Elsevier Editorial System(tm) for Science of the Total Environment

Manuscript Draft

Manuscript Number: STOTEN-D-15-02388R3

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

Article Type: Research Paper

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

Corresponding Author: Prof. Heqing Shen, Ph.D

Corresponding Author's Institution: Institute of Urban Environment,Chinese Academy of Sciences

First Author: Meiping Tian

Order of Authors: Meiping Tian; Heqing Shen, Ph.D

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.

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).

*Revised manuscript with no changes marked
Click here to view linked References

Association of environmental benzo[*a*]pyrene exposure and DNA methylation alterations in hepatocellular carcinoma: a Chinese case-control study

Meiping Tian[1], Benhua Zhao[2], Jie Zhang[1], Francis L. Martin[3], Qingyu Huang[1], Liangpo Liu[1], Heqing Shen[1*]

[1]*Institute of Urban Environment, Chinese Academy of Sciences, Key Lab of Urban Environment and Health, Xiamen, 361021, PR China*

[2]*School of Public Health, Xiamen University, Xiamen, 361102, PR China*

[3]*Centre for Biophotonics, LEC, Lancaster University, Lancaster LA1 4YQ, UK*

[*]**Corresponding author details:** 1799 Jimei Road, Xiamen 361021, PR China;

Tel.: +86-592-6190771; Fax: +86-592-6190771; E-mail: hqshen@iue.ac.cn

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℃ 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 C_{18} 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 C_{18} 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℃ 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.^{TM} 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 χ^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.*

22.4 ± 3.6; *P* =0.01). Smoking and alcohol consumption subjects were higher among the cases as compared to controls. The incidence of HBV and HCV was markedly higher in cases than in controls (43.3% *vs.* 9.1% for HBV; 44.4% *vs.* 21.2% for HCV). The number of liver cirrhosis patients among cases was also higher compared to controls (Table 2).

3.2 GSTP promoter methylation and B[a]P exposure levels

Human *GSTP* promoter region flank (-226 ~ -78) included 17 CpG sites and/or (-226 ~ +84) included 38 CpG sites. To estimate *GSTP* promoter methylation levels, HRM PCR was performed on serum cell-free DNA. To test the sensitivity of the HRM assays, we used the standard dilution series as described in the Materials and Methods section. The HRM assay can offer sufficient sensitivity to distinguish the heterogeneous methylation pattern. This assay was able to detect 1% methylation DNA in a background of unmethylated DNA. The correlation coefficient was 0.9919 (Figure 1). Furthermore, to verify the reliability of the HRM assays on serum DNA, we performed BSP assays to quantify the levels of methylation accurately. Figure 2 demonstrates the concordance of the methylation status by the HRM and BSP assays. We applied HRM for *GSTP* promoter methylation to analyse the *n*=90 HCC and *n*=99 normal serum cell-free DNA. The frequency distribution of *GSTP* methylation status was observed as: methylated (exhibited fully methylated peak, Figure 2), heterogeneous (exhibited only one broad methylated peak, Figure 2) and unmethylated (only exhibited fully unmethylated peak, Figure 2) in cases (42.2%, 11.1% and 44.4%) and controls (6.1%, 11.1% and 77.8%), respectively (*P* <0.001,

Table 2). The median DNA methylation levels of *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 fmol/mg adduct *vs.* 1.51 fmol/mg adduct, P <0.001, Table 3). Meanwhile, we found a positive correlation between *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 fmol/mg) was significantly higher than all unmethylated subjects (1.66 fmol/mg) (P =0.02), while, there was no significance between heterogeneous subjects (3.22 fmol/mg) and unmethylated subjects (1.66 fmol/mg) (P =0.15) as well as methylated subjects (4.35 fmol/mg) (P =0.82) (Figure 3).

Logistic regression was applied to investigate possible associations between *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 *GSTP*-M/H subjects, *i.e.*, methylated and heterogeneous. The AOR for *GSTP*-M/H subjects who exhibited both low and high BPDE-Alb adduct levels were markedly elevated (3.8 with 95% CI 1.3-10.9; 8.4 with 95% CI 2.7-26.6, P <0.001, for trend see Table 4).

3.3 GSTP detoxification in B[a]P exposure

In the *in vitro* study, we investigated whether *GSTP* silencing is accompanied by promoter region hypermethylation. Two human cell (A549 and L02) lines were investigated, and *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

in hepato-carcinogenesis.

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 *GSTP* hypermethylation in the HCC group (53.3%), agreeing with previous studies showing a methylation-associated inactivation of *GSTP* in liver tumours (Lambert et al., 2011, Yang et al., 2003 and Zhang et al., 2005). Additionally, we also found *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 *in vitro* L02 cell study that *GSTP* expression is silenced due to the promoter region hypermethylation. The π class glutathione *S*-transferase (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 *GSTP* expression may result in accumulation of genotoxic agents and elevated oxidative stress, causing DNA damage induction and HCC promotion (Lambert et al.,

2011).

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

This study was financially supported by National Natural Science Foundation of China 2011 foundation (21177123, 21307126) and the related NSFC-RC International Exchange Programme 2013, the Hundred Talent Program of the Chinese Academy of Sciences (CAS) for 2010 on Human Exposure to Environmental Pollutant and Health Effect, CAS/SAFEA International Partnership Program for Creative Research Teams (KZCX2-YW-T08) and the Xiamen Science and Technology Fund (3502Z20122003). We thank all who contributed their samples and participated in this study.

Reference

Autrup H, Daneshvar B, Dragsted LO, Gamborg M, Hansen M, Loft S, Okkels H, Nielsen F, Nielsen PS, Raffn E, Wallin H, Knudsen LE. Biomarkers for exposure to ambient air pollution-comparison of carcinogen-DNA adduct levels with other exposure markers and markers for oxidative stress. Environ Health Perspect. 1999; 107(3):233-8.

Chen JG, Song XM. An evaluation on incident cases of liver cancer in China. Bulletin of Chinese Cancer 2005;1:28-31.

Chen SY, Wang LY, Lunn RM, Tsai WY, Lee PH, Ahsan H et al. Polycyclic aromatic hydrocarbon-DNA adducts in liver tissues of hepatocellular carcinoma patients and controls. Int J Cancer 2002; 99:14-21.

Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61:3225-3229.

Feng Z, Hu W, Chen JX, Pao A, Li H, Rom W, Hung MC, Tang MS. Preferential DNA damage and poor repair determine ras gene mutational hotspot in human cancer.J Natl Cancer Inst. 2002;94(20):1527-36.

Hamilton JP. Epigenetic mechanisms involved in the pathogenesis of hepatobiliary malignancies. Epigenomics, 2010;2(2):233-243.

Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 2007;22:91-103.

Hu X, Herzog C, Zimniak P, Singh SV. Differential protection against benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide-induced DNA damage in HepG2

cells stably transfected with allelic variants of pi class human glutathione S-transferase. Cancer Res. 1999;59(10):2358-62.

Huang ZH, Hu Y, Hua D, Wu YY, Song MX, Cheng ZH. Quantitative analysis of multiple methylated genes in plasma for the diagnosis and prognosis of hepatocellular carcinoma. Exp Mol Pathol 2011;91:702-707.

Islam GA, Greibrokk T, Harvey RG, Ovrebø S. HPLC analysis of benzo[a]pyrene-albumin adducts in benzo[a]pyrene exposed rats. Detection of cis-tetrolsarising from hydrolysis of adducts of anti- and syn-BPDE-III with proteins. Chem Biol Interact 1999;123:133-148.

Iyer P, Zekri AR, Hung CW, Schiefelbein E, Ismail K, Hablas A et al. Concordance of DNA methylation pattern in plasma and tumor DNA of Egyptian hepatocellular carcinoma patients. Exp Mol Pathol 2010;88:107-111.

Johnson N M, Qian G, Xu L, et al. Aflatoxin and PAH exposure biomarkers in a US population with a high incidence of hepatocellular carcinoma[J]. Science of the total environment, 2010;408(23): 6027-6031.

Källberg H, Ahlbom A, Lars Alfredsson L. Calculating measures of biological interaction using R. Eur J Epidemiol 2006;21:571-573.

Ko CB, Kim SJ, Park C, Kim BR, Shin CH, Choi S, Chung SY, Noh JH, Jeun JH, Kim NS, Park R. Benzo(a)pyrene-induced apoptotic death of mouse hepatoma Hepa1c1c7 cells via activation of intrinsic caspase cascade and mitochondrial dysfunction. Toxicology 2004;199(1):35-46.

Kumaki Y, Oda M, Okano M. Quantification tool for methylation analysis. Nucleic

Acids Res 2008;36:170-175.

Li T, Zhao XP, Wang LY, Gao S, Zhao J, Fan YC, Wang K. Glutathione S-transferase P1 correlated with oxidative stress in hepatocellular carcinoma Int J Med Sci. 2013;10(6):683-90.

Lim SO, Gu, JM, Kim MS, Kim HS, Park YN, Park CK, Cho JW, Park YM, Jung G. Epigenetic Changes Induced by Reactive Oxygen Species in Hepatocellular Carcinoma: Methylation of the E-cadherin Promoter. Gastroenterology. 2008;135 (6): 2128-40.

Lambert MP, Paliwal A, Vaissière T, Chemin I, Zoulim F, Tommasino M et al. Aberrant DNA methylation distinguishes hepatocellular carcinoma associated with HBV and HCV infection and alcohol intake. J Hepatol 2011;54:705-715.

Martin M, Herceg Z. From hepatitis to hepatocellular carcinoma: a proposed model for cross-talk between inflammation and epigenetic mechanisms. Genome medicine 2012;4(1):8.

Niu JJ, Su YH, Han YF, Zhao R, Sun XL,Guo F et al. Synergistic effects of different types of smoking and other risk factorson risk of hepatocellular carcinoma in Xiamen, China. Chin J Epidemiol 2010;31:850-855.

Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics. CA Cancer J Clin 2002;55:74-108.

Stanzer S, Balic M, Strutz J, Heitzer E, Obermair F, Hauser-Kronberger C, et al. Rapid and reliable detection of LINE-1 hypomethylation using high-resolution melting analysis. Clin Biochem 2010;43:1443-1448.

Su Y, Zhao B, Guo F, Bin Z, Yang Y, Liu S, Han Y, Niu J, Ke X, Wang N, Geng X, Jin C, Dai Y, Lin Y. Interaction of benzo[a]pyrene with other risk factors in hepatocellular carcinoma: a case-control study in Xiamen, China. Annals of epidemiology 2014;24(2):98-103

Tang WY, Levin L, Talaska G, Cheung YY, Herbstman J, Tang D et al. Maternal exposure to polycyclic aromatic hydrocarbons and 5'-CpG methylation of interferon-γ in cord white blood cells. Environ Health Perspect 2012;120:1195-1200.

Tian M, Peng S, Martin FL, Zhang J, Liu L, Wang Z et al. Perfluorooctanoic acid induces gene promoter hypermethylation of glutathione-*S*-transferase Pi in human liver L02 cells. Toxicology 2012;296:48-55.

Wu HC, Wang Q, Wang LW, Yang HI, Ahsan H, Tsai WY, Wang LY, Chen SY, Chen CJ, Santella RM. Polycyclic aromatic hydrocarbon- and aflatoxin-albumin adducts, hepatitis B virus infection and hepatocellular carcinoma in Taiwan. Cancer Lett. 2007;252(1):104-14.

Xu XF, Cai L, Dai L, Kang TC, Chen SQ. Mortality analysis and the tendency prediction of cancer in Tongan District, Fujian. Strait J Prev Med Methods 2003;9:14-16.

Yang B, Guo M, Herman JG, Clark DP. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. Am J Pathol 2003;163:1101-7.

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

Silencing of glutathione S-transferase P1 by promoter hypermethylation and its relationship to environmental chemical carcinogens in hepatocellular carcinoma. Cancer Lett. 2005;221(2):135-43.

Zhang YJ, Wu HC, Shen J, Ahsan H, Tsai WY, Yang HI et al. Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. Clin Cancer Res 2007;13:2378-2384.

Zhao B, Shen H, Liu F, Liu S, Liu J, Guo F, Sun X. Exposure to organochlorine pesticides is independent risk factor of hepatocellular carcinoma: a case-control study. J Expo Sci Environ Epidemiol 2012;21:601-608.

Zhao BH, Guo F, Liu S, Pan LL. The study on the interactionsbetween polycyclic aromatic hydrocarbon-albumin adducts and variousrisk factors to primary hepatocellular carcinoma. Chin J Prev Med 2010;44:427-432.

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

Click here to download Table: table.docx

Table1. Sequence of primers used for HRM, quantitative RT-PCR and BSP analysias

Gene	Application	Primer sequence (5‘-3’)	Product size (bp)	CpG No.
GSTP	HRM/BSP	F: GGGATTTGGGAAAGAGGGAAAGG R: ACCGCTCTTCTAAAAAATCCC	149	17
GSTP	BSP	F: GGGATTTGGGAAAGAGGGAAAGG R:CCCATACTAAAAACTCTAAACCCC	311	38
CYP1A1	Q-PCR	F: CATCCCCCACAGCACAACA R: CAGGGGTGAGAAACCGTTCA	152	-
GSTP	Q-PCR	F: CGGAGACCTCACCCTGTA R: CGCCTCATAGTTGGTGTAGA	169	-
P53	Q-PCR	F: TCTTCTGTCCCTTCCCAGAA R: AATCAACCCACAGCTGCAC	163	-
DNMT1	Q-PCR	F: TACCTGGACGACCCTGACCTC R: CGTTGGCATCAAAGATGGACA	103	-
DNMT3A	Q-PCR	F: TATTGATGAGCGCACAAGAGAGC R: GGGTGTTCCAGGGTAACATTGAG	111	-
DNMT3B	Q-PCR	F: GGCAAGTTCTCCGAGGTCTCTG R:TGGTACATGGCTTTTCGATAGGA	113	-
HMLH1	Q-PCR	F:TCTCAGGCCAGCAGAGTGAA R: TGTGTGAGCGCAAGGCTTTA	93	-
HMSH2	Q-PCR	F: AATGACTTGGAAAAGAAGATGC R: TTAAAGAAGTCAATTTGCTGTTG	223	-
HPMS2	Q-PCR	F: GCACTGAGCGATGTCACCATT R: TTCCTTATGGCGCACAGGTAGT	171	-
GAPDH	Q-PCR	F:GGAGAAGGCTGGGGCTCAT R: TGATGGCATGGACTGTGGTC	230	-

Table2. Demographics and clinical characteristics in cases (*n*=90) and controls (*n*=99)

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

GSTP methylation status was qualitatively determined by HRM PCR.

[a]M, fully methylated; H, heterogeneous; U, fully unmethylated.

Table3. Serum pollutant concentrations and DNA methylation levels in the participants

Characteristic	Case			Control			*P*
	Mean	SD	Median	Mean	SD	Median	
BPDE-Alb (fmol/mg adduct)	17.71	21.64	1.79	6.48	11.10	1.51	< 0.001
GSTP (%)	12.79	23.38	1.62	2.17	11.16	0.00	< 0.001

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

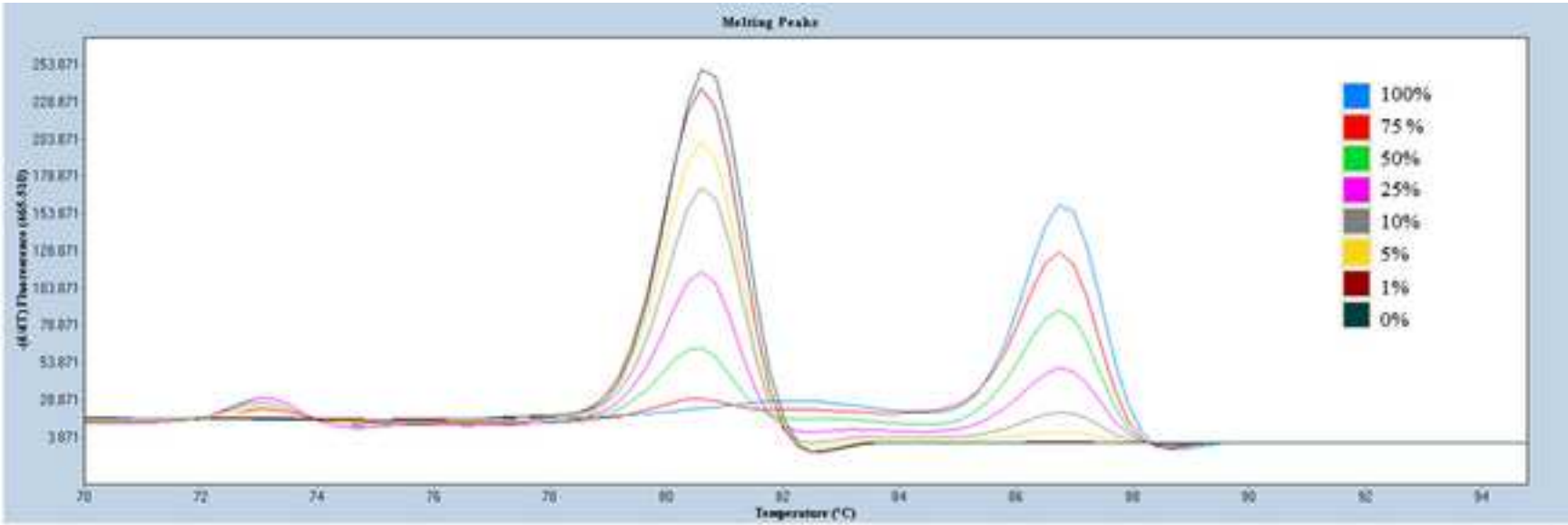
Table4. Multivariate logistic regression analysis of the interactions of GSTP epigenetic vs benzo[a]pyrene exposure in HCC risks

		Case	Control	Crude OR (95% CI)	AOR[a] (95% CI)
GSTP	BPDE-Alb				
GSTP-U	Low	15	41	1.0 (reference)	1.0 (reference)
GSTP-U	High	24	36	1.1 (0.3-3.3)	1.1 (0.3-4.3)
GSTP-H/M	Low	19	7	4.4 (1.8-10.5)	3.8 (1.3-10.9)
GSTP-H/M	High	29	10	7.7 (3.0-19.6)	8.4 (2.7-26.6)
				P-trend < 0.001	*P*-trend < 0.001

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

Figure1

Click here to download high resolution image



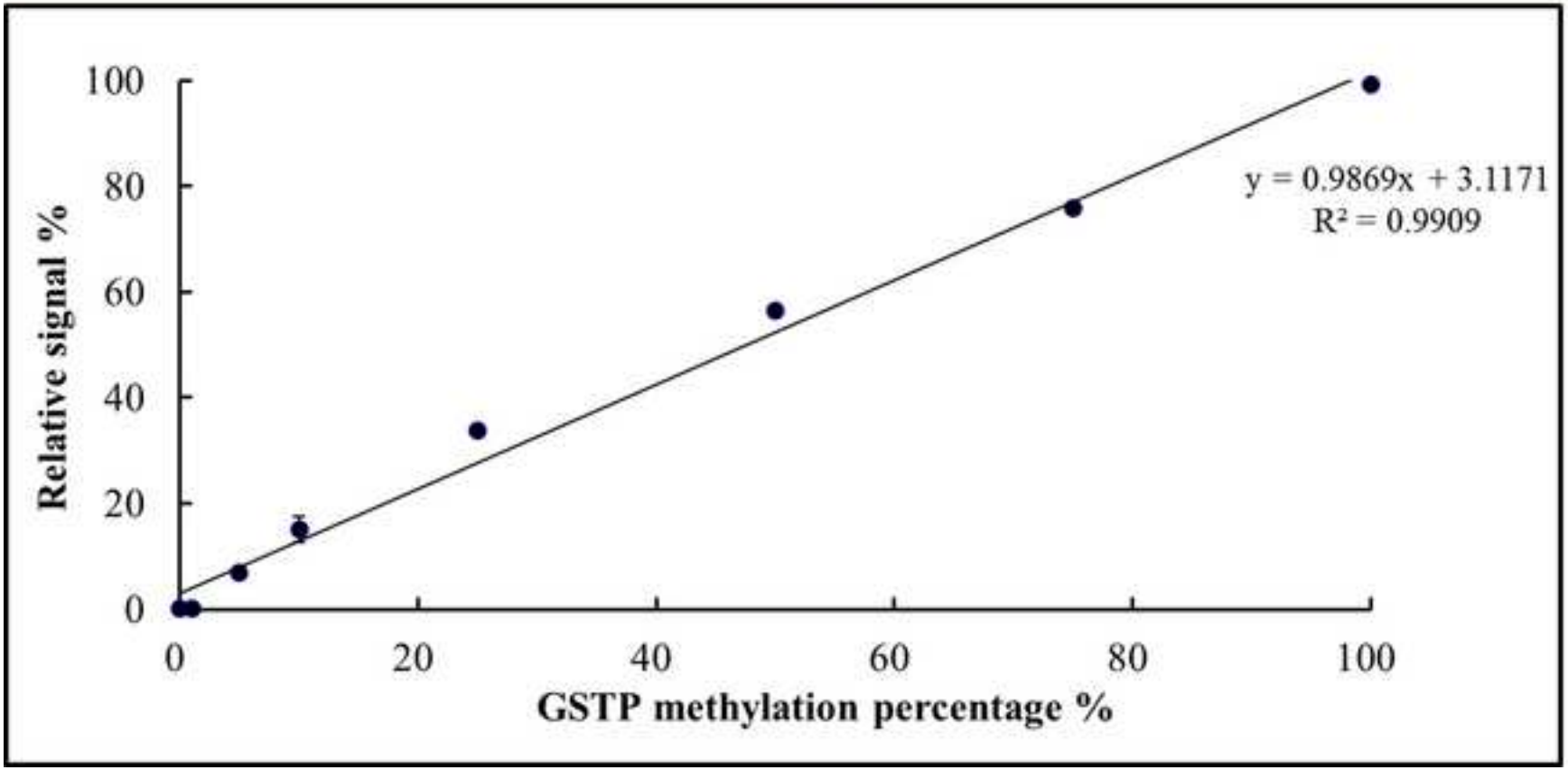


Figure2

Click here to download high resolution image

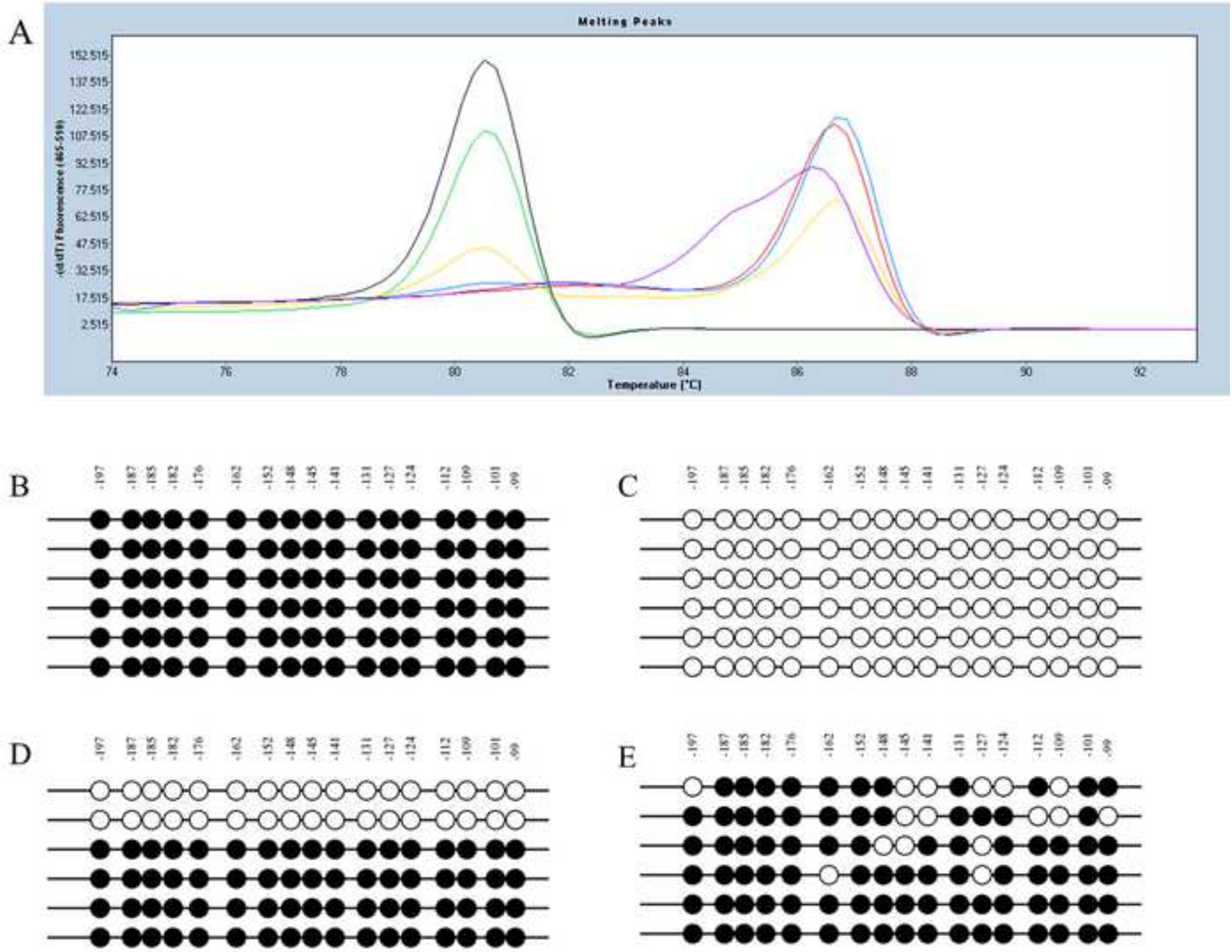


Figure3

Click here to download high resolution image

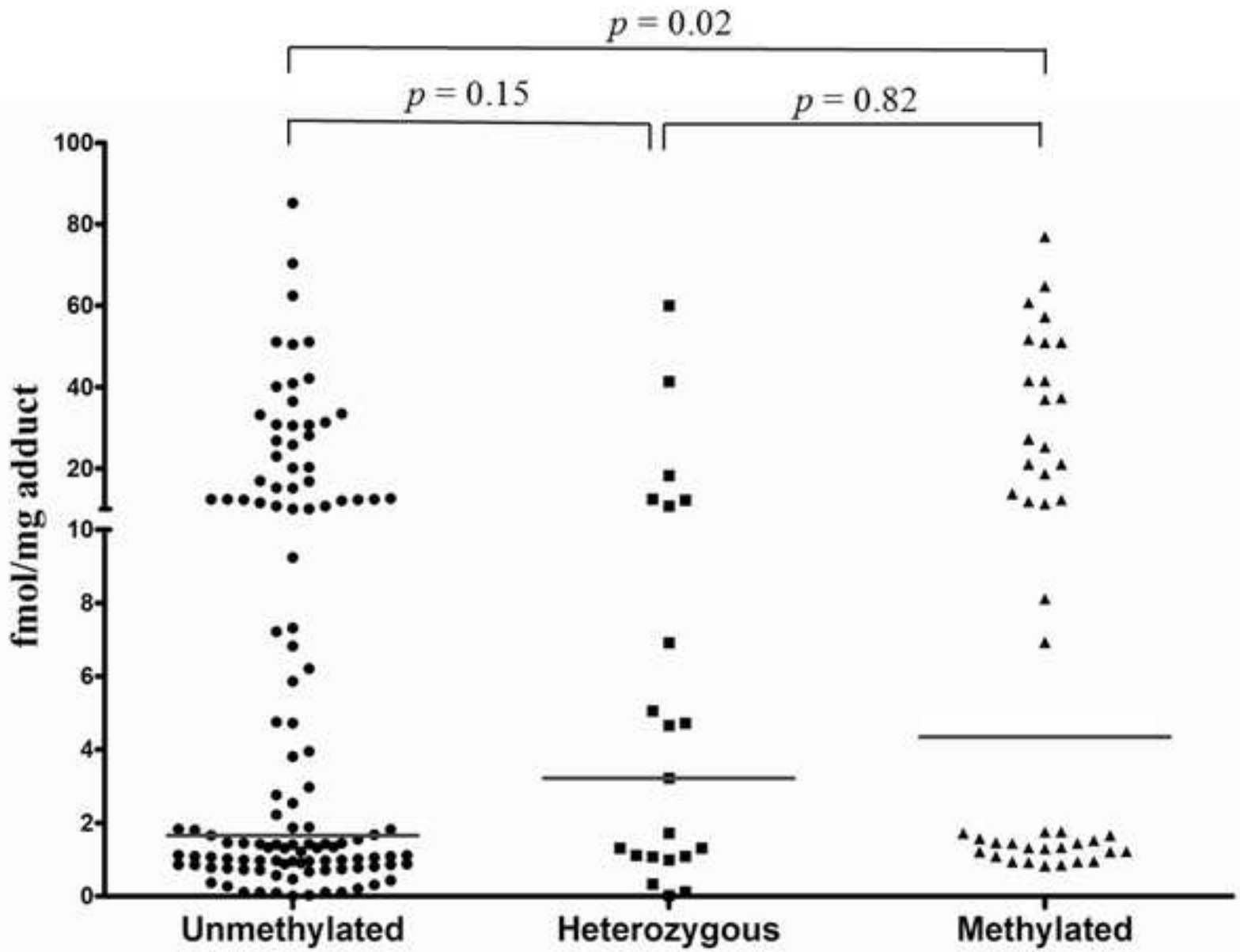


Figure4

Click here to download high resolution image

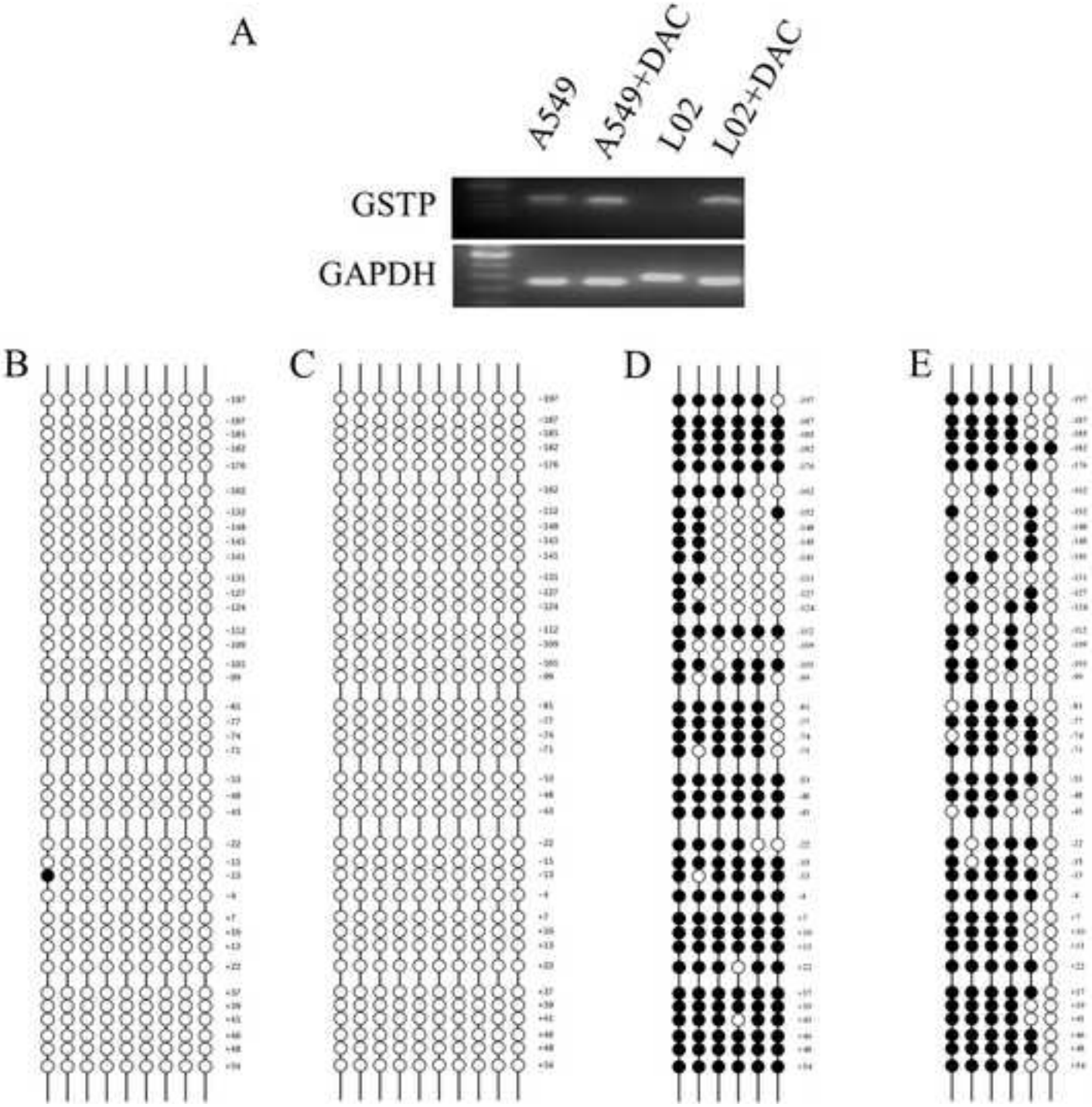


Figure5

Click here to download high resolution image

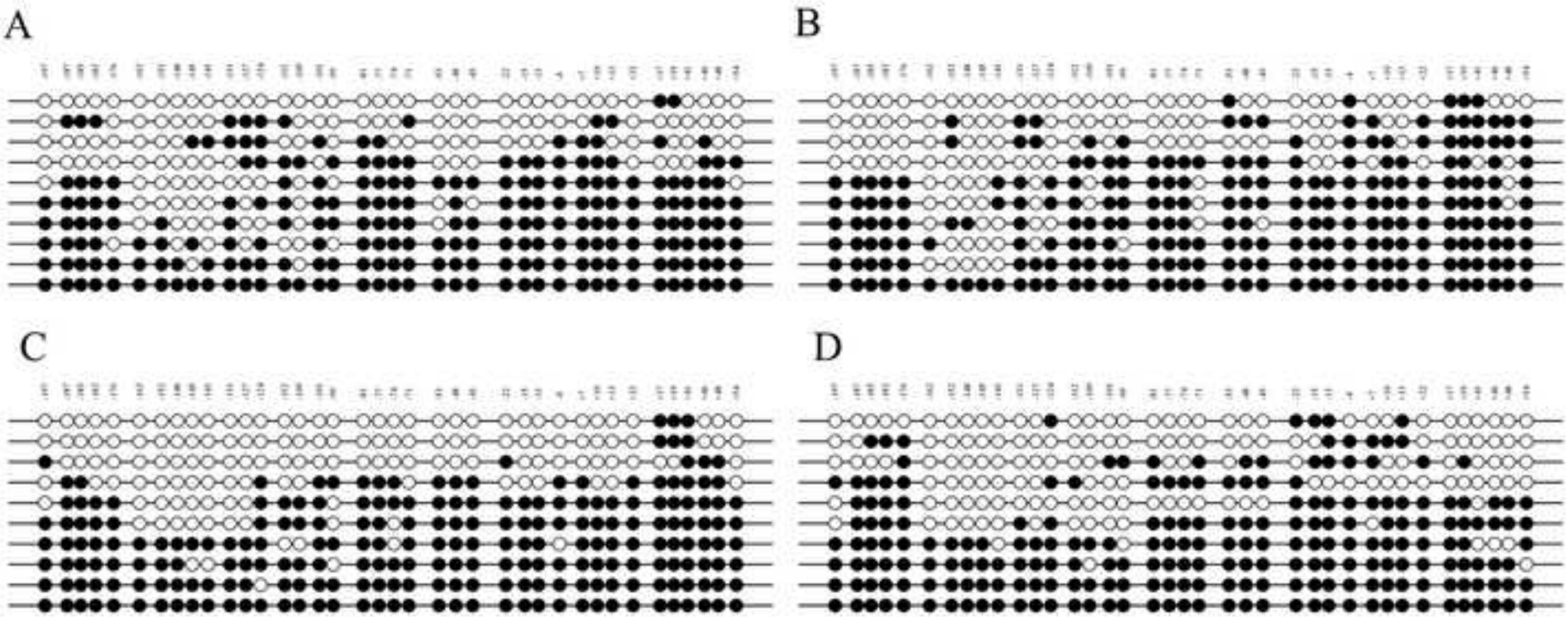


Figure6

Click here to download high resolution image