Environmental Research 171 (2019) 536–545

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Differential DNA methylation in newborns with maternal exposure to heavy metals from an e-waste recycling area

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ARTICLE INFO

Keywords:
E-waste
Heavy metals
Maternal exposure
DNA methylation
Epigenetics

ABSTRACT

This study explored the effects of maternal exposure to e-waste environmental heavy metals on neonatal DNA methylation patterns. Neonatal umbilical cord blood (UCB) samples were collected from participants that resided in an e-waste recycling area, Guiyu and a nearby non-e-waste area, Haojiang in China. The concentrations of UCB lead (Pb), cadmium (Cd), manganese (Mn) and chromium (Cr) were measured by graphite furnace atomic absorption spectrometry. Epigenome-wide DNA methylation at 473, 844 CpG sites (CpGs) were assessed by Illumina 450 K BeadChip. The differential methylation of CpG sites from the microarray were further validated by bisulfite pyrosequencing. Bioinformatics analysis showed that 125 CpGs mapped to 79 genes were differential methylation in the e-waste exposed group with higher concentrations of heavy metals in neonatal UCB. These genes mainly involve in multiple biological processes including calcium ion binding, cell adhesion, embryonic morphogenesis, as well as in signaling pathways related to NFkB activation, adherens junction, TGF beta and apoptosis. Among them, *BAI1* and *CTNNA2* (involving in neuron differentiation and development) were further verified to be hyper- and hypo-methylated, respectively, which were associated with maternal Pb exposure. These results suggest that maternal exposure to e-waste environmental heavy metals (particularly lead) during pregnancy are associated with peripheral blood differential DNA methylation in newborns, specifically the genes involving in brain neuron development.

1. Introduction

Heavy metals are environmental pollutants which are widely utilized in various electronic products and could have potential toxicity to human health. Some typical heavy metals, such as lead (Pb), cadmium (Cd), chromium (Cr) and manganese (Mn) are characteristic contaminants originating from electronic waste (e-waste) (Huo et al., 2007; Zheng et al., 2008; Xu et al., 2015a, 2015b; Ohajinwa et al., 2018; Zeng et al., 2018). Many researchers have reported high levels of these contaminants in the environment and in human biological samples from several e-waste dismantling and recycling areas. These e-wastes impose adverse health effects on local residents, particularly on susceptible populations (pregnant women and newborns) (Guo et al., 2010, 2014; Ni et al., 2014; Heacock et al., 2016; Huo et al., 2014; Shi et al., 2016; Zeng et al., 2016a). Exposure to heavy metals during fetal development has injurious effects on cellular function and might negatively influence health trajectories in later life (Godfrey and Barker, 2001). Examples of prenatal exposure to lead resulting in adverse health outcomes include neurocognitive and behavioral deficits, low birth weight and preterm deliveries, which are associated with the disease risk throughout the later life course (Andrews et al., 1994; Chen et al., 2011; Jelliffe-Pawlowski et al., 2006; Needleman et al., 1990; Rich-Edwards et al., 1997). With respect to cadmium exposure, several studies have reported negative associations between maternal exposure during pregnancy and birth length, weight, head circumference of newborns, and detrimental cognitive developmental effects in later life (Frery et al., 1993; Kippler et al., 2016; Lin et al., 2011; Nishijo et al., 2004; Shirai et al., 2010). In addition, prenatal manganese exposure has also been shown to be associated with neurodevelopmental problems in childhood and health problems later in adulthood (Bailey et al., 2006;

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https://doi.org/10.1016/j.envres.2019.01.007
Received 7 September 2018; Received in revised form 6 December 2018; Accepted 4 January 2019
Available online 06 January 2019
0013-9351/

Ericson et al., 2007; Roels et al., 2012). Animal studies have indicated that chromium-related toxic effects on the embryo and fetus are correlated with decreased birth weight and crown-rump length, retarded fetal development and dead fetuses, while epidemiologic research has shown that high levels of chromium exposure *in utero* can cause higher potential risk of delivering preterm infants and newborns with abnormal birth outcomes (Bailey et al., 2006; Junaid et al., 1995; Pan et al., 2017; Xia et al., 2016).

Interestingly, recent studies have linked early-life exposure to cigarette smoke with DNA methylation alterations in peripheral blood mononuclear cells. Differential methylation of a large number of CpG sites was associated with differential gene expression and disease susceptibility and development (Joubert et al., 2012). In addition, increasing evidence suggests that maternal exposure to heavy metals can modify the epigenetic status of the human genome. For instance, epidemiological studies indicate that prenatal exposure to Pb can inversely affect genomic DNA methylation in umbilical cord blood (Pilsner et al., 2009); maternal Cd exposure is negatively associated with DNA methylation at regulatory sequences of imprinted genes in offspring (Vidal et al., 2015); and preliminary evidence suggests that *in utero* exposure to Mn is associated with defined placental DNA methylation patterns (Maccani et al., 2015). However, very few studies have reported such differential DNA methylation at an epigenome-wide level in newborns whose mothers were exposed to the heavy metals, particularly those originated from e-waste during pregnancy. In the present study, we applied the Infinium HumanMethylation450 Beadchip (450 K; Illumina Inc.) measuring CpG methylation at > 470,000 CpGs to investigate differential DNA methylation patterns of neonatal umbilical cord blood (UCB) between the e-waste exposed group and the reference group. We further explored two differential methylation of CpG sites selected from the microarray in neonatal UCB by bisulfite pyrosequencing. These two CpGs are respectively mapped to genes expressing brain-specific angiogenesis inhibitor 1 (*BAI1*) and Catenin cadherin-associated protein, alpha 2 (*CTNNA2*) which involve in neuron differentiation and development. Based on our results, this study may provide novel insights for maternal exposure to heavy metals (particularly lead) on toxicity health risks of neonatal brain neuron development in an epigenetic-modified way.

2. Materials and methods

2.1. Study areas and subjects

A total of 939 healthy pregnant women was recruited shortly after delivery from June 2011 to September 2012. In brief, five hundred and ninety-three participants living in Guiyu, China were defined as the e-waste exposed group. Guiyu is one of the largest electronic waste sites and known for more than 30-year history of informal e-waste recycling. We considered the other 346 participants living in a region called Haojiang, approximately 31.6 km to the east of Guiyu, without any e-waste recycling activities as the reference group. The two regions share a similar population density and traffic conditions, and the local residents have a similar lifestyle, cultural background and socioeconomic status, as described in our previous studies (Zeng et al., 2017). Neonatal UCB samples were collected into EDTA-K2 anticoagulant tubes shortly after delivery and frozen at −80 °C until analysis. All recruited pregnant women were requested to complete a detailed questionnaire involving information covering maternal age, height and weight, parity, gestational age, maternal smoking and alcohol drinking, family member smoking during pregnancy, pregnancy complications through face-to-face interviews guided by trained research staff. Neonatal birth information including gender, fetal number, birth body mass index (BMI), birth complications and defects were obtained from hospital records. To evaluate the epigenome-wide DNA methylation patterns of newborns, twenty-four neonatal UCB samples (twelve from the e-waste exposed group and twelve from the reference group) were selected from this large population: all samples in the e-waste exposed group with Pb levels over 10 μg/dL and less than 5 μg/dL in the reference group; pregnant woman who had ever smoked, drank alcohol and with birth complications was excluded; newborn was single birth with no birth defects. To validate the differential DNA methylation patterns, two hundred and four neonatal UCB samples (101 from the e-waste exposed group and 103 from the reference group) were also included from this large population with no maternal smoking during pregnancy, single birth, and no birth complications and defects. All subjects gave informed consent and the study was conducted according to the approved guidelines. The protocol was approved by the Ethics Committee of Shantou University Medical College. The work in this study are in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.2. Assessment of heavy metal exposures

Measurements of neonatal UCB Pb, Cd, Mn and Cr concentrations by graphite furnace atomic absorption spectrophotometry (GFAAS) were performed in the Laboratory of Environmental Medicine and Developmental Toxicology at Shantou University Medical College. The procedure for blood sample pretreatment and details of the GFAAS analyses have previously been described in detail elsewhere (Zeng et al., 2016b).

2.3. Epigenome-wide methylation analysis

Twenty-four neonatal UCB samples were selected for the Infinium Human Methylation 450 K Beadchip analysis. For these 24 neonatal UCB samples, genomic DNA was extracted with the DNeasy Blood and Tissue Kit according to the manufacturer's instruction. DNA quality was assessed with the NanoDrop spectrophotometer (NanoDrop Products) (A260/A280 > 1.8 & < 2.1) and also controlled by agarose gel electrophoresis (no dispersion DNA fragment band). One microgram qualified DNA (50 ng/μL) of each UCB sample was bisulfite-converted by EZ DNA Methylation kit (Zymo Research Corporation, Irvine, CA) following manufacturer instructions. The methylation status covering 485,577 CpGs in the human genome was assessed on 500 ng of bisulfite-converted DNA using the Human Methylation 450 K BeadChip according to the Infinium HD Methylation Assay protocol. In addition, the 24 samples were equally loaded in the Methylation Beadchip randomly distributed in order to prevent batch or localization effects. Assays were scanned to generate data (.idat files) for each human subject by Illumina's GenomeStudio and the data (.idat files) were preprocessed using the R (2.15.3) /Bioconductor package [minfi] (The R Project for Statistical Computing, Auckland, New Zealand). Finally, a β-value between 0 (completely un-methylated) and 1 (completely methylated) was generated after preprocessing both control normalization and background subtraction by using Illumina's algorithm, and used to assess the methylation level at each CpG site. To eliminate the sex-specific methylation bias, all CpG loci on sex chromosomes (X and Y) were excluded in analysis (Bibikova et al., 2011; Hansen and Aryee, 2013; Wang et al., 2012).

Bioinformatics analysis was carried out using the Illumina methylation analyzer (IMA) package: a pooled *t*-test, and a filter of absolute values ≥0.14 in average beta difference were employed to identify differential methylation of CpG sites between the e-waste exposed group and the reference group; a *P* value of ≤0.05 was applied as a cutoff; CpG sites with average beta difference ≥0.14 were regarded as hypermethylated & ≤-0.14 being hypomethylated (Wang et al., 2012). A chromosomal distribution graph was generated from R package [minfi]. Hierarchical clustering analysis of the differential methylation of CpGs was visualized through a heat-map using the Multi Experiment Viewer (MEV-4.6.0) Software. The Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) was adopted for gene ontology (GO) and pathway enrichment analysis to portray the

differential methylation patterns between the e-waste exposed group and the reference group.

2.4. Bisulfite pyrosequencing analysis

For the differential methylation of CpGs of interest screened from the microarray, bisulfite pyrosequencing was applied to validate in a total of 204 enrolled UCB samples (101 from the e-waste exposed group and 103 from the reference group). After DNA quality control, qualified DNA were bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). The original sequences of the genes of interest with differential methylation of CpGs were obtained from the microarray. The PCR amplifying and pyrosequencing primers were designed for the sequences by PyroMark Assay Design v2.0 software (Qiagen, Hilden, Germany). The primer sequences for the two CpG sites are shown in Table S1. Bisulfite-converted DNA (1 μg) was amplified using Hot-Start Taq-polymerase, then the amplicons were analyzed by PyroMark Q96 instrument (Qiagen) according to the manufacturer's instructions, and finally the methylation level of each CpG site was quantified using a percentage of C (methylated Cytosine) to C + T (methylated Cytosine + unmethylated Cytosine).

2.5. Statistical analysis

The Kolmogorov-Smirnov test was used to explore the normal distribution of all data. The central tendency and spread of variables were described by the mean ± standard deviation and as the median [interquartile range (IQR)] for skewed distribution. The composition ratio of variables was presented by percentage. The independent-sample *t*-test and Mann-Whitney *U*-test were used to determine the difference between two groups, while the chi-square test was utilized for determining the difference of composition ratio. Spearman's rank correlation analysis was performed to analyze the relationships between the differential methylation of two CpGs and heavy metal levels. Multiple linear regression analyses adjusted for confounding factors (e.g. maternal age, maternal pre-pregnant BMI, family member smoking during pregnancy, gestational age, neonatal gender and birth BMI) were applied to investigate the associations between the differential methylation of two CpGs and heavy metal levels in neonatal UCB. Statistical significance was set as $P < 0.05$ for a two-tailed test. Statistical analyses were performed using SPSS 20.0 for Windows (Chicago, IL, USA).

3. Results

3.1. Demographic characteristics and heavy metal levels of neonatal UCB from the e-waste exposed group and the reference group

A total of 101 pregnant women from Guiyu and 103 pregnant women from Haojiang were enrolled and provided umbilical cord blood for the bisulfite pyrosequencing analysis (Table 1). Maternal age at enrollment ranged from 14 to 41 years old with a mean age ± standard derivation of 27.3 ± 4.5 and 28.0 ± 4.9 years, respectively for the e-waste exposed and the reference group. No significant difference was observed in maternal age, maternal pre-pregnant BMI, family member smoking during pregnancy, maternal drinking alcohol during pregnancy and sex ratio of newborns between the two groups. Gestational age in e-waste exposed group was slightly increased ($P < 0.05$). Neonatal birth BMI were showed a significant difference ($P < 0.05$). Neonatal UCB lead levels in the e-waste exposed group (7.34 ± 2.69 μg/dL) were over 2 times higher than the reference group (3.07 ± 0.84 μg/dL) ($P < 0.001$). Neonatal UCB Cd, Mn and Cr levels did not show significant difference between the two groups. As regards the twenty-four neonatal UCB samples of epigenome-wide analysis, newborns from both groups were matched well according to their maternal age, pre-pregnant BMI, passive smoking status, and neonatal gender distribution and birth BMI. Neonatal UCB Pb levels in the e-waste exposed group (15.894 ± 3.770 μg/dL) was approximately ten times higher than in the reference group (1.795 ± 0.410 μg/dL); significantly higher neonatal UCB Mn (but not Cd or Cr) levels were found in the e-waste exposed group than the reference group, which also indicated a slightly higher Mn exposure *in utero* from the e-waste exposed group (Table S2).

Table 1
Demographic characteristics and heavy metal levels of neonatal UCB from the e-waste exposed group and the reference group in bisulfite pyrosequencing analysis (n = 204).

Variable	Exposed group (n = 101)	Reference group (n = 103)	*P*-value
Maternal characteristics			
Age (years)	27.3 ± 4.5	28.0 ± 4.9	0.273[a]
Pre-pregnant BMI (kg/m^2)	20.05 ± 2.50	19.99 ± 2.50	0.884[a]
Gestational age (weeks)	39.93 ± 0.84	39.47 ± 1.68	0.021[a]
Smoking during pregnancy [n (%)]			
Yes	0 (0)	0 (0)	
No	99 (100)	87 (100)	
Family member smoking during pregnancy [n (%)]			0.224[b]
Yes	54 (61.36)	45 (51.72)	
No	34 (38.64)	42 (48.28)	
Drinking alcohol during pregnancy [n (%)]			0.602[b]
Yes	1 (1.02)	2 (2.30)	
No	97 (98.98)	85 (97.70)	
Newborn characteristics			
Gender [n (%)]			0.778[b]
Male	55 (55.00)	53 (52.48)	
Female	45 (45.00)	48 (47.52)	
Birth BMI (kg/m^2)	12.21 ± 1.24	13.00 ± 2.59	0.006[a]
Lead levels (μg/dL)	7.34 ± 2.69	3.07 ± 0.84	< 0.001[a]
Cadmium levels (μg/L)	0.22 ± 0.21	0.29 ± 0.51	0.187[a]
Manganese levels (μg/L)	54.01 ± 21.88	51.21 ± 16.43	0.302[a]
Chromium levels (μg/L)	5.90 ± 3.30	6.23 ± 5.92	0.631[a]

The values are expressed as mean ± SD or percentage; *P*-value, statistical significance of the differences between two groups.
[a] Independent-sample *t*-test.
[b] Chi-square test.

3.2. Differential DNA methylation patterns between the e-waste exposed group and the reference group

We conducted a high-throughput platform of epigenome-wide DNA methylation analysis to explore the differential DNA methylation patterns in neonatal UCB between the e-waste exposed group and the reference group. After analysis of the microarray, a total of 125 CpG sites were differential methylation (46 were hypermethylated and 79 were hypomethylated) in the e-waste exposed group compared with the reference group ($P < 0.05$). The specific positions of these differential methylation of CpGs on human 22 chromosomes were presented in Fig. 1. No certain chromosomes enriched suggests that the effects of maternal heavy metal exposures on DNA methylation are non-specific for chromosomes. Fig. 2 shows the heatmap concerning hierarchical clustering analysis for the differential methylation of CpGs in 24 samples (12 in front from the reference group and 12 behind from the e-waste exposed group). More hypomethylated CpGs and less hypermethylated CpGs were observed in the e-waste exposed group than in the reference group. These differential methylation of CpGs were mapped to 79 genes (see Tables 2(A) and 2(B)). Among them, 31 were hypermethylated (see Table 2(A)) and 48 were hypomethylated (see Table 2(B)) (the complete details of these genes are shown in Table S3). In addition, of the differential methylation of 79 genes, several genes are involved in differentiation and neuron development, including the gen es expressing brain-specific angiogenesis inhibitor 1 (*BAI1*) and Catenin cadherin-associated protein, alpha 2 (*CTNNA2*) (Tables 2(A) and 2(B), Table S3 and Fig. 3 (B)).

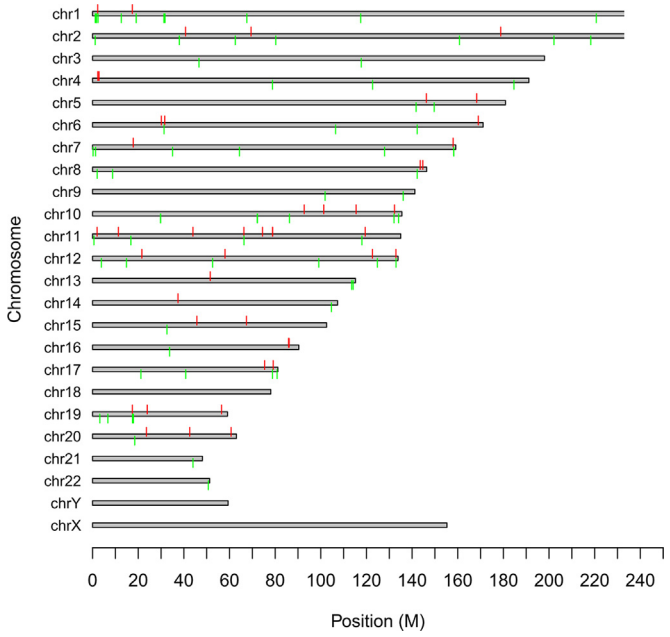

Fig. 1. Chromosomal distribution of the differential methylation of 125 CpG sites between e-waste exposed group and reference group. Little red bars represent hyper-methylated sites, while little green bars are hypo-methylated sites (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

3.3. Functional annotation clustering (FAC) analyses

In order to gain a deeper insight into biological functions of these differential methylation of CpG sites and their associated genes, we conducted functional annotation clustering (FAC) analyses by David Database (6.7, http://david.abcc.ncifcrf.gov/home.jsp). Clusters are enriched through selecting the overrepresented annotation that convey the broadest biologic meaning within each FAC. In general, a smaller *P* value accompanies a more meaningful pathway enrichment cluster which is also more likely to occur abnormally (Uddin et al., 2010). We uploaded the mapped differential methylation of 79 genes (selected "OFFCIAL_ GENE_SYMBOL") as a gene list into DAVID and adopted functional annotation tool (Default setting and Classification Stringency: Medium). Fig. 4 shows the main results of the gene ontology (GO) functional enrichment analysis among the differential methylation of genes between the e-waste exposed and the reference neonates ($P < 0.05$). These results indicate that these differential genes mainly participate in biological processes including regulation of cell adhesion (Cluster 1, e.g. *TDGF1*, *SMAD3*, *NID1*), transmembrane transport (Cluster 4), cell morphogenesis involved in differentiation and neuron development (Cluster 12, e.g. *BAI1, SLIT3, CTNNA2, TGFBR1, ANTXR1*), regulation of apoptosis (Cluster 13), cation transport (Cluster 23); they also have some molecular functions in antiporter activity (Cluster 4, e.g. *SLC8A1*, *SLC26A10*, *TMCO3*) and calcium/metal ion binding (Cluster 6, e.g. *SMOC2*, *SLC8A1*, *GALNTL4*, *SVIL*, *FAM20C*, *PADI2*, *NID1*, *ACTN3*, *SDF4*, *EFCAB4B*, *SLIT3*), as well as in comprising of some cellular components of plasma membrane (Cluster 14, 15) and actin cytoskeleton (Cluster 15, e.g. *MYOM2*, *SVIL*, *ACTN3*, *SEPT9*, *CTNNA2*) and mitochondrion (Cluster 22) (the complete set of all FAC analyses is shown in Table S3). Fig. 3 (A) illustrates the most significant enrichment cluster, and Fig. 3 (B) shows the most critical enrichment cluster of our interest, which contain the gene functional annotations and differential genes. The results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) and BIOCARTA pathway functional enrichment analysis indicates that these differential methylation of genes are involved in several biological pathways, including the KEGG pathway of Adherens junction (*TGFBR1, SMAD3, ACTN3, CTNNA2*) ($P < 0.01$),

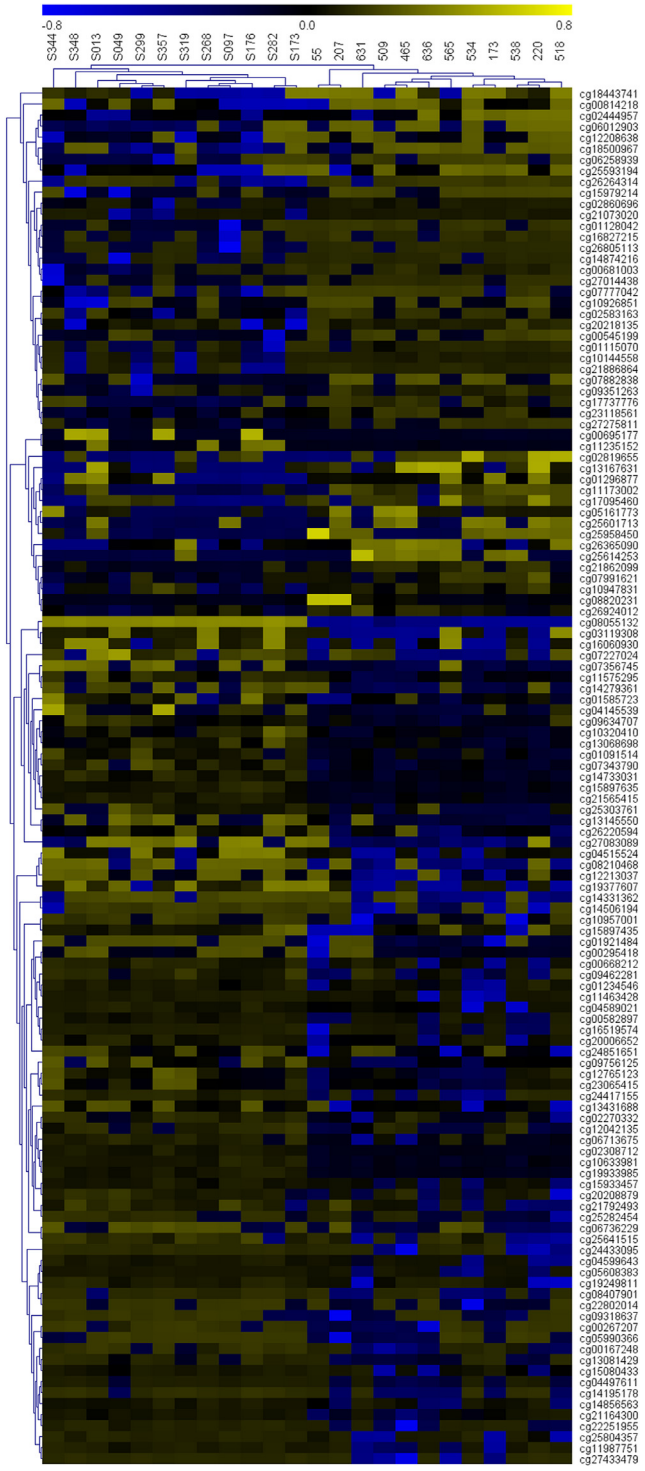

Fig. 2. Hierarchical clustering (Euclidean distance) heat map including the significant differential methylation of CpG sites between reference (12 samples in front) and exposed newborns (12 samples behind). Each colored rectangular box from light blue to light yellow represents that the methylation level of each site is from hypo- to hyper-methylated (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Amyotrophic lateral sclerosis (ALS) (*MAP2K3, CCS, APAF1*) ($P < 0.05$) as well as BIOCARTA pathway including NFkB activation by Nontypeable Hemophilus influenzae (*MAP2K3, TGFBR1, SMAD3*) ($P < 0.05$), TGF beta signaling pathway, and Role of Mitochondria in Apoptotic Signaling, Apoptotic Signaling in Response to DNA Damage and the

Table 2(A)
Differential hypermethylation of genes (31) of newborns between the e-waste exposed group and the reference group ($P < 0.05$).

CpG site	CHR	UCSC_REFGENE_NAME	UCSC_REFGENE_GROUP	Beta. Diff.	*P*-value	CpG site	CHR	UCSC_REFGENE_NAME	UCSC_REFGENE_GROUP	Beta. Diff.	*P*-value
cg21862099	20			0.15	1.67E-03	cg07882838	15	*SMAD3*	Body; TSS1500	0.21	2.64E-02
cg11173002	5	*SLIT3*	Body	0.26	2.68E-03	cg09351263	16			0.15	2.70E-02
cg26924012	15	*SPATA5L1*	TSS1500	0.15	2.78E-03	cg18443741	12	*PYROXD1*	Body	0.34	2.85E-02
cg25958450	19	*ZNF681*	TSS200	0.33	3.93E-03	cg05161773	17	*SEPT9*	5'UTR; Body	0.22	2.96E-02
cg25614253	8	*BAI1*	Body	0.31	4.64E-03	cg25601713	10			0.25	3.09E-02
cg23118561	13			0.14	5.44E-03	cg07777042	8			0.23	3.17E-02
cg27014438	6	*BAT3*	Body	0.21	6.29E-03	cg14874216	12	*LRRC43*	TSS1500	0.19	3.17E-02
cg01128042	10	*CASP7*	Body	0.23	7.00E-03	cg21073020	6	*SMOC2*	Body	0.16	3.17E-02
cg07991621	4	*SH3BP2*	Body; 5'UTR; TSS200	0.15	8.56E-03	cg02583163	10			0.18	3.24E-02
cg21886864	11	*ODZ4*	5'UTR	0.24	1.06E-02	cg00814218	14	*SLC25A21*	Body	0.31	3.34E-02
cg13167631	2	*SLC8A1*	TSS200	0.35	1.09E-02	cg20218135	19	*ABHD8*	Body	0.22	3.46E-02
cg15979214	6	*TRIM10*	Body	0.27	1.18E-02	cg00681003	20	*CST9*	5'UTR;1stExon	0.17	3.54E-02
cg27275811	11			0.14	1.25E-02	cg01115070	11			0.17	3.67E-02
cg10144558	11	*ODZ4*	5'UTR	0.19	1.27E-02	cg18500967	7			0.26	3.75E-02
cg17737776	17	*AATK*	Body	0.18	1.27E-02	cg26365090	20	*TOX2*	5'UTR; Body; TSS200	0.25	4.21E-02
cg02444957	2	*ANTXR1*	Body	0.25	1.42E-02	cg08820231	12	*SLC26A10*	TSS200	0.16	4.25E-02
cg17095460	11			0.28	1.56E-02	cg06258939	11			0.19	4.31E-02
cg00545199	4	*ZFYVE28*	Body	0.2	1.70E-02	cg12208638	11	*ACTN3*	Body	0.23	4.54E-02
cg02860696	12			0.14	1.80E-02	cg16827215	11	*GALNTL4*	3'UTR	0.17	4.66E-02
cg26805113	16			0.21	1.84E-02	cg01296877	1	*MORN1*	Body	0.24	4.69E-02
cg26264314	19	*NLRP5*	TSS200	0.26	1.93E-02	cg10926851	5	*PPP2R2B*	Body; 5'UTR	0.2	4.69E-02
cg10947831	10			0.16	2.27E-02	cg25593194	1	*PADI2*	3'UTR	0.27	4.74E-02
cg02819655	2	*PDE11A*	Body	0.31	2.30E-02	cg06012903	7	*PTPRN2*	Body	0.24	4.76E-02

Caspase Cascade in Apoptosis (Table S4).

3.4. Bisulfite pyrosequencing validation

We performed the bisulfite pyrosequencing experiment for 204 neonatal UCB samples to verify the differential methylation of CpG sites identified by Human 450 K microarray analysis. The median methylation level of *BAI1* (cg25614253) in the e-waste exposed group was 8.00% (6.00%, 44.00%) which was higher than the reference group (7.00% (6.00%, 37.75%), $P < 0.05$). However, a lower median methylation level of *CTNNA2* (cg20208879) was observed in the e-waste exposed group than in the reference group [62.00% (47.00%, 67.00%) vs. 64.00% (59.25%, 69.00%), $P < 0.05$], which were respectively shown in Figs. 5A and 5B.

3.5. Associations between heavy metal exposures and differential methylations in neonatal UCB for bisulfite pyrosequencing validation

The results of Spearman's rank correlation analysis were presented in Table 3. It indicated a positive correlation between neonatal UCB Mn levels and Cr levels ($P < 0.05$), while the neonatal UCB Pb levels were inversely correlated with Cd levels ($P < 0.01$). For the CpG site (cg25614253) of *BAI1*, a positive correlation was observed between UCB Pb levels and its methylation level ($P < 0.05$). However, we noticed UCB Pb levels were negatively associated with *CTNNA*2 (cg20208879) methylation level ($P < 0.01$). There was no significant correlation between neonatal UCB Cd, Mn and Cr levels and the methylation levels of the two CpGs. Multiple linear regression models were applied to assess the associations between heavy metal exposures and differential methylations in neonatal UCB samples, which were adjusted for maternal age, pre-pregnant BMI and alcohol drinking, gestational age, family member smoking during pregnancy, neonatal gender and birth BMI (Table 4). For the CpG site (cg20208879) of *CTNNA2*, an increase in UCB Pb levels was associated with a 1.20 (β = -1.297, 95% CI, −2.135 to −0.265) decrease in methylation level of neonatal UCB.

4. Discussion

This study explored the effects of maternal exposure to a variety of environmental heavy metals originating from e-waste during pregnancy, revealing differential DNA methylation patterns at an epigenome-wide level in UCB of newborns prenatally exposed to e-waste pollutions. Through Human 450 K Beadchip analysis, we identified 125 CpGs with differential methylation which are mainly involved in multiple biological processes including calcium ion binding, cell adhesion, embryonic morphogenesis, as well as in signaling pathways related to NFkB activation, adherens junction, TGF beta and apoptosis. Furthermore, two differential methylation of CpG sites of *BAI1* and *CTNNA2* genes involved in brain neuronal development identified from the microarray were validated. Both of them were correlated to neonatal UCB Pb levels.

We selected the UCB samples for Human 450 K methylation BeadChip analysis mainly according to their corresponding Pb concentrations in each group. UCB Pb concentration in e-waste polluted area, Guiyu was 15.894 μg/dL, which was nearly 9 times higher than in the reference group (1.795 μg/dL). From previous studies, we knew that child blood Pb concentration higher than the reference level of 5 μg/dL recommended by U.S. Centers for Disease Control and Prevention (CDC) need to trigger interventions (Betts, 2012); in addition, Pb exposure levels exceeding the concentration of 10 μg/dL could result in the impairment of neurodevelopment, impose adverse effects on cognitive function, and cause behavioral disturbances, as well as attention deficits in early childhood (Zeng et al., 2016a). Our previous studies conducted in the e-waste recycling area confirmed that higher UCB Pb levels in neonates were associated with lower neonatal behavioral

Table 2(B)
Differential hypomethylation of genes (48) of newborns between the e-waste exposed group and the reference group ($P < 0.05$).

CpG site	CHR	UCSC_REFGENE_NAME	UCSC_REFGENE_GROUP	Beta. Diff.	*P*-value	CpG site	CHR	UCSC_REFGENE_NAME	UCSC_REFGENE_GROUP	Beta. Diff.	*P*-value
cg08055132	1			− 0.87	1.83E-26	cg15933457	2			− 0.14	1.43E-02
cg02308712	12			− 0.15	1.31E-14	cg00167248	3			− 0.20	1.51E-02
cg19933985	5			− 0.15	3.06E-14	cg12213037	1	*SLC35E2*	Body	− 0.28	1.53E-02
cg10633981	11	*C11orf58*	3'UTR	-0.17	8.86E-13	cg13431688	9	*TGFBR1*	Body	− 0.24	1.66E-02
cg15897635	1			− 0.16	6.70E-11	cg25303761	1			− 0.15	1.72E-02
cg21565415	11	*MUPCDH*	Body	− 0.16	7.18E-10	cg12042135	7	*FAM20C*	Body	− 0.14	1.97E-02
cg14733031	19	*B3GNT3*	TSS1500	− 0.18	6.12E-09	cg14856563	1			− 0.14	2.06E-02
cg07343790	13	*ATP11A*	Body	− 0.20	3.78E-05	cg09756125	7	*PTPRN2*	Body	− 0.17	2.07E-02
cg13068698	7	*DPY19L1*	TSS1500	− 0.15	1.59E-04	cg04599643	20	*RBBP9*	TSS1500	− 0.16	2.30E-02
cg01091514	4			− 0.15	6.81E-04	cg05608383	13	*TMCO3*	Body	− 0.15	2.35E-02
cg10320410	7			− 0.15	1.01E-03	cg11235152	1			− 0.15	2.39E-02
cg09634707	10			− 0.14	1.13E-03	cg20006652	6			− 0.16	2.48E-02
cg24417155	17			− 0.24	3.41E-03	cg06736229	1			− 0.22	2.58E-02
cg06713675	4	*EXOSC9*	TSS1500	− 0.18	3.62E-03	cg08407901	21	*SLC37A1*	Body	− 0.19	2.68E-02
cg08210468	2			− 0.39	3.69E-03	cg20208879	2	*CTNNA2*	Body	− 0.17	2.90E-02
cg04497611	17	*TBCD*	Body	− 0.15	4.34E-03	cg00695177	8			− 0.21	3.09E-02
cg12765123	10			− 0.18	4.51E-03	cg07227024	2	*ALS2CR12*	Body	− 0.27	3.17E-02
cg21164300	9			− 0.14	5.07E-03	cg00267207	12	*GUCY2C*	TSS200	− 0.20	3.45E-02
cg07356745	10	*FAM190B*	Body	− 0.28	5.55E-03	cg10957001	12	*APAF1*	Body	− 0.22	3.50E-02
cg14195178	17	*TBCD*	Body	− 0.23	6.02E-03	cg04515524	19	*PLVAP*	TSS1500	− 0.25	3.56E-02
cg03119308	7	*RBM28*	3'UTR	− 0.33	7.21E-03	cg25641515	12			− 0.21	3.58E-02
cg11575295	7	*PTPRN2*	Body	− 0.14	7.31E-03	cg01234546	22	*PLXNB2*	Body	− 0.15	3.64E-02
cg13145550	7			− 0.23	7.84E-03	cg14331362	2			− 0.26	3.87E-02
cg09462281	19	*NCLN*	Body	− 0.19	8.52E-03	cg00295418	8	*MYOM2*	Body	− 0.18	3.89E-02
cg00668212	10	*STK32C*	Body	− 0.20	9.82E-03	cg19249811	10	*SVIL*	Body	− 0.20	4.01E-02
cg27083089	6	*HLA-B*	3'UTR	− 0.34	9.94E-03	cg24851651	11	*CCS*	Body	− 0.21	4.02E-02
cg01921484	8	*SLC45A4*	TSS1500	− 0.29	1.03E-02	cg15897435	4	*MRPL1*	Body	− 0.22	4.07E-02
cg22251955	11	*SCN4B*	Body	− 0.22	1.09E-02	cg04145539	3	*LRRC2; TDGF1*	5'UTR; TSS1500	− 0.18	4.11E-02
cg24433095	12	*EFCAB4B*	5'UTR	− 0.27	1.09E-02	cg11463428	15			− 0.18	4.14E-02
cg19377607	10	*LRRC20*	5'UTR	− 0.34	1.10E-02	cg25282454	1	*SDF4*	Body	− 0.15	4.16E-02
cg23065415	10			− 0.15	1.10E-02	cg22802014	1	*SNRNP40*	3'UTR	− 0.19	4.30E-02
cg16060930	1	*PTGFRN*	Body	− 0.33	1.11E-02	cg09318637	17	*RPTOR*	Body	− 0.20	4.31E-02
cg11987751	1	*SLC35E2*	3′UTR	− 0.19	1.12E-02	cg26220594	1			− 0.14	4.35E-02
cg00582897	5			− 0.14	1.19E-02	cg14506194	2	*DIRC3*	Body	− 0.25	4.40E-02
cg02270332	6			− 0.17	1.20E-02	cg14279361	19	*C3*	TSS1500	− 0.20	4.46E-02
cg05990366	12	*FAM101A*	TSS200	− 0.24	1.27E-02	cg01585723	16			− 0.17	4.53E-02
cg16519574	2	*SNTG2*	Body	− 0.19	1.27E-02	cg21792493	14			− 0.16	4.64E-02
cg25804357	10	*MIR604; SVIL*	TSS1500; Body	− 0.23	1.27E-02	cg04589021	17	*MAP2K3*	Body; TSS1500	− 0.16	4.85E-02
cg27433479	1	*NADK*	Body	− 0.26	1.28E-02	cg15080433	2	*LY75*	Body	− 0.14	4.85E-02
cg13081429	1	*NID1*	Body	− 0.15	1.33E-02						

CHR: Chromosome containing the CpG (Build 37).
UCSC_RefGene_Name: Target gene name(s), from the UCSC database. (Note: multiple listings of the same gene name indicate splice variants.).
UCSC_RefGene_Group: Gene region feature category describing the CpG position, from UCSC. (Features listed in the same order as the target gene transcripts.).
TSS200: Upstream of transcription start site (TSS) ranged from 0 to 200 bp.
TSS1500: Upstream of transcription start site (TSS) ranged from 200 to 1500 bp.
5′UTR: 5′-untranslated regions ranged between TSS and ATG.
Body: Ranged from ATG to stop codon.
3′UTR: 3′-untranslated regions ranged between stop codon and poly A tails.
Beta. Diff: Beta value differences between two groups.

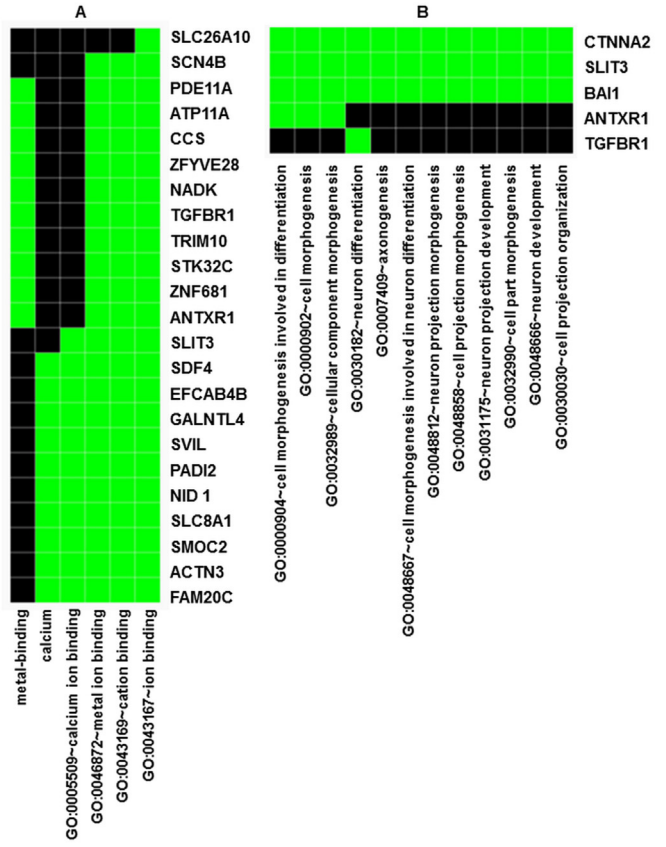

Fig. 3. Sample functional annotation cluster (FAC) heat maps between the e-waste exposed group and the reference group. Displayed are the differential genes and associated annotations for FAC cluster 6 (A) (most significant enrichment cluster) and FAC cluster 12 (B) (most critical enrichment cluster of our interest) determined from the identified differential methylation of gene sets. Each green square block represents a corresponding gene-term association positively reported, while each black square block is a corresponding gene-term association not reported yet. Clusters were enriched through selecting the overrepresented annotation that conveyed the broadest biologic meaning within each FAC (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

neurological assessment (NBNA) scores (Li et al., 2008a, 2008b). Several other studies indicated that high concentrations of Pb exposure during early life could pose serious poisonous effects on brain neurodevelopment by changing its constructions and functions (Bellinger, 2013; Kuehn, 2012; Toscano and Guilarte, 2005). Lots of environmental events (such as pollutants exposure) in early life could involve in changes of epigenetic marks, such as DNA methylation and histone modification, which lead to some possible links between heritable changes in gene expression and adult disease susceptibility and development (Marsit, 2015). Thus, prenatal exposure to environmental chemicals may also be in this manner interacting with the epigenome of newborns which was associated with the outcomes of birth defects or health risk in childhood. Senut et al. (2014) found that genes involved in neurogenesis such as *EFNA2*, *GRIK4*, the motor neuron specification factor *LHX3*, the axonal guidance *PLXN4*, and the neuronal differentiation factor *NEUROG1* were differentially methylated in Pb-exposed differentiating human embryonic stem cells. Sen et al. (2015) identified two genes, *NDRG4* (associated with reduced levels of Brain Derived Neurotrophic Factor (BDNF) in mouse) and *NINJ2* (Nerve Injury Induced Protein 2, involved in regulation of injured sensory and enteric neurons) showed Pb dependent change in DNA methylation status in child's neonatal blood spots exposed in-utero to high Blood Pb levels (≥5 μ g/dL). In the current study, we observed the differential methylation of genes including *TGFBR1*, *BAI1*, *SLIT3*, *ANTXR1, CTNNA2* which were involved in biological processes of axonogenesis, cell morphogenesis involved in neuro-differentiation, neuron projection morphogenesis and neuron development after GO enrichment analysis. However, our study did not show any overlapped differential methylation of genes on neuronal development with their research. As a matter of fact, we yet assessed other heavy metal levels after Human 450 K methylation BeadChip analysis in the present study. We observed the significantly higher concentrations of Mn in the e-waste exposed group (61.62 μg/L) than in the reference group (42.81 μg/L). Recently, prenatal Mn exposure was reported to be linked with the childhood behavioral disinhibition in a population-based study (Tarale et al., 2016). Mn exposure in placenta tissue is associated with some DNA methylation changed genes, such as *CNP*, *EN1*, *ROBO3*, *ZNRF2*, *NEUROD* etc. involved in neuro development and neurogenesis (Maccani et al., 2015). It may be the differential methylation of genes identified from this study are products of mixed effects after prenatal exposure to environmental heavy metals, although we also found different neuro-differentiation, -morphogenesis and -development related differential methylation of genes.

Furthermore, we selected two of them (*BAI1* and *CTNNA2*) to validate the differential methylations identified from the microarray by using bisulfite pyrosequencing in 204 neonatal UCB samples. From the previous studies (Mori et al., 2002; Nishimori et al., 1997; Paavola and Hall, 2012), they confirmed that *BAI1* is highly and specifically expressed in the normal brain. *BAI1* participates in many biological processes including signal transduction, cell adhesion, release of neurotransmitters and synapse formation. *CTNNA2* is a large (about 1 Mb) and conserved gene on chromosome 2. This gene encodes αN-catenin, a sort of cell-adhesion protein served as critical regulator of synaptic plasticity through binding with cadherins, actin cytoskeleton and a brain-expressed a-catenin essential for synaptic contact (Abe et al., 2004; Smith et al., 2005; Terracciano et al., 2011). In the current study, both Spearman's rank correlation analysis and linear regression models confirmed that UCB Pb levels were negatively associated with the hypomethylated CpG site (cg20208879) of *CTNNA2*, while correlation analysis identified a significantly positive correlation between hypermethylated CpG site (cg25614253) of *BAI1* and UCB Pb levels. In addition, it is showed that only the Pb concentration (not Cd, Mn or Cr) of UCB samples from the e-waste exposed group was significantly higher than in the reference group. These data suggest that maternal exposure to Pb through e-waste recycling may be associated with the changes in neonatal UCB of DNA methylation in genes involved in

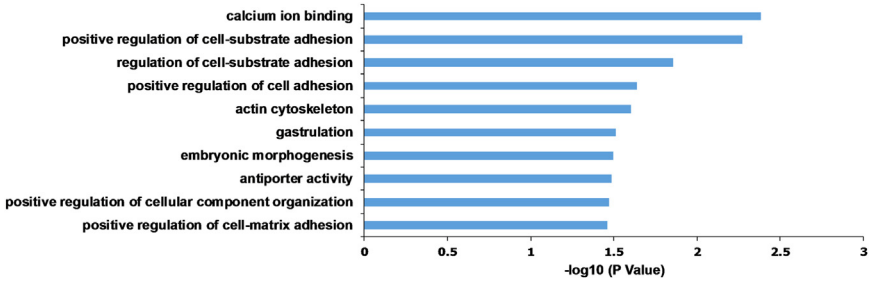

Fig. 4. Gene ontology (GO) functional enrichment analysis of differential methylation of genes between e-waste exposed newborns and reference newborns. Y axis represents differential gene ontology annotation terms, while X axis being the values of -log10 (*P* values) ($P < 0.05$). The smaller *P* value accompanied with gene sets enriches in a more meaningful GO term.

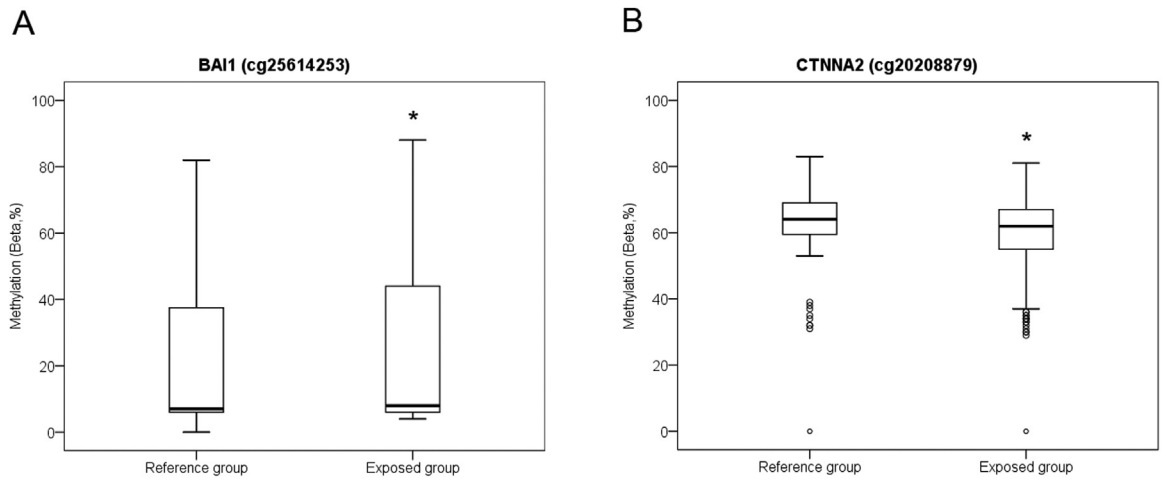

Fig. 5. Validation of *BAI1* (cg25614253) (A) and *CTNNA2* (cg20208879) (B) by bisulfite pyrosequencing in 204 neonatal UCB. Mann-Whitney *U* tests were used for comparisons of DNA methylation levels measured by pyrosequencing at each candidate CpG site between the e-waste exposed group and the reference group. *$P < 0.05$.

Table 3
Spearman's correlation between heavy metal levels and methylation levels of *BAI1* (cg25614253) and *CTNNA2* (cg20208879).

	UCB Pb level	UCB Cd level	UCB Mn level	UCB Cr level	*BAI1* methylation	*CTNNA2* methylation
UCB Pb level	1.000	–	–	–	–	–
UCB Cd level	−0.219**	1.000	–	–	–	–
UCB Mn level	0.109	−0.096	1.000	–	–	–
UCB Cr level	0.042	0.024	0.171*	1.000	–	–
BAI1 methylation	0.162*	0.098	−0.035	0.035	1.000	–
CTNNA2 methylation	−0.191**	−0.022	0.019	−0.039	−0.013	1.000

* $P < 0.05$.
** $P < 0.01$.

Table 4
Multiple linear regression analysis[a] for associations between heavy metal levels and two differentially-methylated genes in neonatal UCB.

	BAI1 methylation (cg25614253) β (95% CI)	*CTNNA2* methylation (cg20208879) β (95% CI)
UCB Pb	−0.054 (−1.496, 1.388)	−1.200 (−2.135, −0.265)*
UCB Cd	−1.490 (−9.662, 6.683)	1.497 (−3.802, 6.796)
UCB Mn	−0.053 (−0.291, 0.186)	−0.001 (−0.155, 0.154)
UCB Cr	0.041 (−0.748, 0.831)	−0.141 (−0.653, 0.371)

* $P < 0.05$.
[a] Adjusted for maternal age, pre-pregnant BMI and drinking alcohol, gestational age, family member smoking during pregnancy, neonatal gender and birth BMI.

neural development pathways. This is also supported by previous research linking lead exposure to child neurodevelopment (Schneider et al., 2013). The results were also essentially consistent with the data from 450 K methylation BeadChip analysis, since we noticed higher Pb and Mn exposure with hypermethylated *BAI1* (cg25614253) and hypomethylated *CTNNA2* (cg20208879). However, the methylation differences between differential methylation of the two CpGs are far larger in 450 K methylation Beadchip analysis than in pyrosequencing analysis. It may be the outcomes of dose or mix effects of the heavy metals, since heavier Pb and Mn burden were noticed between the two groups in 450 K methylation Beadchip analysis than in pyrosequencing validation analysis.

In addition, more of other differential methylation of genes were also observed with higher concentrations of Pb and Mn in neonatal UCB from the e-waste exposed group after 450 K methylation BeadChip analysis. They were mainly involved in multiple biological processes as well as many signaling pathways which were related to toxicity mechanism of Pb and Mn in organism. It is reported that one of the critical aspects of Pb-caused damage in cellular physiology is that Pb can replace multiple polyvalent cations (such as calcium, zinc, and magnesium) in their binding sites. Diverse voltage- and ligand-gated ionic channels such as sodium (Na^+), calcium (Ca^{2+}) are susceptible to such interferences by Pb (Garza et al., 2006). Mn could interfere with the Ca^{2+}-activated ATP production through binding with Ca^{2+}-sensitive sites in mitochondrial metabolic enzymes with more affinity than the Ca^{2+} itself (Gunter et al., 2006). In this study, we found that the genes enriched in Annotation Cluster 6: molecular functions of calcium ion binding, Annotation Cluster 23, 28: biological processes of cation transport and metal ion transport and molecular functions of metal ion transmembrane transporter activity and zinc ion binding, were differentially methylated between the e-waste exposed group and the reference group. Pb could affect the expression, synthesis, and conformational maturation of many cell adhesion signaling molecules, such as the neuronal cell adhesion molecule (Breen and Regan, 1988; Davey and Breen, 1998). In the current study, we found the differential methylation of genes enriched in Annotation Cluster 1, 16: biological processes of positive regulation of cell-substrate adhesion and cell adhesion, and KEGG pathway of Adherens junction. Pb can pass through placenta barrier and accumulate in fetus which impair the growth and development of infant. The genes enriched in Annotation Cluster 13: biological processes of embryonic morphogenesis and in utero embryonic development, were observed differentially methylated in e-waste exposed UCB samples with higher Pb and Mn levels compared with the reference group. In addition, Pb is a genotoxic agent and can induce DNA damage and chromosome abnormalities (Cheng et al., 2012). Pb mainly concentrates in the mitochondria via inhibiting the calcium uptake from outside and promoting calcium release from inside

(Lidsky and Schneider, 2003; Parr and Harris, 1976). Pb is also able to promote the release of cytochrome C into the cytoplasm via opening the mitochondrial permeability-transition pore, finally activates apoptotic cell death (He et al., 2000). Mn could also induce oxidative stress to cause cell death via apoptosis. Mitochondria are an important cellular target in neurotoxicity of Mn. Exposure to Mn causes the release of cytochrome C from mitochondria followed by subsequent loss of mitochondrial electrical potential. Mitochondrial accumulation of manganese results in inhibiting the oxidative phosphorylation and generating the reactive oxygen species (Chtourou et al., 2011). In this study, the genes enriched in Annotation Cluster 9, 13, 22: biological processes of response to hypoxia and oxygen levels, regulation of apoptosis and programmed cell death; cellular components of mitochondrion, mitochondrial membrane and envelope; and BIOCARTA pathway including Role of mitochondria in apoptotic signaling, apoptotic signaling in response to DNA damage and caspase cascade in Apoptosis, were differentially methylated in neonatal UCB of e-waste exposed group with higher prenatal Pb and Mn exposures. Thus, these results indicated that maternal exposure to environmental heavy metals during pregnancy may be associated with the changes in neonatal UCB of DNA methylation in genes involved in their toxicities related mechanisms. In this regard, we think that these external environmental exposures from mothers during pregnancy may affect the DNA methylation patterns of newborn internal. Likely, the variability in methylation of peripheral blood might reflect the cellular response to stimulator, including environmental toxicants. However, future targeted mechanistic research needs to be conducted and validated in more of the above differential methylation of genes with more large-scale population.

Several limitations in current study should also be considered. The e-waste recycling area is a very complicated and mixed environment which we cannot evaluate all the other environmental pollutants, such as organic pollutants in limited amounts of UCB samples. This may weaken the effects explained by heavy metal exposures. Also, the limited sample size restricts the statistical power to seek for more concentrated and accurate differences in methylation between e-waste exposed groups and reference groups although we validated some results in a relatively enlarged population. In addition, the DNA collected to assess the methylation was extracted from the whole UCB of newborns, which is a mixture of multiple blood cell types, so the altered DNA methylation patterns associated with heavy metal exposures may only signify a variability in blood cell composition in this study. Finally, we investigated DNA methylation in peripheral blood which may not be the most relevant target tissue for neurodevelopmental outcomes. It might be the largest limitation in this study.

5. Conclusion

We identified differential methylation patterns in neonatal UCB between the e-waste exposed group and the reference group, since the pregnant women living in the e-waste recycling area were subjected to heavier burdens of heavy metal exposures during pregnancy. We validated differential methylation of two CpGs of *BAI1* and *CTNNA2* involved in brain neuronal development in newborns which were associated with the maternal Pb exposure. It provides us more evidences on the roles of epigenetic-modified changes in impairment of fetal development under maternal exposure to e-waste-originated heavy metals, particularly Pb. Future research aimed at further elucidating the potential roles of the differential methylation patterns in neonatal birth outcomes should be conducted, which will help us better understand interaction networks among maternal heavy metal exposures, epigenetic changes and the developmental origins of disease in newborns from an e-waste recycling area.

Acknowledgments

The authors would like to thank the pregnant women who participated in this study.

Funding

This work was supported by the National Natural Science Foundation of China (21577084, 21876065).

Conflicts of interest

The authors declare that they have no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2019.01.007.

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