Genetic Variations within the Insulin Gene Region are Associated with Accelerated Fetal Growth

HISAO OSADA,1,2 KATSUYOSHI SEKI3 and SOUEI SEKIYA3

1Department of Obstetrics and Gynecology, Juntendo University Shizuoka Hospital, Shizuoka, Japan
2Department of Obstetrics and Gynecology, Chiba University Hospital, Chiba University School of Medicine, Chiba, Japan
3Department of Reproductive Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan

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Size at birth has been proposed to be associated with the risk of type 2 diabetes and cardiovascular disease later in life. It is, however, unclear whether this association is attributed to an unfavorable intrauterine environment or to specific genotypes predisposing both altered fetal growth and common diseases in adult life. The aim of this study was to investigate the associations between the neonatal birth size and the genotypes of polymorphic loci within the insulin gene (INS) region, which is susceptible to diabetes mellitus. We analyzed the genotypes of two polymorphic loci; -23HphI and HUMTH01, in 520 pairs of normal Japanese mothers and their neonates, and compared with the somatoscopic characteristics at birth converted into standard deviation scores (SDS) according to sex, parity and gestational weeks at delivery. It was revealed that neonatal -23HphI T allele and HUMTH01 allele10, which are linked to the INS variable number of tandem repeats (VNTR) class III allele, were associated with increased weight, head circumstance, and length at birth. These associations confirmed that variation within the INS region, most probably at the INS-VNTR, influences fetal growth. Furthermore, the finding that the paternally transmitted -23HphI T allele was exclusively correlated with increased size at birth indicates the involvement of an imprinting mechanism. In conclusion, the INS-VNTR class III allele might accelerate fetal growth in a parent-specific manner.

Etiological studies have proved that birth weight is associated with not only postnatal prognosis but also common disorders in adult life, such as type 2 diabetes, dyslipidemia, hypertension, and atherosclerosis (Barker et al. 1990, 1992; Hales et al. 1991). In the past decade, research has principally focused on the role of the intrauterine environment. It has been hypothesized that undernutrition in utero results in permanent reprogramming of fetal metabolism. In other words, genetic factors were not given a place in this “thrifty phenotype” hypothesis (Hales and Barker 1992). Nowadays, however, many lines of evidence suggest that genetic factors are impor-
tant common determinants of birth weight and adult diseases such as type 2 diabetes (McCance et al. 1994; Lindsay et al. 2000). Therefore, it has been proposed that the phenotype of adult diseases such as type 2 diabetes is a reflection of the genotype and the intrauterine and postnatal environment, with different factors having varying predominance in different individuals (Hattersley and Tooke 1999; Frayling and Hattersley 2001).

Several common gene variants have recently been reported to be associated with alterations in birth size. Dunger et al. (1998) demonstrated that the insulin gene (INS) variable number of tandem repeats (VNTR) locus was associated with altered birth weight, but only in neonates who maintained the same postnatal growth rank from 0 to 2 years old. On the contrary, Castaels et al. (1999) reported an association between birth size and a mitochondrial DNA variant, but only in neonates who changed rank in postnatal growth. Cambien et al. (1998) reported that angiotensin I-converting enzyme gene polymorphism modulates in utero growth. In addition, two recent studies have demonstrated associations between birth size and a DNA variant in a gene encoding a member of the G-protein family of signal transmitting proteins (Hocher et al. 2000; Masuda et al. 2002). Of these gene associations, the INS-VNTR is perhaps most convincing, because it has also been shown to correlate closely with type 2 diabetes (Bennett and Todd 1996; Ong et al. 1999). Furthermore, their association has been demonstrated to be transmitted in a paternal-specific manner (Huxtable et al. 2000). This suggests that the imprinted insulin-like growth factor 2 (IGF2) gene, the expression of which is partly controlled by the INS-VNTR (Paquette et al. 1998), may influence both type 2 diabetes and birth weight through its effects on growth and metabolism.

In the present study, we analyzed the genotypes of two polymorphic loci within the INS region in 520 pairs of normal Japanese mothers and their neonates, and compared the genotypes with various somatoscopic characteristics at birth converted into standard deviation scores (SDS) according to sex, parity and gestational weeks at delivery. This study design allowed us to determine whether parent-of-origin effects are manifested in the relationship between fetal growth and genetic variation within the INS region. We found that paternally transmitted neonatal INS-VNTR class III allele was associated with increased weight, head circumference, and length at birth.

**METHODS**

**Subjects**

Five hundreds and fifty five women with singleton fetus, who were followed antenatally at Chiba University Hospital were initially recruited from weeks 12 to 32 of pregnancy. They were non-smoking Japanese women with no remarkable past histories. Twenty-five women were excluded later because of diseases or circumstances that might independently affect fetal growth. These conditions included preterm delivery (less than 36 weeks' gestation), preeclampsia, pregnancy-induced hypertension, proteinuria, impaired glucose tolerance, pre-existing hypertension, fetal anomalies, and placental or umbilical cord abnormalities. Each participant in the study gave informed consent according to a protocol approved by the local institutional review board.

The final study group consisted of 520 pairs of mothers and their neonates. The mothers had a mean age of 28.0 (range: 17 to 40) years, mean height of 157.7 (range: 144 to 177) cm, mean weight before pregnancy of 52.4 (range: 37 to 95) kg, mean body mass index (BMI) before pregnancy of 21.0 (range: 15.2 to 35.8), mean weight gain during pregnancy of 10.3 (range: -1 to 28) kg. The primipara/multipara ratio was 0.95 and male/female infant ratio was 1.11. The parameters of fetal growth evaluation were birth weight, head circumference, and length. Newborn head circumference was defined as the greatest dimension of the head joining the inter-superior space, frontal tuber and occipital tuber. Newborn length was measured by two persons who positioned the neonate in an extended posture on a measuring board containing a stationary head-board, a movable footboard, and a built-in tape measure. The actual measurements of birth weight, length and head circumference were converted into SDS using the mean and standard deviation (SD) values of the standard somatoscopic curves at birth, by sex and by parity (Japanese Ministry of Health and Welfare Research Group 1983, revised in 1994). For instance, birth weight SDS = (birth weight - mean birth weight of neonates for the corresponding gestational week, sex and parity)/(1 SD of neonates for the
corresponding gestational week, sex and parity).

DNA preparation and genotyping

Maternal and neonatal genomic DNA was prepared from the peripheral blood of mother and umbilical cord blood of neonate, respectively, by the phenol-chloroform procedure.

Polymerase chain reaction (PCR) was performed as follows. The PCR mixture (25 μl) contained 2.5 μl of 10 x PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 2.5 mmol/l MgCl₂), 0.5 μl of each dNTP (10 mmol/l), 0.2 μl of Ampli Taq DNA polymerase (Perkin Elmer Cetus, Emeryville, CA, USA) (5 U/μl), and 1 μl each of the sense and antisense primers. DNA was denatured for 4 min at 94°C, and then subjected to 30 thermal cycles at 94°C (1 min), 65°C (1 min) and 72°C (2 min), followed by a final extension at 72°C (10 min) using a thermal sequencer (Zymoreactor, Atto, Tokyo). The primer sequences were (5′-ATTCAAAGGGTATCTGGGCTCTGG-3′) and (5′-GTGGGCTGAAAAGCTCCCGATAT-3′) for the HUMTH01 locus, (5′-TCCAGGACAGGCTGCATCAG-3′) and (5′-AGCAATGGGCGGTTGGCTCA-3′) for the -23HphI locus.

For the analysis of HUMTH01 polymorphism based on the difference in number of AATG repeat units, the amplified products were electrophoresed on 12.5% polyacrylamide gel and visualized by silver staining method. The -23HphI polymorphism was genotyped for the presence (A allele) or absence (T allele) of the restriction site. Among the neonates in the present study, the frequencies of the three genotypes for the -23HphI polymorphism were A/A: 92.7%, A/T: 7.3%, T/T: 0%.

The HUMTH01 is a [AATG]n tetranucleotide repeat microsatellite and is denoted by the number of tetranucleotide repeats; for example, allele 6 has 6 repeats of AATG. Five (alleles 6, 7, 8, 9, and 10) of the HUMTH01 alleles described by Puers et al. (1993) were observed among the subjects in the present study. The frequencies of these alleles in the neonates were as follows; allele 6: 23.1%, allele 7: 25.9%, allele 8: 4.9%, allele 9: 41.8%, and allele 10: 4.3%. Alleles 5 and 11 were not observed in our subjects, and the variant allele 10-1 was not distinguished from allele 10. Alleles 6, 7, 8, 9, and 10 correspond to HUMTH01 alleles Z-16, Z-12, Z-8, Z-4, and Z, respectively, reported by Bennett and Todd (1996).

Analysis of association between -23HphI genotypes and size at birth

The associations of neonatal or maternal -23HphI genotypes with SDSs of weight, head circumference, and length at birth are shown in Table 1. There were no significant differences in birth weight SDSs between -23HphI A/T and A/A genotypes in both neonates and mothers. No significant differences were also observed for head circumference and length SDSs at birth.

Based on the segregation patterns of -23HphI alleles from mothers to neonates, neonatal -23HphI A/T genotype could be subdivided into 2 genotypes; paternal origin (pat A/T) and maternal origin (mat A/T) of T allele. When a neonate and his or her mother both had -23HphI A/T genotype, we interpreted the allele as having maternal origin because of the extremely low frequency (3.6%) of T allele among Japanese. The birth weight SDSs were significantly greater in -23HphI pat A/T neonates than in -23HphI A/A neonates (p < 0.05).
Similarly, SDSs of head circumference and length at birth were significantly greater in -23HphI pat A/T neonates than in -23HphI A/A neonates ($p < 0.01$ and $p < 0.05$, respectively). In contrast, there were no significant differences in SDSs of the three parameters between -23HphI mat A/T and A/A genotypes.

Analysis of association between HUMTH01 genotypes and size at birth

The HUMTH01 polymorphisms were genotyped for the presence or absence of each allele; for example, 10 (+) genotype contains allele 10, and 10 (−) genotype contains no allele 10. It has been reported that HUMTH01 allele 10 is in linkage disequilibrium with INS-VNTR class III allele (McGinnis et al. 1995; Awata et al. 1997). In sub-

| Table 1. Association between -23HphI genotypes and size at birth. |
|-------------|------|------|----------------|------|------|----------------|------|------|----------------|------|------|
| genoype     | n    | weight | head circumference | length |
|             |      | mean | SD     | mean | SD     | mean | SD     | mean | SD     | p     | p     | p     | p     |
| neonate     |      |      |       |       |       |       |       |       |       |       |       |       |       |       |
| A/T         | 38   | 0.112 | 0.778 | 0.073 | −0.025 | 0.864 | 0.077 | −0.041 | 0.708 | 0.277 |<sup>d</sup> |
| pat A/T<sup>b</sup> | 17   | 0.252 | 0.794 |<sup>0.045</sup><sup>d</sup> | 0.217 | 0.703 |<sup>0.006</sup><sup>d</sup> | 0.252 | 0.665 |<sup>0.016</sup><sup>d</sup> |
| mat A/T<sup>c</sup> | 21   | −0.001 | 0.764 |<sup>0.509</sup><sup>d</sup> | −0.222 | 0.946 |<sup>0.981</sup><sup>d</sup> | −0.278 | 0.665 |<sup>0.507</sup><sup>d</sup> |
| A/A         | 482  | −0.109 | 0.844 | −0.270 | 0.739 | −0.019 | 0.829 | −0.253 | 0.727 |<sup>0.608</sup><sup>c</sup> |
| mother      |      |      |       |       |       |       |       |       |       |       |       |       |       |       |
| A/T         | 38   | 0.041 | 0.823 |<sup>0.225</sup><sup>d</sup> | −0.179 | 0.743 |<sup>0.621</sup><sup>c</sup> | −0.253 | 0.727 |<sup>0.608</sup><sup>c</sup> |
| A/A         | 482  | −0.103 | 0.842 | −0.258 | 0.752 | −0.177 | 0.829 | −0.253 | 0.727 |<sup>0.608</sup><sup>c</sup> |

<sup>a</sup>represented by standard deviation scores (SDS).
<sup>b</sup>paternal origin of T allele.
<sup>c</sup>maternal origin of T allele.
<sup>d</sup>versus neonatal A/A genotype.
<sup>e</sup>versus maternal A/A genotype.
There was no case with homozygous status of -23HphI T allele. $p < 0.05$ are underlined.

| Table 2. Association between HUMTH01 genotypes and size at birth. |
|-------------|------|------|----------------|------|------|----------------|------|------|----------------|------|------|
| genoype     | n    | weight | head circumference | length |
|             |      | mean | SD     | mean | SD     | mean | SD     | mean | SD     | p     | p     | p     | p     |
| neonate     |      |      |       |       |       |       |       |       |       |       |       |       |       |       |
| 10 (+)      | 44   | 0.138 | 0.742 |<sup>0.031</sup><sup>d</sup> | −0.002 | 0.870 |<sup>0.048</sup><sup>d</sup> | 0.033 | 0.634 |<sup>0.037</sup><sup>d</sup> |
| pat 10 (+)<sup>b</sup> | 21   | 0.082 | 0.818 |<sup>0.191</sup><sup>d</sup> | 0.067 | 0.749 |<sup>0.038</sup><sup>d</sup> | 0.105 | 0.735 |<sup>0.067</sup><sup>d</sup> |
| mat 10 (+)<sup>c</sup> | 24   | 0.135 | 0.715 |<sup>0.134</sup><sup>d</sup> | −0.110 | 0.985 |<sup>0.591</sup><sup>d</sup> | −0.085 | 0.583 |<sup>0.372</sup><sup>d</sup> |
| 10 (−)      | 476  | −0.014 | 0.847 | −0.275 | 0.735 | −0.020 | 0.834 | −0.202 | 0.834 | −0.202 | 0.834 | −0.202 | 0.834 |
| mother      |      |      |       |       |       |       |       |       |       |       |       |       |       |       |
| 10 (+)      | 45   | 0.067 | 0.810 |<sup>0.100</sup><sup>c</sup> | −0.144 | 0.786 |<sup>0.560</sup><sup>c</sup> | −0.116 | 0.685 |<sup>0.401</sup><sup>c</sup> |
| 10 (−)      | 475  | −0.108 | 0.843 | −0.262 | 0.747 | −0.188 | 0.833 | −0.188 | 0.833 | −0.188 | 0.833 | −0.188 | 0.833 |

<sup>a</sup>represented by standard deviation scores (SDS).
<sup>b</sup>paternal origin of allele 10.
<sup>c</sup>maternal origin of allele 10.
<sup>d</sup>versus neonatal 10 (−) genotype.
<sup>e</sup>versus maternal 10 (−) genotype.
$p < 0.05$ are underlined.
sequent analyses, therefore, each somatoscopic variable was compared between HUMTH01 10 (+) and 10 (−) genotypes.

The associations of neonatal or maternal HUMTH01 genotypes with SDSs of weight, head circumference, and length at birth are shown in Table 2. A significant difference in birth weight SDSs was observed between neonatal HUMTH01 10 (+) and 10 (−) genotypes (p < 0.05). The head circumference and length SDSs at birth were also significantly greater in neonatal HUMTH01 10 (+) genotype than in HUMTH01 10 (−) genotype (p < 0.05). However, there were no significant differences in these SDSs between maternal HUMTH01 10 (+) and 10 (−) genotypes.

Based on the maternal and neonatal allele combinations, neonatal HUMTH01 10 (+) genotype could be subdivided into 2 genotypes: paternal origin (pat 10 [+]) and maternal origin (mat 10 [+]) of allele 10. When a neonate and his or her mother were heterozygotes with respect to allele 10, we interpreted that allele 10 was of maternal origin because of its extremely low frequency (4.3%) among Japanese. One subject who was homozygous for allele 10 was counted as both pat 10 (+) and mat 10 (+). The head circumference SDS was significantly greater in HUMTH01 pat 10 (+) than in HUMTH01 10 (−) genotype (p < 0.05). In contrast, there was no significant difference in weight and length SDSs between HUMTH01 pat 10 (+) and 10 (−) genotypes. No significant differences were observed in SDSs of the three parameters between HUMTH01 mat 10 (+) and 10 (−) genotypes.

**DISCUSSION**

The -23HphI T allele and the HUMTH01 allele10 were associated with increased size at birth. These associations confirmed that variation within the INS region, most probably at the INS-VNTR, influences fetal growth. Furthermore, the finding that the paternally transmitted -23HphI T allele was exclusively correlated with increased size at birth indicated the involvement of an imprinting mechanism.

The INS-VNTR class I/III genotype has been shown to associate with type I diabetes (Bennett et al. 1995), obesity (Weaver et al. 1992), and polycystic ovary syndrome (Waterworth et al. 1997), and is etiological at least for type I diabetes. The INS-VNTR locus is located 596 bp 5′ to the INS initiation site, and is composed of tandem repetition of a 14-15 bp oligonucleotide consensus sequence (Bell et al. 1982). The INS-VNTR has been divided into three distinct classes I, II and III according to the number of repeats of the oligonucleotide (Bell et al. 1984). The extreme rarity of class II alleles in Caucasian populations effectively reduces the VNTR to a biallelic site.

Since there is 99.6% concordance between the -23HphI alleles and the INS-VNTR alleles in people of European descent, the -23HphI genotype implies INS-VNTR class I/III genotype (Bennett and Todd 1996).

The INS-VNTR class I/III genotype has also been shown to associate with growth in early life. Dunger et al. (1998) followed a cohort of 758 term singletons longitudinally from birth to 2 years. Using -23HphI polymorphism, they detected significant genetic associations with size at birth, and that INS-VNTR class III homozygotes had larger mean head circumference than class I homozygotes. These associations were amplified in babies who did not show postnatal realignment of growth, and were also evident for length and weight at birth. Ong et al. (2004) confirmed the association between the INS-VNTR and head circumference at birth by analyzing a second group of children from their original cohort. They concluded that size at birth is influenced by common genetic variation in INS expression, or in neighboring genes regulated by the INS-VNTR. On the contrary, other studies of an English cohort (Mitchell et al. 2004) and a Finnish cohort (Bennet et al. 2004) were unable to reproduce the associations between class III allele and fetal growth. Our results indicate that INS-VNTR class III correlates with increased size at birth, although the same tight linkage disequilibrium should be confirmed for -23HphI polymorphism in Japanese.

The INS-VNTR III allele has been associated with reduced transcription of insulin gene in fetal pancreas (Bennett et al. 1996; Vafiadis et al. 1996).
and of IGF2 gene in placenta (Paquette et al. 1998). Ong et al. (1999) have proposed that reduced insulin secretion or altered placental nutrient transfer may lead to a muscle-specific insulin resistance (Jensen et al. 1988) in the fetus, and this selective peripheral insulin resistance may enhance the anabolic actions of insulin and thereby promote growth (Amiel et al. 1991). They also suggested that this hypothesis should be supported by data indicating associations of the INS-VNTR class III allele with a number of conditions in which insulin resistance is a major feature (Bennett et al. 1995; Bennett and Todd 1996), such as polycystic ovary syndrome and central obesity.

Analysis of INS-VNTR in type 2 diabetic parents-offspring trios has demonstrated that susceptibility to the disease was exclusively mediated by paternally derived class III allele (Huxtable et al. 2000). Conversely, a protective effect of the paternally inherited INS-VNTR class III allele was reported in type 1 diabetic families in the United States (Pugliiese et al. 1994). These reports suggest that parent-of-origin effects inducing variations at this regulatory element is a significant determinant of diabetes susceptibility. Few studies have been performed to assess the potential role of the parent-of-origin of the INS-VNTR allele for fetal growth and their results remain controversial (Lindsay et al. 2003; Ong et al. 2004). In the present study, we revealed that paternally transmitted INS-VNTR class III allele was associated with increased weight, head circumference, and height at birth by segregation analyses. Our results indicated that INS-VNTR class III allele might also affect fetal growth in a parent-specific manner.

We were able to demonstrate an association between neonatal INS-VNTR class III allele and increased size at birth without the condition of case selection based on evaluation after 2 years of follow-up, which Dunger et al. (1998) adopted. One reason for this difference may be more stringent recruitment criteria (4.5% exclusion rate) in our study. Our enrolment strategy minimized the confounding effects of poor intrauterine environment, and consequently more accurately reflected the effects of fetal genes on size at birth. Another reason for the inconsistent findings by us and Dunger et al. (1998) may be the difference in handling of somatoscopic characteristics. Dunger et al. (1998) analyzed the somatoscopic measurements as absolute values, whereas we analyzed values converted to SDS based on the mean and standard deviation of Japanese fetal growth curves by sex and by parity. The use of SDS adjusts for the variations arising from differences in sex, parity and gestational week, and provides more accurate analysis.

The polymorphic HUMTH01 microsatellite, located in the first intron of the tyrosine hydroxylase (TH) gene is characterized by a tetranucleotide core motif, and alleles are expressed as different numbers of repeat tetranucleotide units (O’Malley and Rotwein 1988). The frequencies of HUMTH01 alleles in the present study were consistent with those in a previous study on Japanese population (Awata et al. 1997). It has been reported that HUMTH01 allele 10 is in linkage disequilibrium with INS-VNTR class III allele and HUMTH01 alleles 9, 7, 6 are associated with INS-VNTR class I subclasses; IS, IM, and IL alleles, respectively (McGinnis and Spielman. 1995; Awata et al. 1997). The association of neonatal HUMTH01 allele 10 with increased size at birth thus reconfirms that INS-VNTR class III allele correlates with increased fetal growth.

In the present study, the parental origin of HUMTH01 allele 10 and -23HphI T allele was determined by comparing mother-neonate pairs without paternal information. This strategy has limitations compared with analysis of complete parents-offspring trios, especially for biallelic polymorphisms. When a neonate is heterozygous for the above alleles and his or her mother also has these alleles, we interpreted the alleles as having maternal origin disregarding the possibility that his or her father also has these alleles. This possible overestimation may be lessened by the extremely low frequencies of these alleles in Japanese population: 3.6% for -23HphI T allele and 4.3% for HUMTH01 allele 10. For instance, 21 of -23HphI mat A/T neonates (Table 1) had 7.3% possibility that their fathers are also hetero-
zygous for T allele. Since a half of them should have paternal origin of T allele, the possible number of \(-23HphI\) pat A/T neonates was calculated to be less than 1 as follows: 21 neonates \(\times 0.073 \times 0.5 = 0.76\) neonates. This strategy could be used for only Japanese population having extremely discordant gene frequency. Although haplotype prevalences of the human insulin gene are thought to be similar across non-African populations (Stead et al. 2003), the \(-23HphI\) genotype distribution in this study is different from those in Caucasian populations (Bennett and Todd 1996; Dunger et al. 1998). It is generally true that traditional transmission testing by the transmission disequilibrium test has increased type I statistical errors when sample from one of the parents is missing. There are still other problems involving the inability of analyzing the effect of untransmitted allele (Bennett et al. 1997). Further studies using complete parents-offspring trios to investigate the correlation between common variance in the INS region and fetal growth are required.

Given the role of insulin and IGF2 in growth and metabolism, gene variations at the \(TH-INS-IGF2\) locus have been expected to explain the observed relationship between intrauterine development and disease in adult life. In the present study we succeeded to demonstrate that at least one pathway contributes toward controlling fetal growth, partially in a parent-specific manner. However, this finding is applied to less than 5% of Japanese population. There should be the possibility that more complex mechanisms including epigenetic regulation are involved in fetal development and progress of diseases in later life. Further studies of this model locus will provide more detailed information about the “thrifty genotype” hypothesis (Neel 1962).

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