The relationship between prenatal exposure to BP-3 and Hirschsprung’s disease

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ABSTRACT

Hirschsprung’s disease (HSCR) is neonatal intestinal abnormality which derived from the failure of enteric neural crest cells migration to hindgut during embryogenesis from 5 to 12 weeks. Currently, the knowledge of environmental factors contributing to HSCR is still scarce. Benzophenone-3 (BP-3) is one of the most widely used UV filters, and has weak estrogen and strong anti-androgenic effects. In order to examine the effect of maternal BP-3 exposure on development of offspring and explore the potential mechanism, we conducted case and control study and in vitro study. In this work, BP-3 concentrations in maternal urine was detected by ultra-high performance liquid chromatography. Besides, we investigated the cytotoxicity and receptor tyrosine kinase (RET) expression in cells exposed to BP-3. The results showed that maternal BP-3 exposure was associated with offspring’s HSCR in the population as well as inhibited migration of 293T and SH-SY5Y cells. What’s more, we discovered dose–response relationship between RET expression and BP-3 exposure dose, and miR-218 and some other genes involved in SLIT2/ROBO1-miR-218-RET/PLAG1 pathway were also related to BP-3 exposure. Therefore, we deduced that BP-3 influenced cell migration via SLIT2/ROBO1-miR-218-RET/PLAG1 pathway. Our study firstly revealed the relationship between maternal BP-3 exposure and HSCR as well as its potential mechanism.

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1. Introduction

Hirschsprung’s disease (HSCR) is a complex congenital disease caused by gene–environment interaction and can lead to intestinal obstruction and chronic constipation (Goldberg, 1984; Amiel et al., 2008). The incidence of HSCR is 1:2000–1:5000 in live births while males are 4 times more susceptible than females (Parisi et al., 2002). HSCR results from enteric neural crest cells’ failing in migrating to certain segments of colon during embryonic development from 5 to 12 weeks (Nishiyama et al., 2012; McKeown et al., 2013; Takahashi et al., 2013). Genetic studies have found that various gene mutations are associated with HSCR, such as receptor tyrosine kinase (RET), EDNRB, GDNF and SOX10. In particular, RET, which encodes a tyrosine-kinase receptor, is the most frequently mutated gene (Angrist et al., 1996; Paratore et al., 2002; Iwashita et al., 2003; Miao et al., 2010). However, the knowledge of environmental factors contributing to HSCR is still quite limited (Gershon, 2010; Lake and Heuckeroth, 2013).
The toxic potential of UV filters that used in a variety of sunscreen and personal care products to attenuate the effects of harmful UV radiation on skin and hair, has attracted public concerns (Okereke et al., 1995). Benzophenone-3 (BP-3) occurs naturally in flower pigments and is synthesized for use in sunscreens, various cosmetic products, and plastic surface coatings and polymers. The application of some personal care products that contain UV filters on the skin can increase the systemic absorption (Gustavsson Gonzalez et al., 2002; Liao and Kannan, 2014). In some cases, as much as 10% of the applied dermal dose was absorbed into the systemic circulation (Janjua et al., 2004). Because of the extensive use of BP-3 in personal care products, human exposure to this compound is widespread. BP-3 was found in >95% of urine samples collected from the U.S. general population, at concentrations ranging from 0.4 to 21,700 ng/mL (Calafat et al., 2008). It is also worth noting that exposure to BP-3 in women is much higher than men (Chen et al., 2012).

BP-3 is a bioactive chemical which is established to be weakly estrogenic and exhibits both intensely anti-estrogenic and anti-androgenic activities in vitro (Schlumpf et al., 2001; Schreurs et al., 2002, 2005). It is reported that BP-3 altered embryo development in insects (Ozaez et al., 2014). Previous study have found prenatal BP-3 exposure is associated with adverse birth outcomes (Wolff et al., 2008; Tang et al., 2013). Therefore, the effects of BP-3 on fetal development need particular concerns.

miR-218 has been extensively studied in pathologies. And it can markedly suppresses cell motility, invasion, and proliferation (Tu et al., 2013; Heckmann et al., 2014). According to a previous report, miR-218 suppresses tumor cell migration through SLIT2-ROBO1 pathway (Alajez et al., 2011). In this study, we conducted a population study to explore the potential effects of BP-3 exposure on HSCR and underlying mechanism in relation to miR-218-mediated pathways was further studied in vitro.

2. Materials and methods

2.1. Study population

We recruited 101 HSCR patients’ mothers and 322 mothers as controls in affiliated hospitals of Nanjing Medical University from October 2009 to May 2014 (Nanjing Medical University Birth Cohort, NJMU Birth Cohort). The Institutional Review Board of Nanjing Medical University approved the protocols. After detailed explanation of the study procedures and clarification of questions raised, the total 423 women signed informed consent forms for the explanation of the study procedures and clarification of questions raised, the total 423 women signed informed consent forms. From the surgical pathology perspective, HSCR is determined by submucosal atrophy which got rid of HSCR or other congenital malformations, and strangulated inguinal hernia without ischemia or necrosis as controls which received non-surgical treatment. Finally, 101 HSCR and 103 control colon tissues were obtained. The tissues were immediately frozen and stored at −80 °C after surgery. All the studies involving human subjects were done under full compliance with government policies and the Helsinki Declaration.

2.2. Chemicals and reagents

BP-3 (CAS NO. 131-57-7, 99.5% purity) was purchased from Dr. Ehrenstorfer GmbH (Bürgermeister-Schlosser, Germany). β-glucuronidase/sulfatase type H-1 from Helix pomatia, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), diethylpyrocarbonate (DEPC) were obtained from Sigma–Aldrich (St. Louis, USA). BP-3 was first dissolved in DMSO and added to the Dulbecco minimal essential medium (DMEM) to final concentrations of 100 μM which was further diluted to lower concentrations for testing. The cells treated with 0.1% DMSO served as controls.

2.3. Measurement of urinary phenols

We measured total (free and conjugated) urinary concentrations of phenols using a sensitive method as previously described (Chen et al., 2012). Briefly, urine samples were incubated in 1 M ammonium acetate buffer solution (pH = 5.0) for hydrolyzation with β-glucuronidase/sulfatase (20,000 units mL−1) overnight. After hydrolysis, the phenols were extracted and preconcentrated with solid phase extraction (500 mg/3 mL, Supelclean, USA), and further detected using ultra-high performance liquid chromatography (Waters, USA)–electrospray ionization tandem mass spectrometry (Waters, USA). The detection was done in the negative ion mode by multiple reaction monitoring. The limits of detection (LODs) were 0.04 ng mL−1. Strict quality control was conducted during the analysis. CR data were collected using an automated chemistry analyzer (7020 Hitachi, Japan), which were obtained for correcting the phenol concentrations caused by urine concentration and dilution.

2.4. Cell culture and BP-3 treatment

Human 293T and SH-SYSY cells were widely used as cell models in research on mechanisms of HSCR (Kawamoto et al., 2003; Vargiolu et al., 2009). Human 293T cells and SH-SYSY cells were obtained from American Type Culture Collection (USA). These cells were cultivated in complete growth medium DMEM (HyClone, USA), supplemented with 10% fetal bovine serum (FBS), 100 units mL−1 penicillin, and 100 μg mL−1 streptomycin at 37 °C, 5% CO2. For chemical treatment, 293T cells and SH-SYSY cells were plated in 6-well, 24-well or 96-well plates and then treated with BP-3 (0.1 μM, 1 μM, 10 μM, and 100 μM) dissolved in DMSO for 24 h. As a negative control, cells were also exposed to 0.1% DMSO alone. 293T cells and SH-SYSY cells were collected and used for gene and protein expression analysis on d 2.

2.5. Cell proliferation assays

Cell viability was assessed by cell counting kit-8 (CCK-8 kit, Beyotime Institute of Biotechnology, China). 293T cells or SH-SYSY cells were seeded at 5000 cells/well in 96-well plates. After 24 h incubation, the cells were treated with BP-3 at concentrations of 0.01, 0.1, 1, 10, 100 and 1000 μM. After 24 h, 100 μL medium solution (content 10% CCK-8) was added and incubated at 37 °C for 1 h. Finally, absorbance was measured on DU-800 Nucleic Acid and Protein Analyzer (Beckman, USA) at 450 nm. The experiment was repeated three times.
2.6. Cell cycle and apoptosis analysis

To estimate if BP-3 could affect the cell cycle and induce apoptosis in 293T cells and SH-SY5Y cells, flow cytometric analysis was used to determine the state of cell cycle and the DNA fragmentation. 293T cells and SH-SY5Y cells were seeded on 6-well plates at a density of about 1 × 10^4 cells per well. Cells were incubated overnight and subsequently exposed to BP-3 (0.1 μM, 1 μM, 10 μM and 100 μM) and control medium containing 0.1% DMSO. After 24 h, cells were washed with PBS and harvested with trypsin. Cells were fixed in 70% ethanol for 2 h or washed in cold PBS, then stained with propidium iodide and annexin V for 30 min protected from light. The fixed/stained cells were analyzed by FACS Calibur Flow Cytometry (BD Biosciences, USA) to quantify cell cycle or cell apoptosis.

2.7. Cell transwell assays

For those cells treated with BP-3 (0, 0.1, 1, 10 and 100 μM), after 24 h, cells (1 × 10^4) were seeded in the upper chamber with serum-free medium. DMEM containing 10% FBS was added to the lower chamber and followed by incubation for 1 d. Cells in upper chamber were stained with crystal violet staining solution (Beyotime Institute of Biotechnology, China), and then counted and photographed under 40 × magnification (five views per well). Migrated cells were counted using Image-pro Plus 6.0 while cell numbers of normal control group were normalized to 1. All experiments were performed in triplicate.

2.8. RNA isolation and quantitative real-time PCR assay

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The RNA pellets were dissolved in ddH2O containing 0.1% DEPC, and quantified by measuring the absorborbity at 260 nm by DU-800 Nucleic Acid and Protein Analyzer (Beckman, USA). We manipulated all real-time PCR reactions on ABI7900 Fast Real-Time System (Applied Biosystems, USA) according to the manufacturer’s instructions for quantification of gene expression. CDNA synthesis for coding genes was performed with 1 μg of total RNA according to the manufacturer’s instructions (Takara, Japan), TaqMan®MicroRNA Assays (Applied Biosystems, USA) were used as the probe for hsa-miR-218 and U6 which acted as an internal control. mRNA levels of RET and other genes were measured using SYBR PCR Master Mix reagent kits (Takara, Japan). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All primer sequences are given in Table S1. The experiments were carried out three times.

2.9. Protein analysis

Total proteins were isolated from cultured cells using RIPA buffer containing protease inhibitors complete, ULTRA, Mini, ethylenediaminetetraacetic acid-free, EASY pack (Roche, Switzerland), while the membrane proteins were extracted from tissues by Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Scientific, USA). The protein samples (100 μg from each group) were separated with 7.5%/12.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. Primary polyclonal antibodies included RET antibody (SC167, Santa Cruz, USA), PLAG1 antibody (BP11647a, Abgent, USA), SLIT2 antibody (ab134166, Abcam, UK) and ROBO1 antibody (MAB7118, R&D, USA). The secondary antibodies with horseradish peroxidase were anti-rabbit, anti-goat and anti-mouse HRP-linked (Beyotime Institute of Biotechnology, China). The bands were visualized using ECL reagent (Millipore, USA). Equal amount of protein loading in each lane was confirmed using GAPDH antibody. The experiment was replicated thrice for each protein.

2.10. Statistical analysis

The data analysis was performed using Stata 9.2 statistical software package (Stata Corp, LP). T test was used to compare the mean age, body mass index (BMI) between case and control groups; if the variances were far from equal, the Wilcoxon signed rank test was used. The chi-squared test was used to evaluate the differences in smoking status and drinking status between case and control groups. All urine samples were divided into three-level variable for statistical analysis. The samples with concentrations <LOD were assigned to the low exposure group, and the remaining samples were divided into 2 groups approximately as the median exposure group and the high exposure group. The samples between detectable concentrations and 0.10 ng mL^−1 were assigned to the median exposure group. The remaining samples were assigned to the high exposure group. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for the estimation of the relationship between HSCR and urinary BP-3 levels by unconditional logistic regression analysis adjusted by BMI which was significantly different between case and control groups. Additionally, we employed urinary creatinine (CR) as a continuous variable to adjust for the urinary dilution.

Statistically significant differences between the treatments and the control were determined by one-way analysis of variance, followed by Dunnett’s multiple comparison test. All tests of statistical significance were two-sided, and the statistical significance was set at p < 0.05.

3. Result

3.1. The association between BP-3 and HSCR in the populations

All of the 423 women participants were ethnic Han-Chinese, including 101 cases and 322 controls. Age, weight and BMI of mother were possible confounders which were related with birth outcome (Fraser et al., 1995; Cnattingius et al., 1998; Kiel et al., 2007). As shown in Table 1, there were significant differences in weight and BMI between cases and controls, which could be due to higher weight of the controls in pregnancy. While no significant differences were identified in smoking status and alcohol consumption. Therefore, we matched age in mothers of HSCR and controls in our study. Weight and BMI were significant in the case and control groups and BMI was adjusted to calculate ORs and CIs between the case and control groups. The distributions of urinary concentrations of BP-3 in the 423 participants are presented in Table 2. BP-3 were detectable in over 50% samples and adjusted ORs for the relationship between phenol exposure levels and HSCR are presented in Table 2. The maximum detection concentration was 400.72 ng mL^−1 and percentiles at 50th, 75th, 90th, 95th were 0.08, 0.17, 0.54, 1.10 ng mL^−1. Compared with women in the lowest exposure group, women in both the median and high BP-3 exposure groups were more likely to give birth of HSCR children, [for BP-3: adjusted ORs for increasing exposure levels = 2.39 (95% CI, 1.10−5.21), 2.61 (95% CI, 1.15−5.92), p-value for trend <0.05] which indicated that maternal BP-3 exposures were associated with the offspring’s HSCR.

The age of 101 HSCR and 103 control groups were 3.62 ± 0.21 and 3.67 ± 0.25 months old, respectively. The body weight were 5.13 ± 0.14 and 5.35 ± 0.12 kg, respectively. There was no statistical difference between cases and controls in age and body weight. The gender rate (Male/Female) of HSCR and control was 79/22 and 83/20, respectively, which matched the common gender rate of this disease in human beings.
The number of migrated cells were significantly lower in 100 μM BP-3 exposure than low exposure in maternal urines. The tables showed significances in both medium and high BP-3 exposure than low exposure in maternal urines. Maternal exposure to chemicals with endocrine disrupting effects can alter the development of progeny, inducing birth defects (Ngalame et al., 2013; Veiga-Lopez et al., 2013). Population study manifests that phthalate exposure of pregnant women during critical window contributes to preterm births (Ferguson et al., 2014). Nevertheless, we got barely research on mammals or primates about BP-3 toxicity in spite of its extensive exposure and potential deleterious effect (Vela-Soria et al., 2011). In our study, sporadic cases (Brooks et al., 2005; de Groot et al., 2006). To evaluate the effect of BP-3 exposure on RET expression, we conducted RT-PCR to detect mRNA expression with BP-3 treatment from 0 to 100 μM doses. As shown in Fig. 2A, similar to HSCR stenosis tissues as reported, RET mRNA expressions were decreased in BP-3 treated 293T and SH-SY5Y cells. Meanwhile, BP-3 reduced RET protein expressions significantly in western blot experiments (Fig. 2B).

### Table 1
Characteristics of participants in this study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HSCR</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother characteristics (n = 423)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (year, mean ± SD)</td>
<td>28.88 ± 4.30</td>
<td>27.48 ± 4.65</td>
<td>0.07*</td>
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<tr>
<td>Parity (%)</td>
<td>65.28</td>
<td>68.96</td>
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<tr>
<td>≥ 1</td>
<td>34.72</td>
<td>31.04</td>
<td></td>
</tr>
<tr>
<td>Education (year, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 9</td>
<td>67.60</td>
<td>69.25</td>
<td>0.38</td>
</tr>
<tr>
<td>10–12</td>
<td>32.40</td>
<td>30.75</td>
<td></td>
</tr>
<tr>
<td>Maternal weight (kg, mean ± SD)</td>
<td>160.41 ± 1.71</td>
<td>161.19 ± 4.03</td>
<td>0.23</td>
</tr>
<tr>
<td>Maternal height (cm, mean ± SD)</td>
<td>173.86 ± 8.19</td>
<td>172.32 ± 10.17</td>
<td>0.01*</td>
</tr>
<tr>
<td>BMI</td>
<td>21.86 ± 3.01</td>
<td>20.9 ± 4.80</td>
<td>0.01*</td>
</tr>
<tr>
<td>Smoking in pregnancy (%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Drinking in pregnancy (%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Weight (kg, mean, SD)</td>
<td>5.13 ± 0.14</td>
<td>5.35 ± 0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>Height (cm, mean ± SD)</td>
<td>160.41 ± 3.71</td>
<td>161.19 ± 4.03</td>
<td>0.38</td>
</tr>
<tr>
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<td></td>
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<td>20.9 ± 4.80</td>
<td>0.01*</td>
</tr>
<tr>
<td>Smoking in pregnancy (%)</td>
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<td>0</td>
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<tr>
<td>Drinking in pregnancy (%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Weight (kg, mean, SD)</td>
<td>5.13 ± 0.14</td>
<td>5.35 ± 0.12</td>
<td>0.39</td>
</tr>
</tbody>
</table>

There was a significant difference both at maternal weight and BMI variables between HSCR mothers and the controls’ mother. In this case our results of BP-3 detection in urine was corrected by BMI. *P < 0.05 indicates significant difference compared with the control group.

3.2. The cytobiology change after BP-3 exposure in 293T and SH-SY5Y cell lines

To examine the effect of BP-3 on cell viability and morphology, 293T cells and SH-SY5Y cells were exposed to various concentrations of BP-3 for 24 h. After that, a significant decrease in viability was observed at the dose of 1000 μM (Fig. S1A). Since cytotoxic effects were not observed during 0.1 μM, 1 μM, 10 μM, 100 μM of treatment, cells were treated with BP-3 at these concentrations in all of the following experiments.

As abnormal migration is the major cause of HSCR, the migration of 293T and SH-SY5Y cell lines after BP-3 treatment were tested with transwell assay. A suppressive effect was observed in both 293T and SH-SY5Y cell lines at the dose of 100 μM (Fig. 1A). The number of migrated cells was significantly lower in 100 μM of BP-3 treatment group (Fig. 1B), which indicates a causal association between BP-3 exposure and HSCR in the population study. We also examined the effect of BP-3 on cell cycle and apoptosis after 24 h exposure using flow cytometry, but no significant differences were observed in both cell apoptosis and cell cycle (Fig. S1B and C).

3.3. The expression of RET was inhibited in BP3 contaminated cell lines

The RET proto-oncogene is considered the major disease-causing locus in HSCR, mutations of which are identified in 15–35% of sporadic cases (Brooks et al., 2005; de Groot et al., 2006). To explore the potential role of miR-218 and PLAG1 in BP-3 induced effect, both gene expression levels were tested by RT-PCR in BP-3 infected 293T and SH-SY5Y cells. The results showed that miR-218 was up-regulated while PLAG1 was down regulated, which were consistent with protein levels (Fig. 2A and B). Therefore, BP-3 treatment might suppress RET and PLAG1 expression through increasing miR-218 expression. As a consequence, SLIT2, the host gene of miR-218, was assessed at mRNA level. The mRNA and protein levels of SLIT2, and its receptor ROBO1 were dose-dependently increased by BP-3 exposure (Fig. 2).

3.4. Effects of BP-3 on miR-218-RET pathway

miR-218 is predicted to target RET as well as PLAG1 by bioinformatics methods in previous research (Tang et al., 2015). To explore the effect of BP-3 exposure on RET expression, we conducted western blot experiments in HSCR, mutations of which are identified in 15–35% of sporadic cases (Brooks et al., 2005; de Groot et al., 2006). To evaluate the effect of BP-3 exposure on RET expression, we conducted RT-PCR to detect mRNA expression with BP-3 treatment from 0 to 100 μM doses. As shown in Fig. 2A, similar to HSCR stenosis tissues as reported, RET mRNA expressions were decreased in BP-3 treated 293T and SH-SY5Y cells. Meanwhile, BP-3 reduced RET protein expressions significantly in western blot experiments (Fig. 2B).

3.4.1. The validation of the miR-218-RET pathway in HSCR colon tissues

To determine whether BP-3 was involved in the pathogenesis of HSCR through miR-218-RET pathway, we detected those gene expressions in colon tissues of HSCR children and controls' at mRNA levels. As shown in Fig. 3, we confirmed the mRNA expression levels of miR-218, SLIT2 and ROBO1 in HSCR were significantly higher than those in controls (P< 7.13 × 10−6, P= 5.03 × 10−5 and 4.12 × 10−6, respectively), while those of RET and PLAG1 were extreme lower (P = 5.47 × 10−9, P = 1.02 × 10−6).

4. Discussion

As far as we know, this is the first study to comprehensively explore the effects of BP-3 exposure (at concentrations that do not induce cytotoxic effects) on HSCR, and the possible underlying mechanism was revealed. We found maternal BP-3 exposure was associated with HSCR. BP-3 suppressed migration in 293T and SH-SY5Y cells. It also decreased RET and PLAG1 and increased miR-218, SLIT2 and ROBO1 both at the mRNA and protein levels.

HSCR is a partially penetrant oligogenic birth defect that occurs when ENS precursors fail to colonize the distal bowel during early pregnancy (Lake et al., 2013). Ganglion migration failure during 5–12 weeks of gestation is one of the leading causes of the disease. Maternal exposure to chemicals with endocrine disrupting effects can alter the development of progeny, inducing birth defects (Ngalame et al., 2013; Veiga-Lopez et al., 2013). Population study manifests that phthalate exposure of pregnant women during critical window contributes to preterm births (Ferguson et al., 2014). Nevertheless, we got barely research on mammals or primates about BP-3 toxicity in spite of its extensive exposure and potential deleterious effect (Vela-Soria et al., 2011). In our study,

### Table 2
Adjusted ORs (95% CIs) for HSCR by exposure level of BP-3 in maternal urines (n = 423)*.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Control/case (NO.)</th>
<th>Adjusted-OR (95% CI)*</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP-3</td>
<td>low</td>
<td>129/37</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>medium</td>
<td>97/33</td>
<td>2.39 (1.10–5.21)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>96/31</td>
<td>2.61 (1.15–5.92)</td>
</tr>
</tbody>
</table>

The tables showed significances in both medium and high BP-3 exposure than low exposure in maternal urines.

*P < 0.05 compared with the low BP-3 exposure level.

* ORs are adjusted for maternal BMI and CR level.
maternal BP-3 exposure levels were extremely consistent with disease risk of offspring’s HSCR. Therefore, maternal BP-3 exposure during critical windows of fetal neural development may affect the development of offspring and induce HSCR.

It has been demonstrated that miRNAs not only regulate multiple protein coding genes, but also control all biological processes (Lin et al., 2012; Liu et al., 2012). It is universally acknowledged that miRNAs are susceptible to environmental exposures, such as bisphenol A and nicotine. miRNA dysfunction may be a mechanism through which toxicants can exert both developmental and carcinogenic effects (Jirtle and Skinner, 2007; Izzotti et al., 2009; Avissar-Whiting et al., 2010; Hou et al., 2012). Studies showed that prenatal chemical exposure could modify the epigenome of specific genes of the offspring and social behavior in the childhood, which
Fig. 3. Validation of population. (A)–(E) The mRNA expression levels of miR-218, RET, PLAG1, SLIT2, ROBO1, in the control and HSCR tissues. ***P = 0.0005, n = 103 controls/101 HSCR. All tests were performed for three times.

gave us clues that prenatal BP-3 exposure might alter gene expressions through epigenetic level (Furlong et al., 2014; Vilahur et al., 2014). Our former study has clarified that increased expression of miR-218 suppressed cell migration and proliferation in 293T and SH-SY5Y cells. Therefore, modified expression level of miR-218 caused by BP-3 exposure may play roles in the pathogenesis of HSCR.

Some evidences confirmed that RET was upregulated by miR-218 in acute myeloid leukemia (Diaz-Beya et al., 2013). Early researches have confirmed that miR-218 inhibits cell migration and proliferation by targeting RET and PLAG1 (Tang et al., 2015). In this study, we firstly demonstrated that BP-3 inhibited cell migration through RET and PLAG1 at the concentration of 100 μM. To ensure the down-regulating of RET and PLAG1 were caused by the enhanced miR-218, we then examined the expression level of miR-218 in both cell lines after the exposure of BP-3. As expected, miR-218 was up-regulated. Considering the relationship between SLIT2 and miR-218 as well as ROBO1, we performed experiments to test the expression levels of both SLIT2 and ROBO1. Our study manifested BP-3 exposure led to higher expression quantities of SLIT2, and conversely lower expression quantities of ROBO1 which acted as its receptor in central nervous. Based on all the cell function experiments, we demonstrated that BP-3 contributed to the pathogenesis of HSCR via regulating genes signal transduction.

A limitation of the present study was the spot urine determination to assess exposure. However, as far as we were aware, all participants in our study had not changed their eating habits, life styles or environments for several months prior to the sample collection. Therefore, their urinary levels of BP-3 exposure are expected to be relatively stable over time. In addition, although the risk of misclassification may still exist, since this would be most likely non-differential, one would expect mainly attenuation of observed exposure–effect associations. Second, this study was the case–control design that limited our strength in making a causal conclusion. Third, although we clarify the fact that maternal BP-3 exposure brought high risk probability to offspring’s HSCR, cell lines are limited in elucidating the mechanism in the present study and chemical exposure in enteric neural crest cells is needed for further investigation. What’s more, other environment factors which may take part in the process of pathogenesis of HSCR are still unknown.

5. Conclusions

In conclusion, our study firstly pointed out that maternal BP-3 exposure may cause offspring’s HSCR through SLIT2/ROBO1-miR-218-RET/PLAG1 pathway. These findings enhanced our understanding of BP-3 effects on embryonic development.

Conflicts of interest

The authors declare no conflict of interest with the study or preparation of the manuscript.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.09.019.

References


