicle control). After three days incubation, all cells were harvested and used for DNA or RNA analyses.

2.4 Serum cell-free and cultured cell DNA extraction, and bisulfite treatment

To remove additional cellular nucleic acids attached to cell debris, aspirated serum samples were centrifuged at 16,000×g for an additional 10 min at 4°C before DNA extraction. Cell-free DNA (i.e., circulating free) in the serum of participants was extracted using the QIAamp DNA minikit (Qiagen, Hilden, Germany), according to manufacturer’s instructions. Approximately, 200 ng DNA isolated from serum was subjected to sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research,
Orange, CA, USA), according to manufacturer’s instructions; unmethylated cytosines are converted to uracil whereas methylated cytosines remain unchanged during the reaction. The modified DNA was eluted in elution buffer and used for quantification of DNA methylation.

2.5 Quantitative DNA methylation measurement

High-resolution melting (HRM) analysis was applied to quantitatively measure the methylation status of GSTP promoter regions. Herein, whole genome PCR amplification of monoclonal plasmid from bisulfite-modified CpGenome Universal Methylated DNA (Chemicon, Millipore, Billerica, MA, USA) and genomic DNA from peripheral blood mononuclear cells of normal individuals were selected as methylation and unmethylation controls, respectively. Both of the control plasmids were confirmed by Sanger sequencing before performing an experiment. Methylation standards were constructed by diluting 100% methylated control plasmid in a pool of unmethylated control plasmid at 100%, 75%, 50%, 25%, 10%, 5% and 0% ratios. PCR amplification and HRM analyses were performed on the LC 480, as adapted from a published protocol (Roche Applied Science, Germany). Primers used for HRM analysis are shown in Table 1. PCR was performed in a final 20 μL volume containing: 1 × ZymoTaq Premix (Zymo, USA), 250 nM of each primer, 2.5 mM SYTO-9 dye (Invitrogen, Carlsbad, USA) and 10 ng bisulfite-treated DNA template. The cycling conditions started with one cycle at 95°C for 10 min, followed by 60 cycles at 95°C for 20 sec each, a touch-down at 64°C to 58°C for 20 sec (1°C/cycle), 72°C for 20 sec, and a HRM step of 95°C for 1 min, 40°C for 1 min, 65°C for 5 sec,
and continuous to 95°C at 25 acquisitions per 1°C. HRM data was analysed by using
gene scanning software (Roche, Germany), as previously described (Stanzer et al.,
2010). The melting curves were processed with normalization and temperature
shifting using LightCycler Software. Raw data were exported from the LightCycler
and used to generate a standard curve by regression analysis. This standard curve was
then used to calculate the methylation levels of samples.

2.6 Bisulfite sequencing PCR analysis

The alternative quantitative measure of GSTP methylation was conducted by
bisulfite sequencing PCR (BSP) (Tian et al., 2012). Genomic DNA was
bisulfite-modified before PCR. GSTP promoter regions were amplified using the
sequencing primer (Table 1). The PCR product containing 38 sites was purified using
a Wizard SV Gel and PCR Clean-up System (Promega, USA), and cloned into
bacteria using the pMD® 18-T Vector (Takara, Japan). Individual clones were grown
overnight and plasmid DNA was isolated with an E.Z.N.A.™ Plasmid Mini Kit
(Promega, USA) and sequenced. Ten to twelve clones were sequenced per group.
Sequence results were analysed online by QUMA (http://quma.cdb.riken.jp) (Kumaki
et al., 2008).

2.7 RNA extraction and RT-PCR

Total RNA was isolated from each cell lines using the High pure RNA isolation
Kit (Roche, Germany), according to the manufacture's protocol. First-stand cDNA
synthesis was performed with one microgram total RNA using PrimeScript RT
reagent Kit with the gDNA Eraser cDNA synthesis Kit (Takara, Japan) employing six
random primers and oligo dT primer. RT-PCR was performed using the SYBR Green Master Mix reagents (Roche, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. Primer sets used for amplification are shown in Table 1.

2.8 Statistical analysis

Statistical analyses were performed using SPSS 18 (SPSS Inc). Variable distributions were analysed by the nonparametric Mann-Whitney U-test for continuous variables and $\chi^2$-test for categorical variables. Due to the non-normal distribution, Spearman correlation coefficients of BPDE-Alb levels with GSTP methylation status were calculated. Unconditional multivariate logistic regression was performed for chemical pollutants, which were significantly ($P < 0.05$) associated with HCC in the univariate analysis. Age, gender, BMI and other important HCC risk factors were adjusted using the likelihood-ratio test. Adjusted odds ratios (AOR) and 95% confidence intervals (CI) were calculated by the maximum likelihood approach.

The quantity assessment of two factors’ interaction was also investigated, as previously described (Källberg et al., 2006 and Zhao et al., 2010).

3. Results

3.1 Demographics and related information

Demographic and clinical characteristics of study participants are shown in Table 2. HCC cases and corresponding controls were well age-matched (57.8 ± 8.4 y vs. 57.9 ± 8.8 y; $P = 0.96$). The case cohort contained a higher proportion of male subjects, and their body mass index (BMI) levels were lower than the controls (21.2 ± 2.8 vs. 21.8 ± 2.8 y; $P = 0.23$).
227 22.4 ± 3.6; \( P = 0.01 \)). Smoking and alcohol consumption subjects were higher among
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229 higher in cases than in controls (43.3% vs. 9.1% for HBV; 44.4% vs. 21.2% for HCV).
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233 3.2 GSTP promoter methylation and B[a]P exposure levels
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247 was observed as: methylated (exhibited fully methylated peak, Figure 2),
248 heterogeneous (exhibited only one broad methylated peak, Figure 2) and
249 unmethylated (only exhibited fully unmethylated peak, Figure 2) in cases (42.2%,
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347 \( P = 0.01 \)).
The median DNA methylation levels of \textit{GSTP} promoter regions in cases (1.62\%) were significantly higher than those of controls (0) \((P < 0.001\), Table 3). B\([a]\)P exposure levels in cases were significantly higher than those of the control group \((1.79 \text{ fmol/mg adduct} vs. 1.51 \text{ fmol/mg adduct}, P < 0.001\), Table 3). Meanwhile, we found a positive correlation between \textit{GSTP} methylation and BPDE-Alb levels in all subjects \((r = 0.13, P = 0.04)\). Nevertheless, the median BPDE-Alb exposure level in all methylated subjects \((4.35 \text{ fmol/mg})\) was significantly higher than all unmethylated subjects \((1.66 \text{ fmol/mg})\) \((P = 0.02)\), while, there was no significance between heterogeneous subjects \((3.22 \text{ fmol/mg})\) and unmethylated subjects \((1.66 \text{ fmol/mg})\) \((P = 0.15)\) as well as methylated subjects \((4.35 \text{ fmol/mg})\) \((P = 0.82)\) (Figure 3).

Logistic regression was applied to investigate possible associations between \textit{GSTP} promoter methylation and B\([a]\)P exposure. In the stratification interaction analysis, after adjustment in the models for confounding variables \(i.e.,\) age, BMI, sex, alcohol consumption, smoking, HBV, HCV and liver cirrhosis, there was a stronger association between BPDE-Alb adduct levels with HCC risk amongst \textit{GSTP-M/H} subjects, \(i.e.,\) methylated and heterogeneous. The AOR for \textit{GSTP-M/H} subjects who exhibited both low and high BPDE-Alb adduct levels were markedly elevated \((3.8 \pm 0.7\;\text{to}\;26.6\;)\) \((P < 0.001)\), for trend see Table 4.

### 3.3 \textit{GSTP} detoxification in \textit{B[a]P} exposure

In the \textit{in vitro} study, we investigated whether \textit{GSTP} silencing is accompanied by promoter region hypermethylation. Two human cell (A549 and L02) lines were investigated, and \textit{GSTP} expression was observed both in vehicle control and
DAC-treated A549 cells; promoter region DNA was almost unmethylated, even following DAC treatment (Figure 4). However, GSTP is silenced in L02 cells and its promoter is hypermethylated (78.5%). After DAC treatment, GSTP expression was restored with a corresponding decrease in promoter region methylation (57.5%) (Figure 4).

To investigate the action of GSTP on detoxification in B[a]P exposure, we further examined the differences in responses to low-dose B[a]P treatment between L02 and DAC-exposed L02 cells. The L02 cell GSTP promoter region exhibited hypermethylation, and gene expression was silenced. Contrary to this, DAC-exposed L02 cell GSTP promoter region hypomethylation was observed; gene expression was significantly induced by B[a]P. However, GSTP promoter methylation status was unchanged by B[a]P exposure (Figure 5). Despite differences in cell models, these observations were associated with similar trends in CYP1A1 expression induced by B[a]P exposure (Figure 6). However, B[a]P exposure significantly induced the detoxification gene (GSTP), oxidative stress response gene (HO-1), cell cycle regulation gene (TP53), DNA methyltransferases genes (DNMT1, DNMT3A and DNMT3B) and DNA mismatch repair genes (HMLH1 and HPMS2) expression in L02 cells, while no significance was found in DAC-treated L02 cells (Figure 6).

4 Discussion

This case-control study demonstrates that BPDE-Alb adducts are significantly ($P$ <0.05) higher in HCC cases than those of controls. Similarly, the PAH detoxification
gene GSTP promoter region is hypermethylated in HCC serum samples. BPDE-Alb adducts are significantly correlated with GSTP methylation levels in this cohort. In addition, risk for HCC development was highest amongst those individuals who exhibited higher levels of BPDE-Alb adducts and GSTP hypermethylation. Our cell model experiments also suggest that loss of GSTP expression via hypermethylation results in elevated B[a]P-induced genomic and epigenomic toxicity.

4.1 Environmental exposure of PAHs in HCC

PAHs are ubiquitous environmental pollutants and emitted into surrounding environment (i.e., air, soil, water, etc.) via incomplete combustion of coal, oil, gas, wood, other carbon-containing organic materials and/or cigarette smoke. These ultimately result in human (or other organism, e.g., wildlife) exposure through direct (water, diet) or indirect (dust, air) routes (Su et al., 2014 and Wu et al., 2007). B[a]P is the prototypical PAH and categorized as a pro-carcinogen. The tumorigenic effects of B[a]P are likely mediated via metabolic activation by cytochrome P450 enzymes and epoxide hydrolase into the highly reactive electrophilic metabolite, BPDE; this ultimately forms adducts through covalent binding to DNA and/or albumin.

PAH-albumin adducts have been used as a sensitive indicator of chronic low-level PAH exposure, due to lack of repair mechanisms for albumin as compared to DNA. PAH-albumin adducts can also reflect different routes of long-term exposure, and may account for inter-individual differences for uptake, distribution, metabolism, and elimination (Wu et al., 2007). PAH-albumin adducts have also been reported to be associated with increased HCC risk for other Taiwanese and Xiamen (China) cohorts.
(Wu et al., 2007 and Zhao et al., 2010). One potential mechanism for PAHs in hepato-carcinogenesis is elevated production of oxidative stress. A previous study found PAH-albumin adducts significantly correlated with urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine, a predominant form of free radical-induced oxidative lesions (Autrup et al., 1999). In agreement with our previous findings, the levels of PAH-albumin and PAH-DNA adducts in HCC blood were significantly higher than those in samples from control subjects (Su et al., 2014 and Zhao et al., 2010). However, this study infers that apart from exposure factors, diverse elimination routes and metabolic fate may exert important roles in determining BPDE-Alb adduct levels in human serum. Likewise, our results also reveal that BPDE-Alb adducts distribution is not gender specific and there appears to be no correlation with cigarette smoking.

4.2 DNA epigenetic alterations in HCC

Epidemiology data suggests that increased oxidative stress from chronic hepatitis virus infection and environmental chemical exposures are involved in the pathogenesis of HCC (Li et al., 2013 and Martin and Herceg, 2012). Viral infection and environmental toxicants generate chronic inflammation and induce DNA damage via reactive oxygen species (Martin and Herceg, 2012). Oxidative stress has also been reported to interfere with epigenetic mechanisms, which can recruit DNMTs and Snail-dependent pathway to promoter hypermethylation of several specific gene promoter regions and silence their expression (Hamilton, 2010 and Lim et al., 2008). Such epigenetic alterations might be early events in carcinogenesis and play key roles...
We employed HRM analysis, a highly sensitive and specific method for detection and quantification of DNA methylation. HRM technology robustly distinguishes promoter region DNA into fully methylated and heterogeneous status. The precision of the HRM quantitative approach was tested by BSP analyses, and both results were highly concordant (Figure 2). We observed a high frequency of \textit{GSTP} hypermethylation in the HCC group (53.3\%), agreeing with previous studies showing a methylation-associated inactivation of \textit{GSTP} in liver tumours (Lambert et al., 2011, Yang et al., 2003 and Zhang et al., 2005). Additionally, we also found \textit{GSTP} hypermethylation in a few control group participants. A possible explanation for these observations could be that epigenetic alternations are early events in carcinogenesis. The age factor may also influence DNA methylation modification (Lambert et al., 2011), and all individuals in the cases cohort were slightly older than controls (57.8 vs. 57.9 y). We also confirmed in an \textit{in vitro} L02 cell study that \textit{GSTP} expression is silenced due to the promoter region hypermethylation. The \pi class glutathione S-transferase (\textit{GSTP}) is a family of phase II enzymes that can conjugate GSH with various environmental etiological factors. It likely plays important roles in protecting cells against genomic damage mediated by a wide range of oxidants and electrophiles from inflammation and/or environmental exposure (Li et al., 2013 and Zhang et al., 2005). The liver is the major organ for metabolism and detoxification, and silencing \textit{GSTP} expression may result in accumulation of genotoxic agents and elevated oxidative stress, causing DNA damage induction and HCC promotion (Lambert et al.,...
It is noteworthy that herein we focused on cell-free serum DNA, which is easily obtained and a non-invasive measure as opposed to traditional biopsied tumour tissue. Serum and/or plasma samples contain circulating cell-free DNA shed from the primary tumour tissue; this is a ready surrogate for genetic and epigenetic studies (Huang et al., 2011). DNA methylation modifications in plasma and/or serum samples are highly concordant with those in matched primary tumour specimens (Huang et al., 2011 and Iyer et al., 2010). This study reports similar results for GSTP hypermethylation frequency (53.3%) when compared to three previous HCC studies (54%, 46% and 54%, respectively) (Lambert et al., 2011, Zhang et al., 2005 and Yang et al., 2003).

4.3 Association between PAH exposure and GSTP methylation

Considering that GSTP plays an important role in reactive electrophile metabolite elimination and antioxidant defence, we further examined the different groups categorized by GSTP promoter region methylation status using a stratified analyses. Subjects exhibiting hypermethylation presented with significantly higher PAH-albumin adducts compared to hypomethylated individuals. Additionally, risk of developing HCC was highest amongst individuals who exhibited hypermethylated GSTP and elevated burden of BPDE-Alb adducts. These results indicate that GSTP plays an important role in PAH detoxification in vivo. Previous in vitro studies have also confirmed that GSTP-transfected HepG2 cells are more resistant to PAH toxicity. GSTP polymorphisms affect such protection, and HepG2 cells transfected with
insert-free vector acquire high levels of PAH-induced DNA damage via formation of PAH-DNA adducts (Hu et al., 1999). Epidemiology also suggests a significant association between GSTP promoter hypermethylation with AFB$_1$-DNA adducts in HCC (Zhang et al., 2005).

To investigate the GSTP protective mechanism against PAH exposure in liver cells, we employed the human liver L02 cell line and DAC-treated L02 cells as an in vitro model, exposed to low-dose B[a]P (≤10 nM). CYP1A1, which converts B[a]P into BPDE during phase I biotransformation, was induced by PAH exposure in both cell models. BPDE may then be detoxified by GSTP during phase II reactions. GSTP can determine BPDE levels, thus influencing resulting genotoxicity. However, detoxification of reactive metabolite was only observed in PAH-induced DAC-treated L02 cells. Our results indicate that GSTP hypermethylation results in gene silencing and loss of its protective function. Thus oxidative stress response gene Heme oxygenase-1 (HO-1), cell cycle regulation gene (P53), DNA methyltransferases genes (DNMT1, DNMT3A and DNMT3B) and DNA mismatch repair genes (HMLH1 and HPMS2) were significantly induced by B[a]P exposure. This infers that GSTP promoter region hypermethylation causes failure in catalysing GSH conjugation of BPDE and results in accumulation of PAH-Alb and/or PAH-DNA adducts.

Herein, selection bias and information bias were stringently controlled for. Potential confounding factors were ruled out using stratified analysis or multiple factor analysis. There were some limitations in the present study. It is a retrospective case-control epidemiological study, which has its inherent limitations and
disadvantages. Due to the relatively small sample size, these findings need to be confirmed in a larger cohort study. Although epidemiological data were complimented with in vitro cell models, it remains difficult to identify the exact chronological order for the elevation in BPDE-Alb adducts and the hypermethylation of GSTP promoter region. However, environmental PAH exposure and GSTP methylation associated with the risk of developing HCC is observed.

In conclusion, we observe that GSTP promoter region methylation in circulating-free DNA isolated from serum is positively associated with HCC risk and may serve as an early epigenetic susceptibility biomarker. In addition, there appears to be an associated effect of environmental PAH exposure and GSTP methylation on risk of developing HCC. However, the interaction between environmental exposure and epigenetic alteration on HCC risk requires further investigation.

Acknowledgments

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Reference


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Zhang YJ, Chen Y, Ahsan H, Lunn RM, Chen SY, Lee PH, Chen CJ, Santella RM.


Figure Legends

Figure 1. High resolution melting assay using serial dilution of methylated plasmid DNA (from 100% to 0%). (A) High resolution melting curve of standard serial dilutions of methylated GSTP promoter region; and, (B) values of relative signal were plotted against the percentage of methylation for each dilution to generate a typical standard curve. All the experiments were performed in duplicate.

Figure 2. High resolution melting (HRM) and bisulfite sequencing PCR (BSP) analysis of GSTP promoter status in representative serum samples. (A) HRM curve of GSTP promoter methylated standard (red), unmethylated standard (green), unmethylated serum (black), methylated serum (yellow), fully methylated serum (blue) and heterozygous methylated serum (purple); (B) Representative BSP analysis of GSTP promoter fully methylated serum; (C) Representative BSP analysis of GSTP promoter unmethylated serum; (D) Representative BSP analysis of GSTP promoter methylated serum; and, (E) Representative BSP analysis of GSTP promoter heterozygous methylated serum. In B-E, each line represents one DNA strand; the numbers at the top of the parallel line identify the location of the cytosine at the CpG sites; the open circles are unmethylated CpGs; the filled-in circles are methylated CpGs.
Figure 3. Distribution BPDE-Alb exposure level in GSTP promoter unmethylated, heterozygous and methylated groups. All individual divided into unmethylated, heterozygous and methylated groups. Bar indicates media, P for Mann-Whitney test.

Figure 4. RT-PCR and BSP analysis of GSTP expression and methylation in A549 cells, DAC-treated A549 cells, L02 cells and DAC-treated L02 cells. (A) GAPDH as internal reference. GSTP is normally expressed in A549 cells, silenced in L02 cells but is restored upon DAC treatment; (B) The CpG methylation rates (%) for GSTP CpGs -226 to +84 in the control and DAC-treated groups for A549 and L02 are shown, DAC significantly decreased GSTP promoter methylation levels and restored GSTP mRNA expression in L02 cells.

Figure 5. BSP analysis of DAC-treated L02 cell GSTP promoter methylation status in response to 24 h different dose of B[a]P exposure. The CpG methylation rates (%) for GSTP promoter -226 to +84 in the DMSO control groups 61.6 ± 9.9% (A); 0.1 nM B[a]P groups 65.5 ± 8.7% (B); 1 nM B[a]P groups 61.1 ± 12.1% (C); and, 10 nM B[a]P groups 60.3 ± 10.7% (D). L02 cells were treated with 5 μM 5-aza-2′-deoxycytidine (DAC) for 2 days prior to exposure, and then exposed to 0.1, 1, 10 nM B[a]P or with DMSO alone (vehicle). In A-D, each line represents one DNA strand; the numbers at the top of the parallel line identify the location of the cytosine at the CpG sites. The open circles are unmethylated CpGs the filled-in circles are methylated CpGs.
Figure 6. Real-time RT-PCR analysis of gene expression in L02 cells (A) and DAC-treated L02 cells (B) in response to B[a]P exposure. Asterisks (*) indicate significant difference ($P < 0.05$) when compared to the control. Standard deviation is showed as the error bars, which arise from triplicate tests for all the four independent experiments.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Application</th>
<th>Primer sequence (5'-3')</th>
<th>Product size (bp)</th>
<th>CpG No.</th>
</tr>
</thead>
</table>
| GSTP  | HRM/BSP     | F: GGGA TTTGGGAAAGGAGGAAAGG  
                         R: ACCGCTCTCTTCTAAAAATCC  
                         | 149                | 17      |
| GSTP  | BSP         | F: GGATTTGGGAAAGGAGGAAAGG  
                         R: CCCATACTAAAATCTCTAAACC  
                         | 311                | 38      |
| CYP1A1 | Q-PCR | F: CATCCCCCAGCACAACA  
                  R: CAGGGGTGAGAAACCCTTTCA  
                  | 152                | -       |
| GSTP  | Q-PCR       | F: CCGAGACCTCCAACCTGGA  
                         R: CGCTCTATAGTTTGGTGAAG  
                         | 169                | -       |
| P53   | Q-PCR       | F: TCTTCTGTCCCTCCAGAA  
                         R: AATCAACCCACAGCTGCAC  
                         | 163                | -       |
| DNMT1 | Q-PCR | F: TACCTGACGACCCCTGACCTC  
                  R: CGTTGGCATCAAAGATGGACA  
                  | 103                | -       |
| DNMT3A| Q-PCR      | F: TATATGAGGCACGACAGAGGA  
                         R: GGGTGTTCCAGGGTAACATTGAG  
                         | 111                | -       |
| DNMT3B| Q-PCR      | F: GGCAAGTTCCCGAGGTCTCG  
                         R: TTGTGATGCTGTGTGGTGA  
                         | 113                | -       |
| HMLH1 | Q-PCR      | F: TCTCAGGCCAGCAGAGTGAA  
                         R: TGTGAGAGCCAGGCCTTTTA  
                         | 93                 | -       |
| HMSH2 | Q-PCR      | F: ATTGACCTTTGGGAAAAAGAAGATGC  
                         R: TAAAAGAGAGCATTGCTGTTG  
                         | 223                | -       |
| HPMS2 | Q-PCR      | F: GCACTGAGGCTGTCCACATT  
                         R: TCTCTTTGCGGCACAGGCTAG  
                         | 171                | -       |
| GAPDH | Q-PCR      | F: GGAGAAGGCTGGGCGCTCAT  
                         R: TGATGGCATTGGACTGGTGTC  
                         | 230                | -       |
Table 2. Demographics and clinical characteristics in cases \((n=90)\) and controls \((n=99)\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case (%)</th>
<th>Control (%)</th>
<th>(\chi^2) (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>76 (84.4)</td>
<td>60 (60.6)</td>
<td>13.3 (&lt;0.001)</td>
</tr>
<tr>
<td>Female</td>
<td>14 (15.6)</td>
<td>39 (39.4)</td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>46 (51.1)</td>
<td>52 (52.5)</td>
<td>0.04 (0.85)</td>
</tr>
<tr>
<td>≥50</td>
<td>44 (48.9)</td>
<td>47 (47.5)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;24</td>
<td>74 (82.2)</td>
<td>70 (70.7)</td>
<td>3.5 (0.06)</td>
</tr>
<tr>
<td>≥24</td>
<td>16 (17.8)</td>
<td>29 (29.3)</td>
<td></td>
</tr>
<tr>
<td>Life style</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoking</td>
<td>40 (44.4)</td>
<td>63 (63.6)</td>
<td>8.4 (0.02)</td>
</tr>
<tr>
<td>Smoking</td>
<td>48 (53.3)</td>
<td>36 (36.3)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2 (2.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>56 (62.2)</td>
<td>75 (75.8)</td>
<td>11.8 (0.03)</td>
</tr>
<tr>
<td>Drinker</td>
<td>32 (35.6)</td>
<td>24 (24.2)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2 (2.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV infection and disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV (-)</td>
<td>51 (56.7)</td>
<td>90 (90.9)</td>
<td>29.2 (&lt;0.001)</td>
</tr>
<tr>
<td>HBV (+)</td>
<td>39 (43.3)</td>
<td>9 (9.1)</td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV (-)</td>
<td>50 (55.6)</td>
<td>78 (78.8)</td>
<td>11.3 (&lt;0.001)</td>
</tr>
<tr>
<td>HCV (+)</td>
<td>40 (44.4)</td>
<td>21 (21.2)</td>
<td></td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cirrhosis (-)</td>
<td>72 (81.1)</td>
<td>99 (100)</td>
<td>20.8 (&lt;0.001)</td>
</tr>
<tr>
<td>Cirrhosis (+)</td>
<td>17 (18.9)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Gene promoter methylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTP-U(^a)</td>
<td>40 (44.4)</td>
<td>77 (77.8)</td>
<td>35.2 (&lt;0.001)</td>
</tr>
<tr>
<td>GSTP-H(^a)</td>
<td>10 (11.1)</td>
<td>11 (11.1)</td>
<td></td>
</tr>
<tr>
<td>GSTP-M(^a)</td>
<td>38 (42.2)</td>
<td>6 (6.1)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2 (2.2)</td>
<td>5 (5.0)</td>
<td></td>
</tr>
</tbody>
</table>

\(GSTP\) methylation status was qualitatively determined by HRM PCR.

\(^a\)M, fully methylated; H, heterogeneous; U, fully unmethylated.
Table 3. Serum pollutant concentrations and DNA methylation levels in the participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Median</td>
</tr>
<tr>
<td>BPDE-Alb (fmol/mg adduct)</td>
<td>17.71</td>
<td>21.64</td>
<td>1.79</td>
</tr>
<tr>
<td>GSTP (%)</td>
<td>12.79</td>
<td>23.38</td>
<td>1.62</td>
</tr>
</tbody>
</table>

Variable distributions were analysed by the nonparametric Mann-Whitney $U$-test.

Table 4. Multivariate logistic regression analysis of the interactions of GSTP epigenetic vs benzo[a]pyrene exposure in HCC risks

<table>
<thead>
<tr>
<th>GSTP</th>
<th>BPDE-Alb</th>
<th>Case</th>
<th>Control</th>
<th>Crude OR (95% CI)</th>
<th>AOR$^{a}$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP-U Low</td>
<td>15</td>
<td>41</td>
<td>1.0 (reference)</td>
<td>1.0 (reference)</td>
<td></td>
</tr>
<tr>
<td>GSTP-U High</td>
<td>24</td>
<td>36</td>
<td>1.1 (0.3-3.3)</td>
<td>1.1 (0.3-4.3)</td>
<td></td>
</tr>
<tr>
<td>GSTP-H/M Low</td>
<td>19</td>
<td>7</td>
<td>4.4 (1.8-10.5)</td>
<td>3.8 (1.3-10.9)</td>
<td></td>
</tr>
<tr>
<td>GSTP-H/M High</td>
<td>29</td>
<td>10</td>
<td>7.7 (3.0-19.6)</td>
<td>8.4 (2.7-26.6)</td>
<td></td>
</tr>
</tbody>
</table>

P-trend < 0.001  P-trend < 0.001

AOR was adjusted for age, BMI, sex, alcohol consumption, smoking, HBV, HCV, liver cirrhosis each other.
Figure 1

The top graph shows melting peaks at different temperatures, with colors representing various methylation percentages (100%, 75%, 50%, 25%, 10%, 5%, 1%, and 0%). The bottom graph illustrates a linear relationship between GSTP methylation percentage and relative signal, with the equation $y = 0.9869x + 3.1171$ and $R^2 = 0.9909$. The data points on the graph are plotted against the indicated ranges.
Figure 2
Click here to download high resolution image
Figure 3

The scatter plot shows the distribution of fmol/mg adduct for unmethylated, heterozygous, and methylated samples. The p-values are as follows:

- Unmethylated vs. Heterozygous: \( p = 0.02 \)
- Heterozygous vs. Methylated: \( p = 0.15 \)
- Unmethylated vs. Methylated: \( p = 0.82 \)