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Gene-environment interactions between *GSTs* polymorphisms and targeted epigenetic alterations in hepatocellular carcinoma following organochlorine pesticides (OCPs) exposure

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ABSTRACT

Exposure to environmental pollutant organochlorine pesticides (OCPs) and the role of tumour suppressor *GSTs* gene polymorphisms as well as epigenetic alterations have all been well reported in hepatocarcinogenesis. However, the interplay between environmental risk factors and polymorphic tumour suppressor genes or epigenetic factors in hepatocellular carcinoma (HCC) development remains ambiguous. Herein, we investigated the relationship of three *GSTs* polymorphisms (*GSTT1* deletion, *GSTM1* deletion, *GSTP1* rs1695) as well as *GSTP1* promoter region DNA methylation and HCC risk with a particular focus on the interaction with OCPs exposure among 90 HCC cases and 99 controls in a Chinese population. Serum samples were analysed for OCPs exposure employing gas chromatography coupled with mass selective detector (GC-MS). *GSTs* polymorphisms and epigenetic alterations were determined using high-resolution melting PCR (HRM PCR) and DNA sequencing. After adjusting for confounders (HBV infection, smoking, alcohol consumption, BMI, age, gender), OCPs exposure and *GSTP1* methylation is significantly associated with elevated risk of HCC, while no significance is observed for *GSTs* polymorphisms. Moreover, the effects of OCPs exposure on HCC risk are more pronounced amongst *GSTP1* (Ile/Val + Val/Val) and *GSTP1* promoter methylation subjects than those who were *GSTP1* (Ile/Ile) and unmethylated subjects. The interactions between OCPs exposure and *GSTP1* genotype as well as *GSTP1* epigenetic status are statistically significant. The current study demonstrates the importance of gene-environment interactions in the multifactorial development of HCC.

1. Introduction

Liver cancer incidence and mortality thereof occurs at high frequencies in many regions around the world (VoPham, 2019). In 2018, it was the 7th most commonly occurring cancer and the 3rd leading cause of cancer-related death (Bray et al., 2018). Hepatocellular carcinoma (HCC) may account for 80–90% of all liver cancers; most cases arise in sub-Saharan Africa, Southeast Asia and China, with > 50% of HCCs occurring in China alone (Parkin et al., 2005). Previous epidemiological studies into HCC aetiology indicate involvement and interplay between environmental carcinogen exposures and tumour suppressor genes (TSGs) genetic or epigenetic factors in its multi-stage process (Chen et al., 2010, Song et al., 2012, Tian et al., 2016, Yang et al., 2003, Ezzat et al., 2005). Most cases of HCC development are associated with oxidative stress and inflammation (Bishayee, 2014, Wang et al., 2016).

Environmental exposures including hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, tobacco smoking, excessive alcohol consumption, aflatoxin B_1, polycyclic aromatic hydrocarbons (PAH) and organochlorine pesticides (OCPs) are associated with a significantly elevated risk of HCC (Tian et al., 2016, VoPham, 2019, Persson et al., 2012). The most abundant OCPs present in the environment are hexachlorocyclohexane (HCH) and dichlorodiphenyltrichloroethane (DDT). Although usage of OCPs has been banned in most countries, residues of HCHs and DDTs still exist in various environmental matrices, and

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humans remain extensively exposed to them *via* the diet (Qian et al., 2017, Aamir et al., 2018). Due to their environmental persistence, long-range transport, bioaccumulation and potential toxicity (Milun et al., 2016), epidemiological and toxicological studies have associated OCPs exposure with elevated levels of reactive oxygen species (ROS) with inflammation generation leading to increased liver cancer risk (Jin et al., 2014a, Pathak et al., 2011, Mustafa et al., 2015, Engel et al., 2019). Our previous work observed that OCPs exposure appears to be an independent risk factor for HCC (Zhao et al., 2012).

The liver plays a central role in xenobiotic metabolism and detoxification. Glutathione *S*-transferases (GSTs) belong to a gene superfamily of Phase II metabolizing enzymes and play essential anticancer roles *via* catalysing glutathione conjugation with electrophiles generated by xenobiotic-induced ROS (Khabaz et al., 2016). The major GSTs enzymes are GSTM1 (mu), GSTT1 (theta) and GSTP1 (pi); genes encoding these enzymes are polymorphic (Khabaz et al., 2016). Polymorphism deletion variants in *GSTM1* and *GSTT1* result in a functional enzyme (GSTM1 non-null and GSTT1 non-null) or complete absence of the enzyme (GSTM1 null and GSTT1 null) (Song et al., 2012). The phenotypic absence of *GSTM1* and *GSTT1* activity is due to homozygous deletion of these genes. The *GSTP1* rs1695 polymorphism is an A (Ile) to G (Val) transition that confers reduced catalytic activity (Yuille et al., 2002). Hypermethylation of *GSTP1* promoter region CpG islands causes loss of gene expression, which is often associated with increased cell vulnerability to carcinogens (Jerónimo et al., 2002). Both polymorphisms and epigenetic modification in *GSTs* may lead to an imbalance in pro- and anti-oxidant systems resulting in the accumulation of ROS that could contribute to cancer incidence, including HCC (Jerónimo et al., 2002, Dastjerdi et al., 2017).

Given that the liver is a target organ for OCPs and it is susceptible to OCPs-induced injury, the detoxifying abilities of liver GSTs plays a key role in OCPs-induced ROS and inflammation. Therefore, OCPs exposure and GSTs detoxification could interact in HCC development risk. However, associations of HCC risk with environmental OCPs exposure and *GSTs* polymorphisms as well as their epigenetic alterations remain unknown. Herein, a case-control study was designed using a hospital-based population. The study participants are residents living in a high HCC incidence belt located in the heartland of the SouthEast coastline of China (Xu et al., 2003). The present study aimed to evaluate the potential interaction of *GSTs* polymorphisms, epigenetic modifications and OCPs exposure in HCC risk.

2. Materials and methods

2.1. Recruitment of study participants

The participants recruited to this study were a subset of subjects in the Xiamen HCC epidemiological survey undertaken between March 2007 to December 2009 (Zhao et al., 2010, 2012). Newly-diagnosed HCC patients before treatment and non-cancer healthy volunteers were recruited from the Zhongshan Hospital of Xiamen University, Xiamen Hospital of Traditional Chinese Medicine and the 174th Hospital of the People's Liberation Army. The purposes of the research were explained to all prospective participants, and written informed consent was obtained from all participants. As previously described (Zhao et al. 2012, Tian et al., 2016), a face-to-face interview was arranged for each participant to collect individual information, which included demographics [*e.g.*, gender, age, height, body weight, history of cigarette smoking, alcohol consumption and, virus infection (HBV)]. Exclusion criteria for participation in the study were as follows: occupational exposed to OCPs, lived in Xiamen < 10 y, or HCC subjects presenting with another cancer type simultaneously. Finally, 90 HCC patients and 99 healthy eligible participants were recruited to this study, and these each provided 5 mL of blood sample for OCPs measurements, *GSTs* genotyping and epigenetic analyses.

2.2. Organochlorine pesticides (OCPs) detection

α-HCH, *β*-HCH, γ-HCH, δ-HCH, *p,p′*-DDE, *p,p′*-DDT, *o,p′*-DDT and *p,p′*-DDD were analyzed by gas chromatography coupled with mass selective detector, as previously described (Sundberg et al. 2006) with some modification. Briefly, 0.5 mL serum was initially spiked with ^{13}C-isotope-labelled standard 2,2′,3,3′,4-pentachlorobiphenyl (PCB-82) as surrogate standard and de-conjugated proteins using 8 M solid urea, then purified by a SPE cartridge (Oasis HLB, Waters) and eluted with methylene chloride, and finally analyzed by a 7890 gas chromatograph coupled to a 5973 mass selective detector (Agilent Technologies, Palo Alto, CA). The DB25ms capillary column (30 m × 0.25 mm × 0.25 μm) was selected for OCPs congener separation. The MS was operated with electron impact ionization in the selected ion monitoring (SIM) mode. The temperatures of the source and the transfer line were 250 °C and 290 °C, respectively; helium gas flow rate of 1.0 mL/min, and oven temperature of 60 °C were used. Briefly, the oven temperature was quickly increased to 200 °C at 30 °C/min, then to 220 °C at 5 °C/min, then to 230 °C at 1 °C/min, and held at 290 °C for 5 min. Two blanks, two quality control (QC) samples (human serum spiked with PCB-82), and two sets of standards were also run concurrently with the unknown samples in each analytical batch. Accuracy and precision were measured for QC in the same analytical batch, and the analytical values of QC samples were within ± 2 SD of the nominal value. The spiked recoveries were 69.3% to 105.4%. The limit of detection for the method was 0.007–0.110 ng/mL [see Electronic Supplementary Information (ESI) Table S1]. The results indicate that the method was highly reproducible and accurate.

2.3. Glutathione S-transferases polymorphism HRM (high resolution melting) and sequencing analysis

DNA in the blood of participants was extracted using the DNA Blood mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. A NanoDrop spectrophotometer (NanoDrop Technologies Inc., USA) was used to measure DNA concentration and purity. Multiple (*GSTM1*, *GSTT1* and *β-globin*) HRM PCR were performed using a Light Cycler 480 (Roche, USA), as adapted from a published protocol (Drobná et al., 2012). *β-globin* was selected as an endogenous control towards excluding poor-quality DNA samples or any inhibitor that might interfere with PCR amplification that would generate false negative results. Primers used for HRM analysis are shown in Table S2 (see ESI). 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 DNA template. The cycling conditions started with one cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 30 s each, 58 °C for 30 s and 72 °C for 30 s. This was followed by a HRM step of 95 °C for 1 min, 40 °C for 1 min, 65 °C for 5 s and a continuous run to 95 °C at 25 acquisitions per 1 °C. HRM data were analyzed using gene melting software (Roche, Germany), "Tm calling" algorithm to determine the presence of melting peaks and their maxima, and specific melting temperatures, as previously described by (Drobná et al., 2012) (see ESI Fig. S1). The HRM results were confirmed with conventional PCR and gel electrophoresis (see ESI Fig. S2). The *GSTP1* rs1695 polymorphism type analysis employed conventional PCR and DNA sequencing (see ESI Figs. S3–S5). Primer sequences are shown in Table S2 (see ESI), and cycling conditions started with one cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 30 s each, 58 °C for 30 s and 72 °C for 30 s, after which PCR products were sequenced.

2.4. GSTP1 promoter region methylation analysis

GSTP1 promoter region methylation status was determined by HRM as reported in our previous published method (Tian et al., 2016) (see ESI Fig. S6–7). Briefly, cell-free DNA was extracted from serum with QIAamp DNA minikit (Qiagen, Hilden, Germany) and DNA modified

with sodium bisulfite using the EZ DNA Methylation Kit (Zymo, USA). Methylation standards were constructed by diluting the 100% methylated control plasmid from bisulfite-modified CpGenome Universal Methylated DNA (Chemicon, Millipore, Billerica, MA, USA) in a pool of unmethylated control plasmid from peripheral blood mononuclear cells of normal individuals. Both control plasmids were confirmed by Sanger sequencing before performing the experiment. HRM PCR amplification with touch-down PCR (primer sequence is shown in Table S2, see ESI); cycling conditions started with one cycle of 95 °C for 10 min, followed by 60 cycles of 95 °C for 20 s each, a touch-down of 64 °C to 58 °C for 20 s (1 °C/cycle), and 72 °C for 20 s. HRM data and DNA methylation status were distinguished by using gene scanning software (Roche, Germany) (Tian et al., 2016, Stanzer et al., 2010) (see ESI Figs. S6–S7). Both homogeneous and heterogeneous DNA methylations were recognized for methylation status.

2.5. Data analysis

Statistical analysis was performed using SPSS 18 (SPSS Inc). Variable distributions were analyzed by nonparametric Mann-Whitney *U* test for non-normal distribution of continuous variables and χ^2-test for categorical variables, and the false discovery rate (FDR) correction was applied to correct for multiple hypothesis tests. Hardy-Weinberg equilibrium (HWE) for *GSTP1* rs1695 polymorphism distribution in control groups was checked by goodness-of-fit tests. Unconditional multivariate logistic regression was performed for chemical pollutants, if significantly associated with HCC in univariate analysis ($P < 0.05$). To examine whether different genotypes and epigenetic status of *GSTs* could modify effects of OCPs exposure in HCC, we divided subjects into low- or high-exposed groups depending on median serum OCPs concentrations. Then, logistic regression was applied to investigate possible interactions between *GSTs* polymorphism and epigenetic modification as well as OCPs exposure. Age, gender, BMI, drinking, smoking, HBV and other important HCC risk factors were adjusted using the likelihood-ratio test. Adjusted odds ratio (AOR) and 95% confidence interval (CI) were calculated by the maximum likelihood approach. Quantity assessment of two factors' interaction was also investigated (Källberg et al., 2006, Zhao et al., 2010). A *P*-trend was determined based on a linear-by-linear association by Chi-square analysis. The constructed interactions model was evaluated by receiver operating characteristics (ROC) analysis and area under the curve (AUC) calculations. For all tests, *P*-values < 0.05 were considered significant.

3. Results

3.1. Study participant demographics

Demographic and clinical characteristics of HCC cases and controls are shown in Table 1. The two groups were well matched by age (mean of 57.9 *vs.* 57.8 y, $P = 0.96$). The proportion of male subjects in the cases group was higher than controls (84.4% *vs.* 60.6%; $P < 0.001$). The body mass index (BMI) levels were lower for HCC cases compared with healthy controls (mean of 21.2 *vs.* 22.4; $P = 0.01$). Rates of smoking and alcohol consumption were higher among cases compared to controls. The incidence of HBV was markedly higher in cases *vs.* controls (43.3% *vs.* 9.1%).

3.2. GSTP1, GSTT1 and GSTM1 polymorphisms plus GSTP1 methylation status

The distributions of *GSTs* polymorphisms and epigenetic modification among cases and controls are given in Table 2. The genotypic frequencies of *GSTP1* rs1695 Ile/Val in controls were consistent with HWE ($\chi^2 = 0.15$, $P = 0.11$). The frequencies of *GSTP1* rs1695 Ile/Val + Val/Val genotypes and *GSTP1* promoter region methylated individuals were significantly higher in HCC patients (66.7% and 53.3%, respectively) in comparison to controls (52.5% and 19.2%, respectively) ($P < 0.05$). Logistic regression analysis demonstrates an association of *GSTP1* rs1695 Ile/Val + Val/Val genotypes with a 1.8-fold increased risk of HCC incidence in the studied population (OR: 1.8; 95% CI: 1.0–3.3, $P = 0.05$). Similarly, *GSTP1* promoter region methylation status exhibited a 4.9-fold increased risk of HCC incidence herein (OR: 4.9; 95% CI: 2.6–9.6, $P < 0.001$). However, after adjusting for confounding variables (*i.e.*, age, BMI, gender, alcohol consumption, smoking and HBV) in the models, only *GSTP1* promoter region methylation status conferred a significantly increased risk of HCC (OR: 4.9; 95% CI: 2.3–10.4, $P < 0.001$). There was no significant difference in frequencies between HCC cases and healthy controls for *GSTP1*, *GSTT1* and *GSTM1* polymorphism (Table 2).

Table 1
Demographics and characteristics of cases ($n = 90$) and controls ($n = 99$).

	Characteristic	Cases	Controls	*P*
General	Age (y)	57.9 ± 8.8[a]	57.8 ± 8.4	0.96
	Body mass index (kg/m^2)	21.2 ± 2.8	22.4 ± 3.6	0.01
	Gender			
	Male	76 (84.4)[b]	60 (60.6)	< 0.001
	Female	14 (15.6)	39 (39.4)	
Lifestyle	Smoking status			
	Non-smoker	40 (44.4)	63 (63.6)	0.02
	Smoker	48 (53.3)	36 (36.3)	
	Not available	2 (2.2)		
	Alcohol consumption			
	Abstinence	56 (62.2)	75 (75.8)	0.03
	Drinker	32 (35.6)	24 (24.2)	
	Not available	2 (2.2)		
Virus infection	HBV			
	HBV (−)	51 (56.7)	90 (90.9)	< 0.001
	HBV (+)	39 (43.3)	9 (9.1)	

[a] Continuous variables: mean values ± standard deviation, *P*-value from *t*-tests.
[b] Categorical variables: numbers and percentages, *P*-values from χ^2 test.

Table 2
Distribution of *GSTs* genetic/epigenetic and test of association of *GSTs* polymorphisms and *GSTP1* DNA methylation in HCC participants *vs.* controls.

Variable	Cases	Controls	OR	95% CI	AOR[a]	95% CI
GSTP1 rs1695						
Ile/Ile	30 (33.3%)	47 (47.5%)	1.0			
Ile/Val + Val/Val	57 + 3 (66.7%)	50 + 2 (52.5%)	**1.8**	**1.0–3.3**	1.7	0.8–3.5
GSTT1						
GST1 null	35 (38.9%)	46 (46.5%)	1.0			
GST1 Non-null	54 (60.0%)	52 (52.5%)	1.4	0.8–2.4	1.5	0.8–3.0
Not available	1 (1.1%)	1 (1.0%)				
GSTM1						
GSTM1 null	50 (55.6%)	66 (66.7%)	1.0			
GSTM1 Non-null	39(43.3%)	32 (32.3%)	1.6	0.9–2.9	1.5	0.8–3.0
Not available	1 (1.1%)	1 (1.0%)				
GSTP epigenetic						
GSTP-U	40 (44.4)	77 (77.8)	1.0			
GSTP-M	48 (53.3)	19 (19.2)	**4.9**	**2.6–9.6**	**4.9**	**2.3–10.4**
Not available	2 (2.3%)	3 (3.0%)				

[a] Adjusted for gender, age, BMI, HBV, smoking status, alcohol consumption. Values in bold indicate statistical significance, $P < 0.05$.

3.3. Organochlorine pesticides (OCPs) exposure levels

Serum OCPs concentration distributions in the studied population are listed in Table 3. The predominant congeners of DDT and HCH were *p,p'*-DDT and *β*-HCH, respectively. The concentrations of ΣDDT were comparatively higher than ΣHCHs. The OCPs concentrations are non-normal distributions. The median levels of *p,p'*-DDT and ΣDDT in HCC

Table 3
Serum DDT and HCH concentrations in the participants.

Cases					Controls				
OCPs (ng/mL)	> LOD	Median	Mean (SD)	IQR	> LOD	Median	Mean (SD)	IQR	P^a
p,p'-DDT	98.9%	43.28	122.23 (206.58)	15.75–127.49	98.0%	32.11	75.79 (146.42)	14.39–67.32	0.08
o,p'-DDT	76.7%	8.97	16.53 (20.06)	0.31–23.39	75.8%	11.03	17.49 (20.16)	0.66–24.21	0.75
p,p'-DDE	98.9%	25.19	31.05 (33.54)	12.22–37.62	98.0%	21.33	24.43 (21.44)	7.84–33.69	0.26
p,p'-DDD	100%	1.16	9.30 (15.33)	0.01–14.15	100%	0.84	8.02 (12.72)	0.01–13.21	0.39
ΣDDT		106.43	179.09 (220.87)	59.59–185.19		93.26	125.74 (157.58)	41.91–144.37	0.08
α-HCH	70.0%	3.11	9.43 (11.58)	0.04–18.64	72.7%	0.99	6.85 (9.56)	0.04–9.11	0.39
β-HCH	100%	32.94	65.82 (80.44)	8.79–105.07	100%	22.77	40.05 (58.58)	5.27–43.36	0.08
γ-HCH	78.9%	1.33	6.15 (8.39)	0.21–11.18	76.8%	1.18	4.93 (9.07)	0.19–6.95	0.39
δ-HCH	80.0%	0.69	5.86 (12.51)	0.21–6.60	78.8%	0.64	2.92 (4.53)	0.11–5.33	0.35
ΣHCH		53.66	87.21 (85.27)	27.49–138.58		37.89	54.71 (61.52)	19.47–63.74	0.03
ΣOCPs		181.93	266.30 (272.49)	113.11–290.28		127.05	180.45 (200.41)	72.07–195.99	0.01

ΣDDT the mass sum of p,p'-DDT, o,p'-DDT, p,p'-DDE and p,p'-DDD, ΣHCH the mass sum of α-HCH, β-HCH, γ-HCH and δ-HCH, ΣOCPs the mass sum of ΣDDT and ΣHCH.

[a] Variable distributions were analyzed by the nonparametric Mann–Whitney *U* test, Statistically significant after FDR correction.

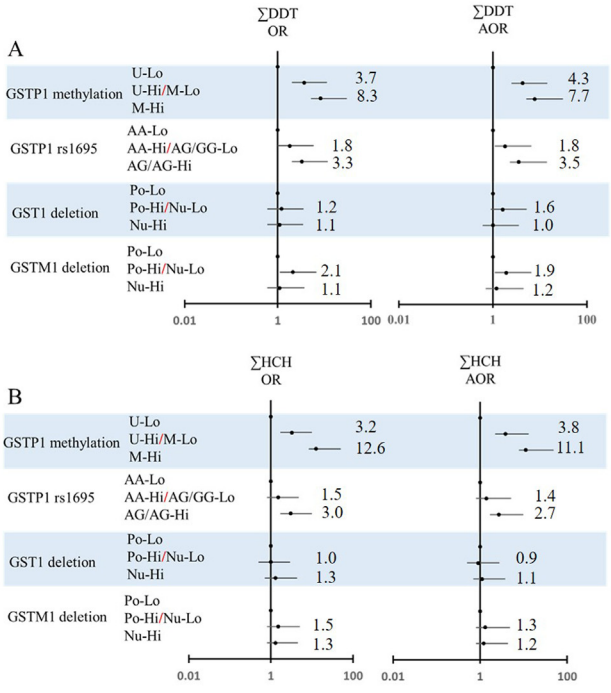
cases (43.28 and 106.43 ng/mL, respectively) were higher than those in controls (32.11 and 93.26 ng/mL, respectively) (probably significant, all $P = 0.08$, P with FDR correction). The *β*-HCH and ΣHCH were also elevated in HCC patients (32.94 and 53.66 ng/mL, respectively) compared to healthy controls (22.77 and 37.89 ng/mL, respectively) ($P = 0.08$ and $P = 0.03$, P with FDR correction). Cases also exhibited significantly higher levels of ΣOCPs than controls (181.93 *vs.* 127.05 ng/mL, $P = 0.01$, P with FDR correction).

3.4. Interactions of GSTs genotype, methypation status and OCPs exposure in HCC

In a stratification interaction analysis, after adjustment for confounding variables (including age, BMI, gender, alcohol consumption, smoking and HBV), significant interactions between *GSTP1* polymorphism, DNA methylation and OCPs were identified (see ESI Tables S3–S4, Fig. 1).

The AOR for participants harbouring a DNA methylated status for *GSTP1* promoter region and exposed to high-level OCPs suggested that HCC risk is 7.8-fold elevated (95% CI = 2.6–23.9, *P-trend = 0.002*) for ΣDDT and 11.4-fold elevated (95% CI = 3.3–39.0, *P-trend = 0.002*) for ΣHCH when compared to reference. Meanwhile, the AOR for *GSTP1* rs1695 Ile/Val + Val/Val polymorphism subjects exposed to high OCPs are markedly elevated, exhibiting a 3.5-fold elevation (95% CI = 1.2–10.5, *P-trend = 0.03*) for ΣDDT and a 2.7-fold elevation (95% CI = 1.0–7.0, *P-trend* = 0.05) for ΣHCH (see ESI Tables S3–S4, Fig. 1). However, no significant interactions for *GSTT1/GSTM1* polymorphisms and OCPs exposure in HCC risk are observed.

To evaluate the HCC prediction interaction model, a receiver operating characteristics (ROC) curve analysis employing SPSS Statistics



C

Low risk Medium risk High risk

OCPs-GSTP1 methylation Interaction		OCPs exposure	
		Low	High
GSTP1	Unmethylation	U-Lo	U-Hi
	Methylation	M-Lo	M-Hi

D

OCPs-GSTP1 rs1695 Interaction		OCPs exposure	
		Low	High
GSTP1	AA	AA-Lo	AA-Hi
	AG/GG	AG/GG-Lo	AG/GG-Hi

Fig. 1. Gene-environment interactions in HCC risk. (A) *GSTs* gene-DDT exposure interactions; (B) *GSTs* gene-HCC exposure interactions; (C) *GSTP1* epigenetic risk-OCPs exposure combination patterns; and, (D) *GSTP1* genetic risk-OCPs exposure combination patterns. OCPs exposure is categorized into high-level (Hi) and low-level (Lo) subsets by their concentration medians. OR (odds ratio) and AOR (adjusted odds ratio) were used to express the relative risk of HCC. The AOR was adjusted for age, gender, BMI, smoking and alcohol consumption along with HBV infection. Low risk was defined as AOR = 1.

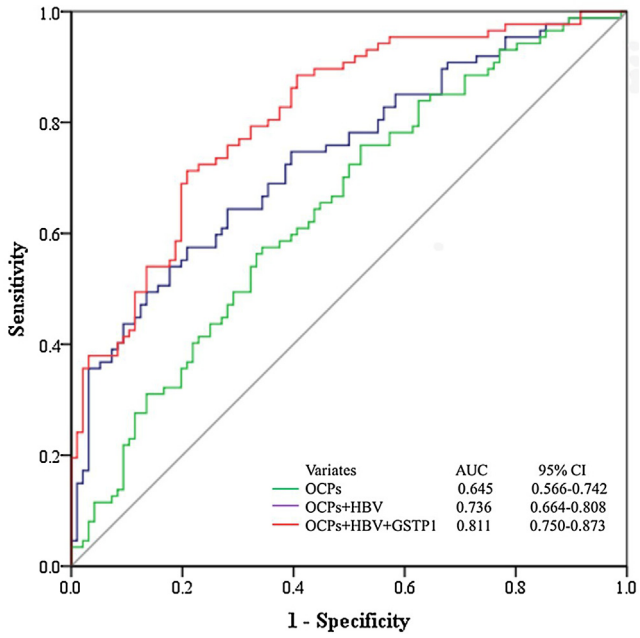


Fig. 2. Receiver operating characteristic (ROC) curves for models to predict HCC. In a combined model, AUC showed higher value in model comprising *GSTP1* genetic as well as epigenetic risks, OCPs exposure and HBV infection (red) than in OCPs combined with HBV (purple) or OCPs (green) model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

19.0 package was performed. The area-under-the-curve (AUC) determined from the ROC curve of a model comprising OCPs exposure, *GSTP1* rs1695, *GSTP1* promoter region methylation status and HBV as predictor variables exhibited higher values (AUC: OCPs + *GSTP1* rs1695 + *GSTP1* M + HBV = 0.81) compared to other models comprising OCPs and HBV (AUC: OCPs + HBV = 0.74) or OCPs (AUC: OCPs = 0.65) (Fig. 2).

4. Discussion

In this HCC case-control study of a Chinese population, we observed indications of gene-environment interactions between *GSTP1* rs1695 polymorphisms plus promoter region methylation alterations and OCPs exposure. On combining OCPs exposure, individuals carrying *GSTP1* allele G (Ile/Val + Val/Val) or promoter region methylated status appear more susceptible to HCC compared to allele A (Ile/Ile) or unmethylated status. Both *GSTP1* allele G (Val) and methylated status results in decreases or deficiencies in *GSTP1* function. Our results indicate the importance of gene-environment interactions in the multifactorial development of HCC.

General human exposure to OCPs occurs mainly *via* daily diet consumption, particularly fish and other fatty foods of animal origin (Wang et al., 2011, Aamir et al., 2018). Consistent with our previous findings in the Xiamen population exposed to OCPs, this subset population appears simultaneously exposed to multiple OCPs and metabolites, in which the predominant isomers of DDT and HCH are *p,p'*-DDT and *β*-HCH, respectively (Zhao et al., 2012). The current Xiamen population appear to be exposed to OCPs levels higher than developed Western countries (Botella et al., 2004, Thomas et al., 2006), but at lower levels than India which is one of the largest consumers of OCPs (Kumar et al., 2006, Nair et al., 1996). Xiamen City is located in the southeast coastal region of China, where OCPs were once used much more heavily and inhabitants tend to consume more seafood than interior cities (Su et al., 2012, Fang et al., 2018, El-Shahawi et al., 2010). The ratio of OCPs congeners can reflect the exposure character. A ratio of *p,p'*-DDT/*p,p'*-DDE > 1 indicates ongoing exposures to *p,p'*-DDT, while a ratio of β-HCH/ΣHCH > 0.5 indicates past exposures (Zhou et al., 2014, Persson et al., 2012). In the present study, the ratio p,p'-DDT/p,p'-DDE (median value = 1.63) reveals that most participants had recent DDT exposures, with higher p,p'-DDT serum concentrations than p,p'-DDE; whereas the ratio of β-HCH/ΣHCH (median value = 0.69) indicates mainly a historical contamination of HCH.

Based on the studied population exposed to high levels of OCPs and recent DDT, we infer that OCPs-contaminated food consumption may be responsible for high exposure risk in the selected aged cohort (mean age 57.8 y). Previous studies note that aged populations exhibit limited OCPs metabolism potency resulting in a higher contaminant burden (Laden et al., 1999, Kim et al., 2018). Our aged population was likely continuously exposed to OCPs over several years before the ban on their production, thus resulting in marked body store accumulation. High OCPs concentrations in groups > 50 y of age have also been reported in breast cancer (Attaullah et al., 2018). Our previous seafood OCPs survey study revealed that these contaminants were still detectable in seafood in Xiamen, and suggests that the Xiamen offshore marine ecological system is still at least partly contaminated as a "fresh source" of DDT exposure (Zhang et al., 2012).

The liver is the major organ for much xenobiotics detoxification; when humans are exposed to DDT, it can be further metabolized to DDE or DDD in this tissue. Amongst the parenchymal organs, the liver is the primary target site of OCPs accumulation (Liu et al., 2017). Animal toxicological exposure experiments show that OCPs (DDT or DDE) lead to HCC and other liver tumours development (Rossi et al., 1983; Turusov et al., 1973). However, an inconsistency is that it has been observed that DDT exposure could possible hormetic effects on HCC, whereby low-dose DDT exposure may generate inhibitory effects on hepatocarcinogenesis (Sukata et al., 2002). Increasing numbers of epidemiological studies to date report the potential association between exposure to OCPs and increased HCC risk (McGlynn et al., 2006; Persson et al., 2012; Zhao et al., 2012; VoPham et al., 2015). However, most studies show no association between self-reported and/or occupational exposure to pesticides and liver cancer risk (VoPham et al., 2017; Porru et al., 2001). Due to high OCPs exposure levels, positive associations between OCPs exposure (including DDT and HCH) and HCC incidence risks are observed herein, including with adjustment for confounding factors (age, BMI, gender, alcohol consumption, smoking and HBV).

Pesticides are metabolized in the liver and it is hypothesized that they contribute to liver carcinogenesis mainly through mechanisms of cell adhesion alterations and oxidative stress (Jin et al., 2014b, 2014c). Polymorphisms in *GSTs* play an important role in susceptibility to oxidative stress and inflammation-related diseases, including cancer (Jerónimo et al., 2002, Dastjerdi et al., 2017). Regarding *GSTs* polymorphism associations with HCC incidence risks, the existing literature is inconsistent (Song et al., 2012). *GSTM1* or *GSTT1* null genotype association with HCC risks depends on ethnicity, whereby significantly increased HCC risks are observed in East Asians and Indians, but no significance is found among Caucasians and African populations (Song et al., 2012, Shen et al., 2014). *GSTP1* Ile105Val polymorphisms are considered a potential susceptibility risk for HCC in individuals < 57 y (Chen et al., 2010). However, Chen et al. (2012) performed a meta-analysis and found that *GSTP1* Ile105 Val polymorphism is not associated with HCC risk in Asian populations. The results of the current study suggest that *GSTs* polymorphisms are not associated with susceptibility to HCC in this Xiamen population, after adjusting for confounding factors (HBV, age, BMI, smoking and alcohol consumption). Other than environmental and genetic factors in HCC development, epigenetic modulations are also considered an important early event in carcinogenesis and may play a key role in hepatocarcinogenesis. In accordance with previous studies (Lambert et al., 2011; Zhang et al., 2005), aberrant *GSTP1* hypermethylation is noted in our studied population. In our previous study, we confirm that *GSTP1* promoter region

hypermethylation results in silencing of gene expression in an *in vitro* liver cell model (Tian et al., 2016). Herein, *GSTP1* promoter region DNA methylation is significantly associated with elevated HCC risk with or without confounding factor adjustment analysis.

Given that the relationship between OCPs exposure and *GSTs* polymorphsims in HCC risk remains controversial, we hypothesize that OCPs exposure may target susceptible individuals. In other words, a considerably lower cumulative exposure to OCPs is sufficient for carriers of an "at-risk" genotype to develop HCC. It is widely accepted that hepatocarcinogenesis is a multifactorial progress; HCC development requires environmental factors acting in a genetically susceptible individual. Exploring gene-environment interactions in relation to HCC risk may be valuable because positive interactions would identify the pollutants with which the gene interacts as disease-causing exposures; such insights into HCC aetiology could point to environmental interventions for disease prevention. Several previous epidemiological studies imply that HCC risk of AFB_1 varies with the exposed individual's detoxifying capacity, where the interaction between serum AFB_1-albumin adduct levels and *GSTs* genotype is statistically significant (Sun et al., 2001). Also, pesticides exposure enhances Parkinson's disease risks with *GSTs* playing a role (Longo et al., 2013, Pinhel et al., 2013). Because enzymes coded by *GSTT1* are involved in tobacco carcinogen metabolism, a significant biological interaction is observed between *GSTT1* and smoking in HCC risk (Boccia et al., 2015). This study indicates OCPs (including DDT and HCH) exposure significantly interacts with *GSTP1* rs1695 (Ile105Val) polymorphism in HCC risk. The same tendency is found in *GSTP1* DNA methylation.

The present study shows *GSTP1* and OCPs gene-environment interaction in HCC; however, the underlying mechanisms remain unclear. Considering the GSTs enzyme involved in xenobiotic metabolism detoxification and that *GSTs* promoter DNA methylation status also tends to be modified with xenobiotics exposure, we investigated whether *GSTs* polymorphisms or epigenetic alterations are associated with serum OCPs residues, or whether OCPs exposure influence *GSTP1* methylation status. As shown in Tables S5–S8 (see ESI), there is no significant correlation between OCPs residue and GSTs polymorphisms as well as *GSTP1* epigenetic alterations in any groups (*P*-value with FDR correction). Meanwhile, the OR of *GSTP1* DNA methylation is not significantly different between low and high OCPs exposure subjects Tables S9 (see ESI). However, we observe that individuals carrying *GSTP1* methylation status exhibit weak potency for DDT dehydrochlorination metabolism (ratio of DDE/DDE + DDT) in all subgroups (see ESI Fig. S8). DDT dehydrochlorination reaction is a major detoxification route that has been found in some insect species and mammals, and GSTs are the major family of detoxification enzyme catalyzing reduced glutathione (GSH) binding to DDT thus converting such agents into the non-toxic lipophilic metabolite DDE (Li et al., 2017, Clark and Shamaan, 1984). According to our present findings, a plausible interaction mechanism, at least partly, is that *GSTP1* DNA methylation status regulates enzyme function and then influences OCPs detoxification metabolism; hypermethylation results in an increased OCPs-exposure HCC risk.

Due to OCPs' hydrophobic structure and long half-lives in the body, they can cause cancer development *via* the induction of oxidative stress and genetic mutations (Mortazavi et al., 2019). Our results are in concordance with the study of *GSTs* polymorphism association with heart failure disease, showing that *GSTP1* Ile105Val polymorphic variants may determine individual susceptibility to oxidative stress, inflammation and endothelial dysfunction in heart failure, while no significance is observed in *GSTT1* and *GSTM1* polymorphisms (Simeunovic et al., 2019). Regarding the interaction effects on HCC risks among different *GSTs* family genes being distinct, we hypothesize that different GSTs family are primarily involved in their specific xenobiotics metabolism, and thus generate various extents of covalent protein binding. *GSTM1* can detoxify active metabolites of PAH, *GSTT1* is required for 1,3-butadiene and ethylene oxide detoxification, and *GSTP1* is involved in preventing cell vulnerability to oxidant and heterocyclic amine carcinogens (Gorukmez et al., 2016). In a previous insect DDT resistance study, results indicate that GSTD6 has the highest interaction affinity with DDT among 23 GSTs (Aravindan et al., 2014). *GSTP1* has been found to have the highest activity towards GSH conjugation of diclofenac metabolites compared to six other GST isoforms in human liver cytosol (den Braver et al., 2016). The effects detected between the two strata for both *GSTP1* genotypes and epigenetic status are consistent for OCPs exposure, suggesting that interactions are unlikely to occur by chance. Meanwhile, ROC area was 0.81 implying combined HBV infection, OCPs exposure, *GSTP1* polymorphism and *GSTP1* epigenetic alterations as predictor variables that might be useful for the prediction of HCC.

Herein, interactions of *GSTs* polymorphisms, epigenetic alterations and environmental OCPs exposure in HCC incidence risks were systematically investigated. However, some limitations are present. Firstly, our study is a retrospective case-control epidemiology research; selection bias is unavoidable, such as HBV infection in the cases, even with adjustment for confounder risk. Furthermore, the statistical power of this study is restricted due to the relatively small sample size. The results need to be confirmed in a larger cohort study. Finally, given that this Chinese population has high levels of OCPs exposure and specific *GSTs* genotype variation distributions, it is unclear whether extrapolation of our findings to other nationalities or regions can be made.

In conclusion, our findings suggest that environmental OCPs exposure is associated with elevated HCC incidence risk, especially among *GSTP1* rs1695 Ile/Val + Val/Val and *GSTP1* promoter region methylated individuals. Our study provides supportive evidence that *GSTP1* might affect susceptibility to OCPs exposure related to HCC development risk. However, the interaction between environmental exposure and genetic polymorphisms as well as epigenetic alteration in HCC risk needs to be more deeply investigated.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105313.

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