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Epigenetic Analysis of Neurocognitive Development at 1 year of Age in a Community-Based Pregnancy Cohort

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This work is dedicated to the memory of Grant Somes, our mentor, colleague, and the founder of the CANDLE study, whose energy and scientific vision inspired and originated this project.

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Conflict of Interest Julia Krushkal, Laura Murphy, Frederick Palmer, J. Carolyn Graff, Thomas Sutter, Khyobeni Mozhui, Collin Hovinga, Fridtjof Thomas, Vicki Park, Frances Tylavsky, and Ronald Adkins declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study. No animal studies were carried out by the authors for this article.

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Abstract

Multiple studies show that molecular genetic changes and epigenetic modifications affect the risk of cognitive disability or impairment. However, the role of epigenetic variation in cognitive development of neurotypical young children remains largely unknown. Using data from a prospective, community-based study of mother-infant pairs, we investigated the association of DNA methylation patterns in neonatal umbilical cord blood with cognitive and language development at 1 year of age. No CpG loci achieved genome-wide significance, although a small number of weakly suggestive associations with Bayley-III Receptive Communication scales were noted. While umbilical cord blood is a convenient resource for genetic analyses of birth outcomes, our results do not provide conclusive evidence that its use for DNA methylation profiling yields epigenetic markers that are directly related to postnatal neurocognitive outcomes at 1 year of age.

Keywords

Genome-wide association study; DNA methylation; Neurocognitive development; Umbilical cord blood

Background

Cognitive development of young children is a highly complex process, which is influenced by a sophisticated interplay among genetic, epigenetic, and non-genetic factors in both perinatal and postnatal periods (Haworth and Plomin 2010; Stromswold 2006). Among many non-genetic influences on neurocognitive development are exposures during pregnancy; fetal growth restriction, fetal hypoxia, and placental dysfunction; preterm birth; postnatal exposures to biological and chemical agents; nutritional status; parental education and intelligence; the environment and physical surroundings; composition and socio-economic status of the child's family; and social, behavioral, and cultural influences (Benton 2010; Dickens and Flynn 2001; Gottlieb 1996; Hack et al. 2005; Ivanovic et al. 2004; Rees et al. 2008; Shonkoff and Phillips 2000). Numerous aspects of cognitive development and functioning are also affected by genetic factors, which are suggested to be involved in

general cognitive ability, verbal and nonverbal intelligence, language development and skills, mathematical ability, reading ability, dyslexia, developmental delays, learning impairment, intellectual disability, and neuropsychiatric disorders (Bishop 2009; Deffenbacher et al. 2004; Docherty et al. 2010; Hayiou-Thomas 2008; Kaufman et al. 2010;Macleod et al. 2012; Posthuma etal. 2005; Spinath et al. 2004a, b; Sullivan et al. 2012;The SLI Consortium 2002; Wainwright et al. 2006). Genetic association studies suggested that multiple common and rare genetic variants contribute to normal variation in cognitive abilities of children and adults or are associated with pathophysiology of cognition or with neuropsychiatric traits (Haworth and Plomin 2010).

While recent population-based and family-based studies have made significant advances in identifying genetic variants associated with cognitive development and outcomes, much less is understood about association of epigenetic variation with the normal range of cognitive functioning. Epigenetic modification patterns, such as DNA methylation, are heritable across cell divisions; they may be partially altered in response to prenatal and postnatal exposures to environmental influences and physiological and gestational variation (Heijmans et al. 2009; Relton and Davey Smith 2010). Epigenetic modifications play a substantial role in the regulation of fetal development, including neurodevelopment, neuronal differentiation, and brain regulation, and in the pathobiological mechanisms of neurological and psychiatric disorders (Houston et al. 2013; Millan 2013; Nicholls 2000; Riccio 2010; Stadler et al. 2005; Wang et al. 2012b).

As part of a complex interplay of molecular mechanisms involving epigenetic modifications and chromatin remodeling, DNA methylation and demethylation are dynamic processes which are intrinsically involved in neuronal development, neuronal proliferation and survival, learning, and memory formation; they have also been associated with age-related cognitive decline and a variety of neurological disorders (Bender and Weber 2013; Covic et al. 2010; Lister et al. 2013; Liu et al. 2009; Moore et al. 2013; Numata et al. 2012; Portela and Esteller 2010; Riccio 2010; Urdinguio et al. 2009). DNA methylation involves reversible methylation of cytosines in CpG dinucleotides—changes that have been proposed as an epigenetic mechanism of regulation of gene expression (Rakyan et al. 2004; Song et al. 2005; Wu et al. 2010) including differential expression in different regions of the brain (Gibbs et al. 2010). Substantial variation in DNA methylation patterns exists among individuals and tissues, which have been associated with a variety of human diseases and physiological traits (Feinberg et al. 2010; Rakyan et al. 2004; Wilson et al. 2007).

Multiple studies provide evidence for the importance of DNA methylation in brain development and the pathobiology of neurological diseases (Houston et al. 2013; Millan 2013). Abnormal patterns of DNA methylation have been associated with the fragile X syndrome, intellectual disability, autism spectrum disorders, and other disorders that involve changes in cognitive functioning (Grafodatskaya et al. 2010; Nguyen et al. 2010; Portela and Esteller 2010; Urdinguio et al. 2009; Wang et al. 2010). A study of genetic variation in DNA methyltransferase genes, products of which are involved in DNA methylation in the brain and developing neural tissues (Riccio 2010), identified a significant association of the DNMT3L 11330 C/T polymorphism with overall intelligence at 11 and 70 years, suggesting the importance of DNA methylation pathways in human intelligence (Haggarty et al. 2010).

Rett syndrome, a clinical genetic disorder associated with epigenetic abnormalities, involves intellectual disability and neurological complications due to genetic mutations in the methyl-CpG binding domain protein (MeCP2) (Riccio 2010; Sun et al. 2011; Urdinguio et al. 2009).

A number of genes and genomic loci expressed in the developing brain are imprinted, i.e., the expression of either their paternal or maternal allele is silenced or demonstrates parental bias, due to the extensive methylation of cytosines in CpG dinucleotides (Keverne 2013). Some clinical syndromes caused by imprinting abnormalities, including Beckwith-Wiedemann, Silver-Russell, Prader-Willi, and Angelman Syndromes, exhibit impaired cognitive function or neurodevelopment (Nicholls 2000). The effects of DNA methylation in imprinted and non-imprinted genomic loci in neural tissues and the developing brain strongly indicate the importance of DNA methylation in cognitive functioning and the need for epigenome-wide association studies (Rakyan et al. 2011) to discern the role of epigenetic variation in cognitive development.

While epigenetic factors appear to have an important role in early cognitive development, direct measurement of the effects of DNA methylation on early cognitive development in healthy infants is complicated by the lack of ability to directly measure DNA methylation patterns in the brain. On the contrary, neonatal umbilical cord blood can be easily obtained following delivery. While tissue-specific variation of DNA methylation patterns, the importance of DNA methylation in neuronal differentiation, and suggested temporal patterns of DNA methylation in different regions of the brain are well documented (Covic et al. 2010; Riccio 2010; Sun et al. 2011), an intriguing question arises whether umbilical cord blood may contain DNA methylation marks which may be associated with cognitive outcomes later in development. Such association could be due to common exposure to maternal or environmental factors which may affect cognitive outcomes. Rauh et al. (2011, 2006) demonstrated that prenatal exposures to chlorpyrifos, an organic pesticide, measured using newborn umbilical cord plasma, were predictive of psychomotor and mental development in 3-year-old children, assessed using Bayley Scales of Infant Development II; these exposures also correlated with Working Memory Index and Full-Scale IQ in 7-year-old children, who were assessed using the Wechsler Intelligence Scales for Children (WISC-IV). Similarly, DNA methylation in umbilical cord blood has been associated with levels of maternal exposure to lead, a developmental neurotoxin (Pilsner et al. 2009). Additionally, maternal smoking status during pregnancy and the levels of cotinine in newborn umbilical cord blood as a result of maternal smoking are correlated with global DNA hypomethylation in umbilical cord blood (Guerrero-Preston et al. 2010). In a separate study, maternal smoking during pregnancy was associated with global hypomethylation and gene-specific changes in DNA methylation patterns measured in buccal cells from kindergarten and first grade children (Breton et al. 2009). In addition, DNA methylation patterns in newborn umbilical cord blood were found to be associated with body size and composition at 9 years of age (Relton et al. 2012). These findings suggest possibilities that DNA methylation patterns in umbilical cord blood may be reflective not only of the newborn's intrinsic epigenetic profile but also of its prenatal exposures and in utero influences, and that such patterns may be predictive of postnatal childhood measures obtained later in life. Additionally, patterns of variability in DNA methylation may precede the differentiation of hematopoietic and neural tissues, which could also suggest a possibility for associations

between DNA methylation patterns in umbilical cord blood and neurocognitive outcomes (Rakyan et al. 2011).

In this study, we attempted to detect an association between DNA methylation patterns in umbilical cord blood with cognitive measures in 1 year old infants. We used data from 168 African American and Caucasian American infants which was collected as part of the Conditions Affecting Neurocognitive Development and Learning in Early childhood (CANDLE) Study and a study ancillary to CANDLE which investigated molecular determinants of fetal growth. CANDLE is a prospective community-based study in Shelby County, TN (Palmer et al. 2013; Volgyi et al. 2013). It involves generally healthy mothers and their infants followed longitudinally from the mothers' second trimester of pregnancy. The dataset includes detailed measures of behavioral and environmental covariates for both mothers and offspring. We describe analyses of epigenetic molecular patterns, measured in umbilical cord blood collected at birth, in relation to early cognitive development at one year of age, as measured at each child's first CANDLE clinic visit (CV1). The three phenotypic outcomes used in our study for assessment of early language and general cognitive development were Cognitive (nonverbal) and Communication (Receptive and Expressive) raw scores measured using the Bayley Scales of Infant and Toddler Development-Third Edition (Bayley-III) Screening Test (Bayley 2006).

Methods

Study participants

The subjects of this study (Tables 1, 2) were 168 CANDLE mother-infant pairs who were included in an ancillary study of molecular determinants of birth weight variation and had both umbilical cord genome-wide DNA methylation measures and cognitive measures at 1 year of age. CANDLE is a prospective longitudinal study of early cognitive development which extends from the second trimester of pregnancy until the child reaches age 4 (Palmer et al. 2013; Volgyi et al. 2013). CANDLE recruited 1,503 healthy pregnant women between 16.0 and 28.0 weeks of gestation, who had normal singleton fetal pregnancies and lived in Shelby County, TN (Palmer et al. 2013). The selection criteria for our study included the following inclusion criteria for the CANDLE study and for the ancillary study of birth weight variation (Adkins et al. 2011a, b): maternal age 18–40 years; singleton pregnancy; gestational ages of 35–42 weeks; complete data on birth weight and maternal pre-pregnancy weight; and absence of several complications, specifically sexually-transmitted disease or other maternal infections, diabetes, chronic illnesses, oligohydramnios, preeclampsia, placental abruption, tocolytics, and cervical cerclage, or other pregnancy complications. The study did not include any siblings, and therefore each family was represented by a single mother– child pair. We included predominantly mother-newborn pairs with self-declared race of either Caucasian or African-American, although one pair in which a mother identified her race/ethnic background as "Other" was also included in our study due to a delay in self-reporting of mixed ancestry (Table 1).

While the primary focus of the CANDLE study is child cognitive development, it has collected detailed information on maternal psychosocial status; family demographics and environment; caregiver functioning; caregiver-child interaction; maternal, newborn, and

postnatal anthropometric measures; and clinical information. Maternal and family data were collected at the second and third trimesters of pregnancy. During a home visit at 4 weeks postpartum (home visit 1 or HV1), CANDLE study personnel collect child health updates. The mother and the child make annual visits to the study clinics for cognitive, psychosocial, and clinical assessments. Additional health information from participants and other updates are collected using telephone interviews, which are regularly scheduled between annual visits. Demographic and phenotypic data on the mothers and newborns were also abstracted from clinical records. This research was approved by the Institutional Review Board of the University of Tennessee Health Science Center, and informed consent was obtained from all mothers.

Measures of infant cognitive and language development

To assess cognitive outcomes at the child's CV1 (at approximately one year of age; Table 3), we administered the Bayley-III (Bayley 2006). The Bayley-III includes items psychometrically selected from the more comprehensive Bayley Scales of Infant Development, Third Edition (Bayley 2006). The Bayley-III is a widely accepted developmental assessment instrument for children ages birth to 42 months (Bradley-Johnson and Johnson 2007; Sattler 2008; Vig and Sanders 2007). Internal consistency and test–retest reliability coefficients of the Bayley-III for infants' Cognitive, Receptive Communication, and Expressive Communication subtests are high to very high (Nunnally and Bernstein 1994), ranging from 0.76 to 0.93 (Bayley 2006). The validity of the Bayley-III, examined by determining its classification accuracy with the Bayley Scales of Infant Development, Third Edition scaled scores, showed correlations between the Bayley-III Cognitive, Receptive Communication, Expressive Communication subtests and the Bayley Scales of Infant Development, Third Edition comprehensive scales of 0.93, 0.95, and 0.95, respectively (Bayley 2006). Although concerns about the Bayley-III underestimating developmental delay have been reported in certain specific clinical samples (Acton et al. 2011; Anderson et al. 2010), these concerns have not been reported by others (Wild et al. 2013).

For this study, the Bayley-III was utilized to minimize infant and parent fatigue. Although the Bayley-III has five subtests, we selected the Cognitive (nonverbal), Receptive Communication, and Expressive Communication subtests as phenotypic outcomes in this study. These subtests were chosen due to content similarity with other measures of cognitive development which are used later in childhood. Subtest scores are used to determine if the child's scoring is in the lowest risk or Competent category, the Emerging Risk category, or the At Risk category. At 1 year of age, the Bayley-III Cognitive items focus primarily on short term visual memory, functional play, and nonverbal problem solving. Receptive Communication items include pointing to common objects or pictures of actions in a picture book, as well as responding to commands, while expressive communication items quantify emitted sound and sound combinations at 1 year of age.

A rigorous training was established to maintain Bayley-III reliability. After graduate coursework in preschool assessment and child development, the cognitive examiners attended didactic instruction on the Bayley-III. Inter-rater reliability attained through direct

observation of test administration and scoring yielded reliability coefficients equal to or greater than 0.90 on all subtests.

Statistical analysis of non-genetic variables

The primary phenotypic outcomes in all regression models were Bayley-III raw scores at CV1, which assessed children's primary cognitive outcomes when they were approximately at their 1st birthday (age range 10–17 months). These phenotypic measures included each child's Bayley-III raw scores for the Cognitive, the Receptive Communication, and the Expressive Communication subtests. The child's age in months was included in statistical modeling as a potential covariate. Additional measures screened for significance in their roles affecting Bayley-III raw scores were (1) maternal psychosocial measures, demographic, and medical history collected at enrollment in the 2nd trimester of pregnancy clinic visit (M1) at >15 weeks, including mother's self-reported race, type of health insurance, maternal education, marital status, family income, maternal age at enrollment, mother's pre-pregnancy BMI, total number of pregnancies, tobacco use during pregnancy, and measures of the Temperament Evaluation of Memphis, Pisa, Paris, San Diego (TEMPS) questionnaire (Akiskal et al. 2005); (2) maternal psychosocial measures, demographic, and medical history collected in the 3rd trimester of pregnancy clinic visit (M2) at 27–42 weeks including data from the Traumatic Life Events Questionnaire (Kubany et al. 2000), the Rosenberg Self-esteem Scale (Rosenberg 1965), the Social Support Questionnaire (SSQ) (Sarason et al. 1987), and Brief Symptom Inventory (BSI) (Derogatis 1993); (3) clinical and anthropometric measures collected in the study hospitals at delivery (M3), including newborn birth weight, length, and head circumference, child's gender, Apgar scores at 1 and 5 min, gestational age, level of resuscitation needed at birth, highest level of care needed after birth, number of pregnancy complications, and number of days spent in neonatal intensive care unit; (4) maternal psychosocial measures and household information collected during HV1 or the home visit at 4 weeks postpartum including data from the Knowledge of Infant Development Inventory questionnaire (MacPhee 1981), from the Edinburgh Postnatal Depression Scale (Cox et al. 1987), and from the Household Questionnaire developed by the CANDLE study; and (5) child and maternal data collected at CV1 at approximately the child's first birthday including maternal BSI and EPDS measures, mother's intelligence measures using the Wechsler Abbreviated Scale of Intelligence (Wechsler 1999), Parenting Stress Index-Short Form (PSI-SF) (Abidin 2002), Child Abuse Potential Inventory (CAPI) (Milner 1986), the Teaching Scale (Sumner and Spietz 1994), Brief Infant-Toddler Social and Emotional Assessment (BITSEA) (Briggs-Gowan and Carter 2006), and a measure of the level of the child's special needs using the Children with Special Health Care Needs Screener (Bethell et al. 2002). Additional description of measures collected by the CANDLE study is provided by Palmer et al. (2013).

Statistical analyses were performed using Stata versions 10.1 and 11 (Stata Corporation, College Station, TX, USA) to identify non-genetic covariates which significantly affected Bayley-III raw scores. Additional statistical testing of non-genetic covariates for confounding was performed using R x64 2.15.1. The dataset included 168 mother-newborn pairs (Tables 1, 2 and 3), for whom both umbilical cord DNA methylation data and the year 1 clinic visit (CV1) data were available. Separate linear regression models were built for raw

scores of Bayley-III Cognitive, Receptive Communication, and Expressive subtests and regression diagnostics were performed to ensure normality of residuals (Chen et al. 2003). Potential covariates were screened in each model using forward stepwise regression, with p value <0.05 used as a threshold for inclusion in the final non-genetic models. Among any highly correlated covariates with p values satisfying this threshold, the covariate with the lowest p value was included in the final model.

DNA methylation analysis

As part of our studies of molecular factors influencing fetal growth (Adkins et al. 2011a, b), DNA methylation was measured in umbilical cord blood samples of CANDLE newborns. Procedures for sample collection and DNA methylation measurement have been described in detail elsewhere (Adkins et al. 2011a, b). Briefly, newborn blood was collected in purple top (EDTA) tubes from the umbilical cord at delivery. After centrifugation of whole blood, the buffy coat was collected and frozen. Subsequently, genomic DNA was isolated from the buffy coat derived from 10 mL of whole blood using Wizard genomic DNA purification reagents from Promega Corp. or their Maxwell 16 automated nucleic acids extractor and stored at −20 °C in Tris–EDTA buffer.

Bisulfite conversion of 750 ng of genomic DNA was performed using EZ DNA Methylation reagents (Zymo Research). Samples were processed according to manufacturer's specifications and hybridized and scanned on the Humanmethylation27 BeadChip (Illumina Inc.) in 9 batches of 24 samples using the Illumina BeadStation. The Humanmethylation27 BeadChip has probes for 27,578 specific CpG dinucleotides assigned to 14,495 loci. The raw data were processed using the Methylation module (version 1.7.0) of the Illumina GenomeStudio (version 2009.1) software. The level of methylation of each CpG is represented by a beta value (ranging from 0 to 1), which is calculated as the level of the fluorescence for the probe specific for 5-methylcytosine divided by the fluorescence from the probes for both the methylated and unmethylated C at that position. For quality control, probes with detection p values $\geq 10^{-3}$ were dropped from that individual. Additionally, one probe with a median detection p value $>10^{-6}$ across newborns was dropped from all individuals. The final dataset included methylation measures for 27,577 genome-wide CpG probes. The level of methylation at a small number of CpG sites was validated by pyrosequencing by an external laboratory, and the measures from the array and pyrosequencing were highly correlated ($p \leq 0.008$) (Adkins et al. 2011b).

One hundred sixty eight samples, for which both DNA methylation measures and CV1 data were available, were analyzed for association of DNA methylation with CV1 cognitive assessments. A logit transformation of DNA methylation values was performed prior to regression analysis (Rakyan et al. 2011). Association analyses of DNA methylation data were performed using Stata 10.1 and 11 while adjusting for significant non-genetic covariates (Table 4). We also adjusted for race, which had been associated with DNA methylation differences in our sample and in other studies (Adkins et al. 2011a; Wang et al. 2012a; Zhang et al. 2011). In addition, to account for possible batch effects, we included the nine methylation batch numbers as factors in all regression analyses of methylation data. To account for gender-specific DNA methylation differences of the X-chromosome (Adkins et

al. 2011a), gender was included as a covariate in regression analysis of all X chromosome probes for all Bayley-III outcomes. Gender was not included in the association analyses of autosomal DNA methylation data, because it was not found to be significant in stepwise forward regression of the baseline non-genetic variables. Stata regression diagnostic tests (Chen et al. 2003) were applied to ensure normality of residuals for all top CpG hits identified in regression analysis of methylation data.

To determine genome-wide significance, a Bonferroni correction was performed on a nominal α of 0.05, which resulted in significance threshold of $p < 1.89 \times 10^{-6}$ for individual CpG probes. We also calculated frequentist q-values by employing the qq-value software for Stata (Newson 2010), while using software options for Bonferroni as well as Simes (Simes 1986) correction procedures. This approach adjusts individual p-values by establishing family-wise error rate (FWER; Bonferroni correction option) or false discovery rate (FDR; Simes correction option) from the input vector of uncorrected *p* values (Newson 2010). Finally, CpG probes which did not achieve epigenome-wide significance but had $p < 1.0 \times 10^{-4}$ were considered of potential interest, and the functional roles of the genes containing these probes were investigated using searches of Information Hyperlinked over Proteins or IHOP[1] (Hoffmann and Valencia 2004), GeneCards[2] (Stelzer et al. 2011), and Online Mendelian Inheritance in Man (OMIM)[3] online databases and search engines, as well as PubMed literature searches at the National Center of Biotechnology Information.[4]

Results and discussion

Non-genetic covariates associated with cognitive and language outcomes

The results of regression analyses of non-genetic covariates in Bayley-III outcomes are presented in Table 4. As developmental measures, Bayley-III raw scores increase with age. Therefore, it is not surprising that the child's age (between 10 and 17 months) at CV1 was a strong predictor of all three Bayley-III outcomes (Cognitive, Receptive Communication, and Expressive Communication). Similarly, the BITSEA autism social skills subscale, also a measure of global social skill development, was a strong predictor of all three Bayley-III outcomes.

For the Bayley-III Cognitive scores, the non-genetic variables associated with outcomes included the presence of a congenital malformation (in two out of 168 infants, or 1.19 %). Other variables reflect maternal factors including whether or not the mother had high school education and mother's BSI raw and T scores for Psychoticism assessed at CV1. Interestingly, maternal pre-pregnancy BMI was a significant ($p = 0.0113$ in stepwise forward regression, Table 4, and $p = 0.001$ when regressing on pre-pregnancy BMI alone) negative predictor associated with Bayley-III Cognitive scores. This result is in agreement with an earlier study of African American mother–child pairs by Neggers et al. (2003) which found pre-pregnancy BMI to be a significant negative predictor of child's IQ and nonverbal ability at approximately 5.3 years of age. It is possible that pre-pregnancy BMI may serve as

[1] http://www.ihop-net.org
[2] http://www.genecards.org/
[3] http://omim.org/
[4] http://www.ncbi.nlm.nih.gov/

a proxy for other maternal or demographic factors which may negatively affect a child's cognitive development.

For the Bayley-III Receptive Communication scores, the non-genetic variables associated with outcomes include the child's Apgar score at 5 min after birth and the birth length z score. Other associated variables include maternal and family factors including income category of $45,000–64,999 versus other income categories, the Parenting Stress Index-Short Form parent–child dysfunctional interaction percentile (PSI-SF PCDI) at CV1, maternal irritability score measure from the TEMP temperament assessment at M1, and three maternal psychosocial BSI measures which included maternal BSI T score for Phobic Anxiety scale assessed at CV1, maternal BSI raw Positive Symptom total score at M2, and maternal BSI raw score for hostility scale at M2.

For the Bayley-III Expressive Communication outcomes, significant covariates reflected family factors such as SSQ average number of support individuals and the raw score of problems with family from the CAPI parent questionnaire. The maternal BSI raw score for Paranoid Ideation scale assessed during prenatal M2 study visit to the clinic in the third trimester of pregnancy and the BSI T score for Anxiety assessed at the one year CV1 visit were both significantly associated with Bayley-III Expressive Communication scores. Additional significant non-genetic covariates included the mother's capacity to be sensitive to her child during an observed teaching episode and dysfunctional parenting indicated by the PSI-SF PCDI at CV1. This is consistent with adverse effects of parental stress on language ability of children aged 2–5 years (King et al. 2005).

As discussed above, among socioeconomic demographic measures, some categories of maternal education and income (Table 1), which were analyzed as factors, were found to influence Bayley-III Cognitive and Receptive Communication scores. Maternal education at the high school level was associated ($p = 0.0074$) with Bayley-III Cognitive scores, whereas income category of $45,000–64,999 as compared to other income categories was associated with Bayley-III Receptive Communication scores ($p = 0.0162$). Maternal education and income were very highly correlated in our dataset (Fisher's exact test $p < 10^{-3}$) and confounded the analysis by race (association between race and income categories by Fisher's exact test, $p < 10^{-3}$). In our baseline regression analyses, race was not a significant predictor of any Bayley-III cognitive outcomes. However, it was included in analyses of methylation data (below) to account for potential differences in methylation levels.

DNA methylation analysis

When using genome-wide Bonferroni correction procedures, no CpG achieved genome-wide significance with $p < 1.89 \times 10^{-6}$ for any of the Bayley-III outcomes. Similarly, no CpG had a q-value <0.05. When a more liberal p value threshold of 1×10^{-4} was used to identify suggestive CpGs with possible weak effects, in analyses of Bayley-III Cognitive and Expressive Communication scores, none of the CpGs achieved that threshold. Analysis of Receptive Communication scores identified four top CpGs with $p < 10^{-4}$, which are reported in Table 5. These include CpG probes in *IGF2BP2*, *WIPI2*, *SCN2A2*, and *CDS2*. In addition, the CpG probe cg14244577 in the *DDX19B* gene has $p = 1.006 \times 10^{-4}$. Of these genes, *IGF2BP2* is the insulin-like growth factor 2 mRNA-binding protein gene 2. While

this gene is considered to be a susceptibility gene for diabetes 2 mellitus, and it may interact with fetal malnutrition and metabolic programming (van Hoek et al. 2009), genetic variation in this gene and variation of its expression levels have also been reported to be associated with schizophrenia in a Han Chinese population (Zhang et al. 2013b). Another gene with a possible connection to brain functioning is *SCN2A2*, mutations in which have been reported in patients with a spectrum of epilepsies and infantile seizures (Ogiwara et al. 2009). Rare mutations in this gene in patients with severe mental decline or familial autism have also been reported (Kamiya et al. 2004; Weiss et al. 2003). The relevance of CpG probes in the genes *WIPI2, CDS2*, or *DDX19B* to cognitive development is unclear, and these findings may not represent functionally important DNA methylation sites.

These results suggest a very weak association of Bayley-III Receptive Communication scores at one year with DNA methylation in newborn umbilical cord blood, which does not provide a strong support for a hypothesis that methylation differences present in the umbilical cord blood at birth may be predictive of early cognitive development. Interestingly, while our use of umbilical cord blood did not yield any strong associations, Zhang et al. (2013a) used peripheral blood of adult patients to identify DNA methylation changes in several candidate genes which were associated with childhood adversity, which suggest that some epigenetic changes in peripheral blood are associated with brain processes during childhood.

The absence of strong associations may be not only due to tissue specificity, but also due to temporal changes, because DNA methylation was measured at birth while cognitive outcomes were measured approximately one year later. Multiple studies suggested that changes in DNA methylation during fetal development are transient in nature, that there are substantial differences in tissue-specific differentially methylated regions between fetal and adult tissues, and that progressive methylation changes occur in an age-dependent manner (Christensen et al. 2009; Numata et al. 2012; Poulsen et al. 2007; Yuen et al. 2011).

It is important to note that the generalizability of the weak associations identified in our study beyond variability of very early cognitive measures in an unselected normal population with healthy pregnancies is unknown. Accordingly, these associations do not necessarily imply predictive value for future neurodevelopment or psychiatric disorders. Due to racial and ethnic differences in DNA methylation patterns, the generalizability of reported findings to other populations also remains to be investigated, although the possible neuropsychiatric effect of the product of the *IGF2BP2* gene was reported in the Han Chinese population (Zhang et al. 2013b).

In this study, we reported information about the genes involved in DNA methylation associations based on searches of biomedical literature and online genetic databases and search engines. Not all of the gene roles listed in Table 5 are necessarily relevant to variation in early cognitive outcomes in an unselected human population. Similarly, some of the functions of these genes are likely not present in current annotations and literature. Furthermore, because of the relative scarcity of the CpG probes from the microarrays used in this study, they could not capture all epigenome-wide methylation variation.

It is possible that the results of our study did not achieve significance at the epigenome-wide level due to insufficient sample size of the cohort, particularly if some epigenetic changes have small effect sizes on cognitive outcomes. Recent genetic studies have suggested involvement of hundreds of genes in common traits such as height (Lango Allen et al. 2010). As with other common traits, many genes have been implicated in clinical manifestations of neurodevelopment disorders such as autism spectrum disorders, schizophrenia, bipolar disorder, intellectual disability, or developmental delays (Betancur 2011; Bilder et al. 2011; Sullivan et al. 2012). While the majority of evidence for the involvement of multiple genes has so far emerged from genetic studies of DNA variation, if individual CpG methylation variants are only weakly associated with cognitive outcomes, such methylation variants would not achieve significance in association analyses in a dataset of this size. However, the number of individuals in our dataset is comparable to or exceeds the size of datasets in many other epigenetic studies of complex traits which used the Illumina Humanmethylation27 BeadChip, and several analytical studies have suggested that this size generally provides adequate power to identify functionally associated CpGs in relevant tissues (Ahn and Wang 2013; Chen et al. 2013; Zhuang et al. 2012). Consistent with these studies, when we employed regression estimates for the four CpGs in Table 5 (full model R^2 of between 0.5665 and 0.5962, reduced model R^2 of 0.5063), we estimated using the powerreg function within Stata that our dataset of 168 infants would have 45.2–86.8 % power to detect an association using a Bonferroni corrected threshold of $\alpha = 1.89 \times 10^{-6}$. We also estimated that 80 % power at this level of significance can be achieved with a dataset of 154–239 infants, and therefore the sample size used in our study was possibly not the main cause for the lack of observed strong associations. We speculate that the reasons for the absence of strong associations may be tissue-specific differences in methylation patterns between umbilical cord blood leukocytes and brain neuronal cells, and the temporal differences between the measures of DNA methylation at birth and cognitive outcomes at 1 year.

We focused our analyses on association of methylation probes with individual Bayley-III outcomes. Many other genetic and epigenetic factors such as common and rare single nucleotide variation, copy number variation, microRNA variation, DNA hydroxymethylation, and histone modifications also likely play important roles in early cognitive development. Future sequencing-based genome and transcriptome studies and novel epigenetics studies, including studies of appropriate tissues in animal models, and investigation of pleiotropic effects (Andreassen et al. 2013) of epigenetic and genetic variants may be able to more precisely pinpoint the functional epigenetic modifications which may be contributing to variation in cognitive learning outcomes. The results of our study suggest that if any common DNA methylation signature marks between the neonatal umbilical cord blood and brain tissues exist, their effects may be too weak to be detected when investigating subsequent cognitive outcomes at 1 year of age, and that epigenetic studies of early neurocognitive development may benefit from the use of other tissue samples which may be more directly relevant to brain function.

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Abbreviations

ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorders
BITSEA	Brief Infant-Toddler Social and Emotional Assessment
BSI	Brief Symptom Inventory
Bayley-III	Bayley Scales of Infant and Toddler Development-Third Edition Screening Test
CANDLE	Conditions Affecting Neurocognitive Development and Learning in Early Childhood
CAPI	Child Abuse Potential Inventory
CSHCN	Children with Special Health Care Needs
CANTAB	Cambridge Neuropsychological Test Automated Battery
CV1	Clinic visit 1
DZ	Dizygotic
EPDS	Edinburgh Postnatal Depression Scale
EWAS	Epigenome-wide association study
FDR	False discovery rate
FWER	Familywise error rate
HV1	Home visit 1 by the CANDLE study staff to the mother at 4 weeks postpartum
KIDI	Knowledge of Infant Development Inventory
M1	Mother’s clinic visit at enrollment in the second trimester
M2	Mother’s second clinic visit in the third trimester of pregnancy
M3	Birth at hospital
MeCP2	Methyl-CpG binding domain protein
PSI-SF	Parenting Stress Index-Short Form

PSI-SF PCDI	Parenting Stress Index-Short Form Parent–Child Dysfunctional Interaction
SSQ	Social Support Questionnaire
TEMPS	Temperament evaluation of Memphis, Pisa, Paris, San Diego
TLEQ	Traumatic Life Events Questionnaire
UTHSC	The University of Tennessee Health Science Center
WASI	Wechsler Abbreviated Scale of Intelligence

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Table 1

Summary of sociodemographic and anthropometric measures for mothers (N = 168)

Measure	Mean ± standard deviation or % (number)
Age	26.92 ± 5.30 years
Pre-pregnancy BMI	27.12 ± 6.94
Race	
African American	53.57 % (90)
Caucasian	45.83 % (77)
Other	0.60 % (1)
Hispanic	1.19 % (2)
Maternal education	
College degree	30.36 % (51)
Graduate or professional	14.29 % (24)
High school diploma or GED	38.69 % (65)
Technical school	11.31 % (19)
Less than high school	5.36 % (9)
Marital status	
Separated	1.19 % (2)
Never married	30.95 % (52)
Living with partner	16.07 % (27)
Married	50.00 % (84)
Marital status not reported	1.79 % (3)
Annual income	
<$5,000	11.31 % (19)
$5,000–24,999	20.24 % (34)
$25,000–44,999	19.05 % (32)
$45,000–64,999	16.07 % (27)
>$65,000	30.36 % (51)
Not reported	2.98 % (5)
Medicaid or TennCare insurance[a]	44.64 % (75)
Tobacco use	8.93 % (15)

[a] TennCare is the Medicaid waiver program for the State of Tennessee

Table 2

Summary of infant measures at birth (N = 168)

Measure	Mean ± standard deviation or % (number)
Age at clinical visit 1 (months)	12.39 ± 1.25
Gestational age (weeks)	38.99 ± 1.31
Gender	
Male	51.79 % (87)
Female	48.21 % (81)
Birth weight (g)	3357.43 ± 486.81
Birth length (cm)	50.25 ± 2.83
Birth head circumference (cm)	34.13 ± 2.32
Apgar 1 min	8.14 ± 0.96
Apgar 5 min	8.97 ± 0.20

Table 3

Summary statistics of Bayley-III raw scores at one year clinic visit (CV1) (N = 168)

Measure	Mean ± standard deviation
Cognitive subtest	16.95 ± 2.05
Receptive communication subtest	11.54 ± 2.02
Expressive communication subtest	12.75 ± 1.97

Table 4

Significant non-genetic variables associated with Bayley-III scores at one year of age (CV1 visit), included as covariates in epigenome-wide association analyses

Variable	Data collection time	*p* Value	Beta
Variables associated with Bayley-III Cognitive subtest scores			
Child's age (months)	CV1	<0.0001	0.69
Whether or not a child had a congenital malformation[a]	M3	<0.0001	−6.89
Mother's high school education versus other education categories	M1	0.0074	−0.72
Mother's BSI raw score for Psychoticism scale[a]	CV1	0.0079	1.85
Child's autism social skills subscale of BITSEA	CV1	0.0102	0.19
Mother's pre-pregnancy BMI	M1	0.0113	−0.07
Variables associated with Bayley-III Receptive Communication subtest scores			
Child's autism social skills subscale of BITSEA	CV1	<0.0001	0.17
Maternal BSI T score for Phobic BSI Anxiety scale	CV1	0.0013	−0.07
Birth length z score	M3	0.0041	0.23
Child's age (months)	CV1	0.0046	0.55
Child's Apgar score at 5 min	M3	0.0146	2.83
Income of $45,000–64,999 versus other categories	M1	0.0162	−1.03
Maternal BSI raw positive symptom total	M2	0.0187	0.05
Maternal BSI raw score for Hostility scale	M2	0.0197	0.74
Mother's TEMPS score on irritable scale	M1	0.0322	−0.32
PSI-SF Parent–Child Dysfunctional Interaction percentile	CV1	0.0440	−0.02
Variables associated with Bayley-III expressive communication subtest scores			
Child's age (months)	CV1	<0.0001	0.66
Child's autism social skills subscale of BITSEA	CV1	<0.0001	0.27
Maternal sensitivity to cues from the Teaching Scale	CV1	0.0003	0.27
PSI-SF Parent-Child Dysfunctional Interaction percentile	CV1	0.0014	−0.02
Maternal BSI raw score for Paranoid Ideation scale	M2	0.0074	−0.80
SSQ average number of support persons	M2	0.0106	−0.21
Maternal BSI T score for Anxiety scale	CV1	0.0146	0.06
CAPI problem with family scale raw score	CV1	0.0212	−0.31

Shown are variables associated ($p < 0.05$ in forward stepwise regression, out of 74 non-genetic variables tested) with infant's raw Bayley-III scores measured at CV1, CANDLE study 1 year cognitive assessment visit

Beta partial regression coefficient, *BITSEA* Brief Infant-Toddler Social and Emotional Assessment, *BMI* body mass index, *BSI* Brief Symptom Inventory, *CAPI* Child Abuse Potential Inventory, *EPDS* Edinburgh Postnatal Depression Scale, *HV1* home visit 1 by the CANDLE study staff to the mother at 4 weeks postpartum, *M1* mother's clinic visit at enrollment, *M2* mother's second clinic visit in the third trimester during pregnancy, *M3* birth at hospital, *PSI-SF* parenting stress index-short form, *SSQ* Social Support Questionnaire, *TEMPS* Temperament Evaluation of Memphis, Pisa, Paris, San Diego

[a] Reported are regression values for the presence of a congenital malformation

[b] Mother's BSI T score for Psychoticism scale was also significantly ($p = 0.0115$) associated with Bayley-III Cognitive outcomes. Due to a strong correlation between thevalues of mothers' raw BSI score and mothers' T score, only the raw score variable, which had a higher significance, was included as a covariate in DNA methylation analyses

Table 5

Highest ranked CpGs associated with Bayley-III Receptive Communication scores at one year of age ($p < 0.0001$)

CpG	Region	Gene	Gene role	CpG Island	*p* Value	Beta	b	R^2
cg24450631	3	*IGF2BP2*	Insulin-like growth factor 2 mRNA binding protein 2 isoform b	Yes	2.48×10^{-5}	1.39	0.35	0.58
cg20592700	7	*WIPI2*	WD40 repeat protein interacting with phosphoinositides 2	Yes	7.34×10^{-5}	1.18	0.40	0.57
cg15513137	2	*SCN2A2*	Sodium channel; voltage-gated; type II; alpha 2	No	7.92×10^{-5}	−1.32	−0.38	0.57
cg25984124	20	*CDS2*	Phosphatidate cytidylyltransferase 2	No	9.72×10^{-5}	2.10	0.44	0.60

P value regression *p* value for a methylation probe, *Beta* partial regression coefficient, *b* standardized partial regression coefficient, R^2 coefficient of determination (ratio of "explained variance" to total variance) for the full multiple regression model which included non-genetic covariates and the logit transformed methylation beta-value for a probe (one at a time)