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Association of prenatal organochlorine pesticide-dichlorodiphenyltrichloroethane exposure with fetal genome-wide DNA methylation

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ABSTRACT

Aims: To investigate whether intrauterine organochlorine pesticide (OCP)-dichlorodiphenyltrichloroethane (DDT) exposure could lead to epigenetic alterations by DNA methylation with possible important lifetime health consequences for offspring.

Main methods: We used Illumina Infinium HumanMethylation 450 K BeadChip to explore the pattern of genome-wide DNA methylation containing > 485,000 gene sites in cord blood of 24 subjects in a 12 mother-newborn pairs birth cohort. Based on the genome-wide DNA methylation data, we chose one potential gene, *BRCA1*, to verify the results in another group comprising 126 subjects.

Key findings: We identified 1,131 significantly different CpG sites which included 690 hypermethylation sites and 441 hypomethylation sites in the DNA methylation level between case and control group. The identified sites were located in 598 unique genes. In subsequent validation studies, we found that the DNA methylation level of the identified CpGs of *BRCA1* increased with increased exposure to dichlorodiphenyltrichloroethane (DDT) and the level of gene expression in the identified CpGs of *BRCA1* decreased with increased exposure to dichlorodiphenyltrichloroethane (DDT).

Significance: The results indicated that epigenetic processes played a possible role in the development of fetuses affected by maternal OCP-DDT exposure. Early prenatal exposure to DDT may affect fetal *BRCA1* gene methylation, and increased exposure leads to a higher DNA methylation level and lower gene expression level.

1. Introduction

In the life course of an individual, environmental risk factors interact with the individual biological system at all times and one of the important gateways is pregnancy. The epigenome may have been changed by environmental pollutants in the early stages of pregnancy [1]. Even traces of chemical pollutants may have an adverse impact on the development of the fetal immune, nervous and reproductive systems [2].

Dichlorodiphenyltrichloroethane (DDT) is a common environmental organochlorine pesticide (OCP) with a long half-life. It can accumulate in adipose tissue and exert biological amplification effects. DDT exposure during the neonatal period may heavily influence lifetime health because DDT will pass through the placenta from mother to newborn, causing poor birth outcomes [3]. How did prenatal exposure to DDT increase the opportunity of suffering from an illness was not well known. Clinical and epidemiological studies have indicated that DNA methylation played an important role in the association of higher risk of

Abbreviations: OCP, organochlorine pesticide; DDT, dichlorodiphenyltrichloroethane; p,p′-DDT, p,p′-dichlorodiphenyltrichloroethane; o,p′-DDT, o,p′-dichlorodiphenyltrichloroethane; p,p′-DDD, p,p′-dichlorodiphenyldichloroethane; o,p′-DDD, o,p′-dichlorodiphenyldichloroethane; p,p′-DDE, p,p′-dichlorodiphenylethylene; o,p′-DDE, o,p′-dichlorodiphenylethylene; *BRCA1*, breast cancer susceptibility gene 1; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; DMCpGs, differentially methylated CpG sites; BMI, body mass index; *QR*, quartile range; *CI*, confidence interval; *OR*, odds ratio

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disease in later life with environmental exposure in early life [4,5,6,7]. If prenatal DDT exposure has effect on fetal heath later in life, how these effects are recorded through epigenetics such as DNA methylation? The current knowledge of this aspect was still very limited and no systematic studies and reports have been reported.

To study the possible causal relationship between prenatal exposure to DDT and the individual life course of disease risk, a combination of methods—epidemiology, bioinformatics and epigenetics was used to comprehensively analyze the relationship between prenatal exposure to DDT and genome-wide DNA methylation in cord blood. Next, to confirm the results, we chose breast cancer susceptibility gene 1 (*BRCA1*), which was reported to be a tumor suppressor gene with an important relationship with breast cancer. Promoter methylation of the *BRCA1* gene in sporadic breast cancer was found to lead to a decrease in or absence of mRNA and protein expression levels, leading to the occurrence of breast cancer [8]. In addition, many studies have shown that organochlorine pesticides are associated with the occurrence of breast cancer [9,10,11,12,13], but the mechanism is still not clear. We suspect that the exposure of prenatal organochlorine pesticide may lead to fetal *BRCA1* gene methylation in the early life of fetus and decrease *BRCA1* gene expression, which increasing the risk of breast cancer in the later life of the fetus. If we can confirm that prenatal organochlorine pesticide exposure affects fetal *BRCA1* gene methylation and expression, our results would contribute new evidence for that DNA methylation may be considered a key mechanism of “fetal origins of adult disease” (FOAD). We would also offer new clues for the mechanistic study of organochlorine pesticides increased risk of breast cancer.

2. Materials and methods

2.1. Ethics statement

All participants signed the information consents before they were included in the cohort. The study was performed according to the World Medical Association Declaration of Helsinki, and the procedures were approved by the ethics committee of the School of Public Health, Xiamen University.

2.2. Study subjects

Our research was based on a birth cohort established in Xiamen, China. We chose eligible pregnant women from Lianhua Hospital in Xiamen, China, during July 2014 to February 2015. A questionnaire contained the information about name, age, education degree, family income, height, pre-pregnancy weight, prenatal weight, smoking habits, alcohol consumption and other pregnancy behaviors was necessary. Eventually, 150 mother-newborn pairs were enrolled in this study.

The inclusion criteria were as follows: (1) resident in Xiamen for more than 1 year; (2) natural pregnancy; (3) single birth; (4) gestational age ≤ 16 weeks at the time the maternal health card was issued.

The exclusion criteria were as follows: (1) a history of endocrine or metabolic diseases, liver or kidney disease, blood system diseases, genetic diseases, or occupational contact with toxic substances; (2) a history of genetic diseases in the spouse; (3) receiving hormone therapy; (4) the use of assisted reproductive technologies, such as in vitro fertilization.

2.3. Sample collection and DDT exposure detection

The umbilical cord blood (5 CC) was collected during parturition using EDTA tubes (3 ml, Vacuette® Blood Collection Tube, K2 EDTA; Greiner Bio-One, German), and stored at −80 °C. The concentrations of DDT containing 6 homologs in the umbilical cord blood were detected by capillary Gas Chromatography (GC) with electron capture detection (ECD; Shimadzu) and an HP/DB-1 capillary column (15.0 m–530 mm, 0.5 mm; Agilent, USA).

2.4. Genome-wide DNA methylation analysis

According to the detected DDT level (the sum of the concentrations of the 6 DDT homologs), we chose 12 fetuses of the highest exposure concentration as the case group and another 12 fetuses who have the lowest level of DDT exposure as the control group, matched by the mother's age, pre-pregnancy and prenatal weight, education degree, as well as the neonate's sex, birth height, birth weight and Apgar score. There were no preterm-labor or low-birth-weight children. DNA was isolated from 24 cord blood samples using QIAamp DNA Blood Mini Kit (Qiagen, 51306, German). Illumina Infinium HumanMethylation 450 K BeadChip (Illumina, San Diego, CA, USA) was used to measure genome-wide DNA methylation.

2.5. Validation study

Our birth cohort contained 150 mother-newborn pairs. In addition to 24 cases for genome-wide DNA methylation analysis, we chose the rest of 126 fetal cord blood samples to further study the relationship between DDT and *BRCA1* gene methylation. According to the result of genome-wide DNA methylation, cg25067160 and cg25067162, found in TSS1500 in the *BRCA1* gene, were chosen to be the next candidate sites.

2.5.1. DNA extraction and pyrosequencing technology

Genomic DNA was isolated from the cord blood of 126 fetuses using QIAamp DNA Blood Mini Kit (Qiagen, 51306, German). Sodium bisulfite modification of the DNA was performed using EpiTect Bisulfite Kit (Qiagen, 51306, German). Primer Premier 6 and Oligo 7.0 software were used for primer design according to the objective sequence. The primer sequences were as follows: forward: TTAGAAATTGTAGTTTTA TGGAGAGG; reverse: CATAACACTCCAATCCATAACTATTAAC. The conditions of PCR were as follows: 95 °C for 3 min; 35 cycles of 94 °C for 25 s, 60 °C for 25 s and 72 °C for 25 s; an elongation step of 72 °C for 5 min. After PCR was completed, 5 μl PCR products were taken with 1% agarose gel electrophoresis, 150 V, 100 mA, 20 min electrophoretic observation. The level of DNA methylation of the identified CpG sites was then detected by pyrosequencing. The sequencing primers were as follows: GGAAATTTAGTGGATA. PyroMark Q96 ID platform and PyroMark CpG Software 1.0.11 (Qiagen, German) were used to make a quantitative pyrosequencing analysis.

2.5.2. RNA extraction and TaqMan RT-qPCR

Total RNA was isolated from the cord blood of 126 fetuses using Trizol reagent (Invitrogen, San Diego, CA, USA). The conditions of PCR were as follows: reverse transcription 45 °C for 10 min; predegeneration 95 °C for 10 min; 40 cycles at 95 °C for 15 s and 60 °C for 50 s. The TaqMan probe was used to quantify the expression levels of *BRCA1*. All samples were normalized to internal control Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and the level of gene expression was calculated according to the relative quantification method. The primer sequences were as follows: *BRCA1*-F: CGATTGCGTCGACGGAGAC, *BRCA1*-R: CAAGTAGTCTTGTAAGGTCAGTGGC, *BRCA1*-P: AACTACGA GTGCGCAGACATGGGC; *GAPDH*-F: TATTGGGCGCCTGGTC, *GAPDH*-R: GACGGTGCCATGGAATT, *GAPDH*-P: TTAACTCTGGTAAAGTGGATATT GTTGCC.

2.6. Statistical analyses

R software 3.2.0 and SPSS 17.0 were used to perform the statistical analyses. The participants' anthropometric characteristics were described as the mean ± standard deviation. We used *t*-test and χ^2 test to identify difference in these characteristics between case and control group. β value was used to present the methylation level of each CpG site, which was calculated from methylated (M) and unmethylated (U) allele intensity; the ratio of fluorescent signals $\beta = M/[M + U + 100]$