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Association Between Paternally Inherited Haplotypes Upstream of the Insulin Gene and Umbilical Cord IGF-II Levels

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ABSTRACT: The insulin (INS) and IGF 2 (IGF2) genes are in close proximity to each other and undergo maternal imprinting during fetal growth. We investigated the association between maternal and umbilical cord IGF 2 protein (IGF-II) levels and single nucleotide polymorphisms (SNPs) in the INS and IGF2 genes in 207 healthy African-American mother-newborn pairs. No association was found between maternal IGF-II levels and polymorphism in the INS-IGF2 locus. A significant association was found between newborn IGF-II levels and two SNPs (rs3842738 and rs689) at the 5′ end of the INS-IGF2 locus. Analyses of haplotypes inferred from these two SNPs demonstrate a significant relationship between paternally transmitted haplotypes and newborn IGF-II levels, but no association with maternally transmitted haplotypes. **(*Pediatr Res* 62: 451–455, 2007)**

Being small for gestational age is correlated with predisposition to various illnesses, although this association remains controversial (1,2). Full-term, low birth weight infants are at least five times more likely to die in the first year and are second only to premature infants in their rates of morbidity and mortality (3,4). As adults, individuals born small for gestational age (SGA) are at elevated risk of pregnancy-induced hypertension (5), gestational diabetes (6), chronic hypertension (7), type 2 diabetes (8,9), and cardiovascular disease (10).

The relationship between birth size and adult predisposition to chronic disease has been heavily debated. According to the "fetal origins" (11) hypothesis, an inadequate uterine environment results in long-term alterations in organ structure and function and in hormonal milieu that make the individual more susceptible to disease. The existence of genetic factors common to both reduced fetal growth and disease predisposition is another explanation. This hypothesis is supported by an inverse correlation between paternal mortality and offspring birth size that is only slightly weaker than that between maternal mortality and offspring birth size (12,13), even after adjusting for lifestyle risk factors and socioeconomic status. Imprinted loci, which include several genes with direct roles in pathogenesis (*i.e.* insulin, insulin-like growth factor 2 gene (IGF2), insulin-like growth factor gene receptor (IGF2R)), may comprise a key part of this connection.

Insulin-like growth factor 2 protein (IGF-II) is expressed abundantly in trophoblast and fetal endothelial cells (14). The IGF2 gene resides within a block of genes at 11p15 that undergo the silencing (or imprinting) of the allele inherited from one of the parents during fetal development. In the case of IGF2, the allele inherited from the mother is imprinted (15) before implantation, around the eight-cell stage (16). Several lines of evidence indicate that IGF2 and other genes at 11p15 have significant effects on fetal growth. Loss of imprinting at 11p15, including biallelic expression of IGF2, is associated with the prenatal overgrowth of Beckwith-Wiedemann Syndrome (17) and with some cases of postnatal overgrowth (18,19). Conversely, hypomethylation of the telomeric imprinting center region (ICR1) is found in a proportion of pre- and postnatally growth-retarded individuals with Silver-Russell Syndrome. The hypomethylation of ICR1 appears to result in biallelic expression of the nearby paternally imprinted H19 gene with consequent under expression of IGF2 (20–22). In the mouse, disruption of IGF2 expression during development results in growth retarded pups (23,24). Conversely, transgenic doubling of IGF2 expression in the mouse results in fetal overgrowth (25).

It has been difficult to identify a clear connection between IGF-II levels in pregnancy and newborn size. Several studies have found a significant reduction in umbilical cord IGF-II levels in small for gestational age newborns (26–29), while a similar number of studies have found no relationship (30–33). Part of the inconsistency in the relationship between birth weight and fetal IGF-II levels may be attributable to variation in the ratio of IGF-II to the soluble portion of the IGF-II receptor, which promotes the degradation of IGF-II but is rarely measured in studies of birth weight (34).

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Abbreviations: IGF-II, insulin-like growth factor 2 protein; **IGF2,** insulin-like growth factor 2 gene; **INS,** insulin gene; **SNPs,** single nucleotide polymorphism; **VNTR,** variable number of tandem repeats locus

The goal of the present study was to test the relationship between single nucleotide polymorphisms (SNPs) in the insulin gene (INS)-IGF2 locus and circulating levels of IGF-II in the mother and newborn. The paternal allele of IGF2 accounts for most of the production of the IGF-II protein (15). Therefore, we tested the association between circulating IGF-II levels and polymorphism in the INS-IGF2 locus, as well as the possibility that the effect of SNPs alleles differs according to the parent of origin of the alleles.

MATERIALS AND METHODS

Subjects. Informed consent was acquired from 207 healthy African-American women with uncomplicated pregnancies upon admission for delivery at the University of Mississippi Medical Center (Jackson, MS) and the Regional Medical Center at Memphis (Tennessee). Only subjects who delivered healthy newborns were retained in the study. Inclusion criteria were: singleton pregnancy, >36 wk gestation, 18–35 y old. Exclusion criteria were chosen to eliminate a large number of additional contributing factors to birth weight variation and included, among others: smoking, diabetes (type 1, type 2, gestational), hypertension/vascular disease, preeclampsia, autoimmune disease, infectious disease (*e.g.* hepatitis, HIV), sickle-cell disease, uterine infection, illicit drug use, and birth defects. The following maternal characteristics potentially related to newborn size were recorded: parity, age, body mass index (BMI), and weight gain during pregnancy. The Institutional Review Boards of both institutions approved this study.

Genotyping. Maternal blood was collected during labor, umbilical cord blood was collected at delivery, and DNA was extracted. Six SNPs were selected by several criteria, including allele frequencies in excess of 10%, previous association with obesity- or diabetes-related traits, and linkage disequilibrium with the INS variable number of tandem repeats locus (VNTR) (35–39) (Fig. 1, Table 1). SNPs were genotyped using either TaqMan Assay by Demand or Assay by Design (rs3842748 and rs3213233; details available upon request) kits from Applied Biosystems Inc. At least two representatives of each genotype at each site were sequenced to verify the accuracy of the TaqMan assay and to resolve any ambiguous genotypes.

IGF-II measurements. Maternal and fetal cord blood plasmas were assayed for IGF-II concentration using an ELISA assay developed with components from R & D Systems (Minneapolis, MN). Because IGF binding proteins present in plasma may compete for IGF-II binding in ELISA, an acid/ethanol extraction of the samples was performed (40,41) to separate binding proteins from IGF-II, followed by centrifugation to remove the binding proteins. Five microliters of plasma, 2.5 μL of PBS + 1% BSA (fraction V), and 62.5 μL of acid/ethanol (0.25 N HCl in 87% ethanol) were mixed and allowed to stand for 30 min. The pH was adjusted to 7.6 by addition of 22 μL of 0.8M Tris, and the samples were centrifuged in a microcentrifuge for 10 min in a cold room at 10,000× *g*. Twenty microliters of the supernatant was assayed for IGF-II in the presence of 80 μL of Krebs Ringer phosphate (KRP) buffer to achieve a final ethanol concentration below 15% because higher concentrations of ethanol were found to interfere with the assay. Blanks and standards were assayed in the presence of 20 μL of an acid-ethanol extract of KRP buffer +1% albumin with the same concentration of ethanol as in the samples from subjects. The final reaction volume was 100 μL. The plasma samples were assayed in duplicate, and the average of the two values was used as the protein concentration. The coefficient of variation for most samples was <5%. Plasma samples for which the coefficient of variation was 10% or greater were repeated. Only 15% of the samples required repetition because of a high coefficient of variation. ELISA used 0.3 μg/well of a mouse monoclonal anti-human IGF-II obtained from R & D Systems (MAB292, clone #75015), while the detection antibody was 0.025 μg/well of a biotin-labeled goat polyclonal anti-human IGF-II (BAF 292 from R & D Systems). The standard was recombinant human IGF-II (R & D Systems, 292-G2-050). The protocol was that recommended for use with R&D Douset ELISA development procedures.

Statistical analysis. Characteristics of the SNPs are summarized in Table 1. Birth weight Z scores (SD from the mean birth weight), specific for gestational age, gender and ethnicity, were calculated based on U.S. Natality Statistics for 2001 and 2002 (National Center for Health Statistics).

Regression equations were selected by a backward selection process (42) in which all nongenetic variables were included in the model. The dependent variables used were maternal or newborn IGF-II concentration. With a sample size of 207 and a critical value of 0.05, we had power of 0.8 to detect a difference in IGF-II levels of 0.38 SD under an additive genetic model and 0.41 SD under a dominant genetic model (43). Fetal IGF-II values were normally distributed and required no transformation. Maternal IGF-II values were log-transformed. Among the nonsignificant partial regression coefficients, the variable with the smallest t statistic was removed and a new regression equation calculated. This process was repeated until a set of variables with significant partial regression coefficients was identified. Variables encoding the number of copies of the minor allele of each SNPs or number of copies of each haplotype in each individual were individually added to the regression equation to test for a significant association with IGF-II levels. The initial nongenetic variables used in regression on maternal IGF-II levels were newborn nearest week of gestation, parity, newborn birth weight Z, maternal BMI, and maternal height. The initial nongenetic model for regression on newborn IGF-II levels included nearest week of gestation, parity, gender, birth weight Z, and maternal BMI.

Haplotypes were inferred for the newborn SNPs exhibiting significant association with umbilical cord IGF-II levels using the program SNPHAP (http://www-gene.cimr.cam.ac.uk/clayton/software). Regression was performed with respect to the number of copies of each haplotype (*i.e.* 0, 1 or 2). For 162 mother-newborn pairs, the maternal and paternal origins of haplotypes could be directly inferred based upon the maternal and newborn haplotype combinations. In 45 cases, the mother and newborn were heterozygous for the same pair of haplotypes. In these cases, the parental origin of haplotypes could not be unambiguously inferred. ANOVA was performed to test for differences in mean umbilical cord IGF-II levels according to the parental origin of each haplotype, with the inclusion of birth weight Z score as a continuous covariable. In the case of ambiguous haplotype transmissions, both parental possibilities were included and given a weight half that of the unambiguous transmissions, resulting in a doubling of the variance for those observations. Separate analyses were performed for unambiguous transmissions and for a combination of ambiguous and unambiguous transmissions.

RESULTS

Association of SNPs with circulating IGF-II levels. Maternal IGF-II levels covered a much broader range than did fetal levels (0.07–2.0 *versus* 0.24–0.68 μg/mL) and had a geometric mean (to compensate for extreme outliers) 20% higher than fetal levels (0.44 *versus* 0.53 μg/mL). None of the nongenetic variables tested (weeks gestation, parity, newborn Z score, maternal BMI, and maternal height) were found to be significant predictors of maternal IGF-II levels. Additionally, in regression analyses none of the maternal single nucleotide polymorphisms were found to be significantly associated with maternal IGF-II levels (Table 2). Among newborns, weeks gestation, parity, gender, and maternal BMI were excluded as significant predictors of newborn IGF-II levels. However, birth weight Z score was significantly associated with fetal

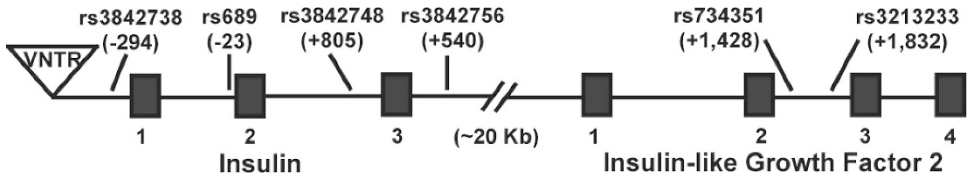

Figure 1. Location and dbSNP accession numbers of single nucleotide polymorphisms. SNPs are numbered relative to the start codon (ATG) of the nearest gene, in conformance with the traditional numbering used for this locus.

Table 1. *Locations and minor allele frequencies (MAF) of the single nucleotide polymorphisms surveyed in this study*

dbSNP	Location†	Gene	Alleles‡	Newborn MAF	Maternal MAF
rs3842738	−294	INS	C/G	0.44	0.43
rs689	−23	INS	T/A	0.25	0.25
rs3842748	+805	INS	G/C	0.27	0.29
rs3842756	+1428	INS	G/A	0.12	0.14
rs734351	+540	IGF2	T/C	0.26	0.23
rs3213233	+1832	IGF2	C/G	0.10	0.12

† Relative to the start codon of the nearest gene (INS or IGF2).
‡ Minor (less frequent) allele is listed second.

IGF-II levels ($p = 0.02$). Among the SNPs, neither of the sites located within the IGF2 gene (rs734351 and rs3213233) were associated with IGF-II levels, nor were two of the SNPs within the INS (rs3842748 and rs3842756). However, the two SNPs located the furthest 5′ within the INS-IGF2 locus (rs3842738 and rs689) did exhibit significant ($p = 0.02$) association with fetal IGF-II levels.

Haplotypic association with fetal IGF-II levels. Newborn haplotypes were inferred for the two SNPs exhibiting individual association with newborn IGF-II levels. Due to a high degree of linkage disequilibrium between the two sites (scaled covariance D' = 0.93) (44), one of the four possible haplotypes was not predicted while the remaining three haplotypes had frequencies ranging from 24 to 44%. Regression performed on the number of newborn copies of each haplotype and birth weight Z score indicated that one haplotype (G at rs3842738 and T at rs689) was associated with a reduction of 0.02 μg/mL per copy of the haplotype, whereas its complement (C at rs3842738 and A at rs689) was associated with an increase of 0.03 μg/mL per haplotype copy (Table 3). The third haplotype (CT) exhibited no association with newborn IGF-II levels.

Comparison of average fetal IGF-II levels according to the haplotype inherited from the mother found no significant difference among maternally inherited haplotypes (Table 4). Comparisons of fetal IGF-II levels among paternally inherited haplotypes found significant differences among the paternal haplotypes when either unambiguous or all possible transmissions were considered ($p \leq 0.01$). Paternal haplotype GT is associated with the lowest average IGF-II levels (0.40 μg/mL; 0.24–0.63 μg/mL) and haplotype CA is associated with the highest average levels (0.47 μg/mL; 0.28–0.68 μg/mL).

Table 2. *Regression analyses of the relationship between SNPs variation and maternal and fetal IGF-II levels*

	Regression coefficient†		
	Fetal		
	Genotype	Birth weight Z	Maternal genotype
rs3842738	−0.02*	0.02	−0.01
rs689	0.03*	0.02*	−0.02
rs3842748	0.01	0.02*	−0.02
rs3842756	0.01	0.02*	−0.02
rs734351	0.00	0.02*	−0.02
rs3213233	0.01	0.02*	0.03

$p < 0.05$. † Change in plasma IGF-II level per each copy of the less common allele or per unit change in birth weight Z score.

Table 3. *Regression analyses of the relationship between fetal IGF-II levels and the number of 5' insulin haplotypes*

Haplotype sequence†	Frequency	Haplotype β (p value)‡	Birth weight Zβ (p value)
GT	44.4	−0.02 (0.02)	0.02 (0.05)
CT	31.5	0.00 (0.73)	0.02 (0.02)
CA	24.1	0.03 (0.03)	0.02 (0.04)

† SNPs rs3842738 and rs689, in that order.
‡ β value indicates unit change in plasma IGF-II levels per copy of the haplotype or unit change in birth weight Z score.

DISCUSSION

We have found a significant effect of paternally inherited haplotypes upstream of the insulin gene on umbilical cord IGF-II levels. This observation is consistent with previous analyses demonstrating an unusually strong genetic component to IGF-II expression. Based on an analysis of middle-aged to elderly mono- and dizygotic twins, Harrela *et al.* (45) estimated an overall genetic heritability of 66% for IGF-II levels. The intra-twin correlation for dizygotic twins was about 50% that of monozygotic twins ($r = 0.34$ *versus* $r = 0.66$). A major factor in the intra-twin correlations may be the influence of shared, paternally derived INS VNTR alleles or SNPs haplotypes. Specifically, monozygotic twins share the same paternal SNPs and VNTR alleles, while dizygotic twins on average share the same paternal allele 50% of the time. In addition to environmental and epigenetic influences, the relative frequency of allele sharing of paternally derived variants between mono- and dizygotic twins may explain a significant proportion of their correlations in circulating IGF-II levels.

Furthermore, the pattern of association we observed between IGF-II levels and INS VNTR allele size classes in linkage disequilibrium with the genotyped SNPs is in agreement with the observations of Paquette *et al.* (46), who found that term human placentas had higher levels of IGF2 mRNA expression in the presence of the class I alleles compared with the longer class III alleles. This is consistent with our observation because haplotype CA (Table 2) is in high linkage disequilibrium with class I alleles based on the work of Stead *et al.* (35), and umbilical cord IGF-II levels are positively associated with the number of copies of that haplotype. Additionally, our results further suggest that it is the paternal origin of the INS-IGF2 allele that is relevant to IGF-II levels because maternally transmitted haplotypes exhibit no association with newborn IGF-II levels. This can be explained by the fact that the paternal alleles of INS and IGF2 account for the vast majority of RNA expression in several tissues key to fetal growth regulation, such as the yolk sac (47) and placenta (15).

Previously Adkins *et al.* (47a) found that a paternally transmitted haplotype composed of three SNPs (G at rs3842738, T at rs689, G at rs3842748) at the 5′ end of the INS-IGF2 locus conferred a 7-fold increased risk of small for gestational age birth compared with the haplotype CAC. Here, we have found that paternally transmitted haplotypes com-

Table 4. *ANOVA on differences in cord blood IGF-II levels according to parental origin of 5′ insulin haplotypes.*

	Paternal haplotype						Maternal haplotype					
	Unambiguous transmissions†			All transmissions			Unambiguous transmissions			All transmissions		
	Mean IGF-II (SD)	F	*p*	Mean IGF-II (SD)	F	*p*	Mean IGF-II (SD)	F	*p*	Mean IGF-II (SD)	F	*p*
GT	0.40 (0.10)	4.65	0.01	0.42 (0.10)	5.57	0.004	0.43 (0.10)	0.13	0.88	0.44 (0.10)	0.27	0.76
CT	0.45 (0.10)			0.45 (0.09)			0.44 (0.10)			0.44 (0.10)		
CA	0.47 (0.11)			0.48 (0.10)			0.45 (0.11)			0.45 (0.10)		

† $n = 162$ for unambiguous transmission; $n = 207$ for all transmissions.

posed of two (rs3842738 and rs689) of those SNPs are associated with umbilical cord levels of IGF-II, with the lowest levels observed with haplotype GT, as would be expected given that haplotype *GT*G is associated with reduced birth weight. All three SNPs exhibit high linkage disequilibrium ($D' > 0.92$) with one another; therefore, it is not certain why rs3842748 is not associated with both phenotypes (birth weight Z and IGF-II levels). It may be significant that the GT haplotype at rs3842738 and rs689 defines a distinct cluster of haplotypes (J, K, L, T, W, and Z, plus M, which has a back mutation from G to C) entirely of African origin (35). Possibly, the insulin VNTR substructure and/or a SNPs in high frequency among those haplotypes directly influences IGF-II production, while rs3842748 or a SNPs highly correlated with it is more directly involved in fetal growth regulation or insulin production. Indeed, there is evidence that rs689 is functional during the processing of the INS transcript (48) and that not only IGF2 SNPs but also subclasses of the INS class I VNTR alleles are associated with adult male BMI and circulating IGF-II levels (49–51). Quite possibly, the full explanation for our observations is a combination of a direct functional role of some SNPs and their high linkage disequilibrium with the functionally important INS VNTR alleles.

In contrast to newborns, we found no association among maternal IGF-II levels, newborn size, maternal anthropometry, or SNPs genotypes, despite evidence for a strong genetic component to IGF-II levels (45). The lack of a genetic association may be due to one or both of two factors. First compared with newborns there is likely to be a much greater environmental contribution to IGF-II levels among adults. For example, the duration and stress of labor, as well as endocrinological changes associated with parturition, may affect IGF-II levels and confound detection of genetic influences. This may partly explain the observation of positive correlation between maternal IGF-II levels and newborn size in some studies (26,52) but not others (53,54). Second the predominant influences on the level of IGF-II may be driven by different promoters and polymorphic sites in newborns and adults (49–51,55), and we may not have surveyed those sites that are important for adult IGF-II production.

Imprinting and other epigenetic controls of gene expression are key mechanisms through which the maternal and paternal genomes can influence the rate of fetal growth. Our demonstration that genetic polymorphism and paternal transmission of maternally imprinted loci is associated with variation in IGF-II levels suggests that imprinting may be a major influence on fetal growth regulation. On this basis, a fruitful avenue for future research would be to determine the role of polymorphisms and parental origin of other imprinted growth-regulating loci on the rate of fetal growth.

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