Pediatric Research (2009) 66(2):135-139.

Paternal Allele of IGF2 Gene Haplotype CTG Is Associated With Fetal and Placental Growth in Japanese

Nagaya, Ken ; Makita, Yoshio ; Taketazu, Genya ; Okamoto, Toshio ; Nakamura, Eiki ; Hayashi, Tokitsugi ; Fujieda, Kenj

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Running title: *IGF2* polymorphism in fetal growth

*Ken Nagaya, Yoshio Makita, Genya Taketazu, Toshio Okamoto, Eiki Nakamura, Tokitsugi Hayashi, Kenji Fujieda,

Department of Pediatrics, Asahikawa Medical College, Asahikawa, Hokkaido, 078-8510, Japan
Telephone: +81-166-68-2481
FAX: +81-166-68-2489

Address all correspondence and requests for reprints:
Ken Nagaya, MD
E-mail: nagaya5p@asahikawa-med.ac.jp
Ground mail: Department of Pediatrics, Asahikawa Medical College, 2-1-1-1 Midorigaoka-higashi, Asahikawa, Hokkaido, 078-8510, Japan
Telephone: +81-166-68-2481
FAX: +81-166-68-2489

Statement of financial support: This work was supported by KAKENHI (17591137); Grant-in-Aid for Scientific Research (C) by Japan Society for the Promotion of Science (JSPS).

Category of study: Clinical study

Word count of abstract: 186 words

Word count of manuscript: 4085 words

Abstract

IGF-II associates with feto-placental growth in rodent and human. We determined three tag-SNPs (single nucleotide polymorphisms) to investigate haplotype frequency of *IGF2* relative to size at birth in 134 healthy Japanese infants. In addition, a total of 276 healthy infants were investigated to determine if common genetic variation of *IGF2* might contribute to feto-placental growth using haplotype analysis. Further, quantitative methylation analysis of the *IGF2/H19* was performed using the MassARRAY Compact system. In the initial study, the frequency of haplotype CTG from the paternal allele in small for date (SFD) infants was significantly higher than that in non-SFD infants ($p = 0.03$). In a second study, the CTG haplotype infants exhibited significantly lower birth length, weight and placental weight compared to non-CTG infants. Further, the number of infants less than -1.5SD (standard deviation) birth weight in CTG haplotype was higher than those in non-CTG infants. There was no significant difference in the methylation status of *H19/IGF2* in the two haplotypes. In conclusion, inheriting the *IGF2* CTG haplotype from a paternal allele results in reduced feto-placental growth, but it is not associated with the methylation status of *IGF2/H19*.

Abbreviations: AFD (appropriate for date), HFD (heavy for date), SFD (small for date), SRS (Silver-Russell syndrome)

Introduction

Numerous factors have been identified as influencing size at birth. Fetal growth can be determined by maternal, placental and fetal factors. Although abnormalities of these factors may cause IUGR, it is difficult to clarify the etiology for the majority of IUGR.

Intrauterine hormones and growth factors play an important role for regulation of fetal growth. Of those, the IGF family has been implicated in the pathogenesis of IUGR (1). IGF-I and IGF-II, as well as their receptors, are synthesized in a number of fetal and placental tissues and are involved in autocrine/paracrine stimulation of cellular proliferation and differentiation in development (2). The birth weight of the *IGF1* knockout mouse is 60% of wild type and that of the *IGF1 receptor* knockout mouse is 75% of wild type (3). This is consistent with the reduced birth weights seen in human with mutations or deletions in *IGF1* (4) and *IGF1 receptor* genes (5). Further, polymorphisms of the *IGF1* gene are related to pre- and postnatal growth (6). The birth weight of the *IGF2* knockout mouse is also 60% of wild type and their placental weights are 75% of wild type (7). In rodents, it is clear that IGF-II is associated with fetal growth, especially placental growth (8). In human, *IGF2* is located at chromosome 11p15.5, which contain a cluster of imprinted genes that are crucial in the control of fetal growth. This cluster

includes paternally expressed genes (such as *IGF2* and *KCNQ1OT1*) and maternally expressed genes (such as *H19* and *CDKN1C*) (9). Studies of several transgenic or knockout mouse models (3,7,8,10), and Beckwith-Wiedemann syndrome (BWS) (11) or Silver-Russell syndrome (SRS) (12) in humans, indicate that this region has a key role in fetal development. However, it is not clear whether common genetic variations of *IGF2* contribute to feto-placental growth, the way some studies showed an association between polymorphisms of the *IGF1* gene and size at birth (6,13,14). Therefore, we investigated the relation between *IGF2* gene polymorphisms and feto-placental growth using haplotype analysis.

Materials and Methods

Subjects

We first investigated haplotype frequency of *IGF2* relative to size at birth in 134 pairs of healthy Japanese infants and their mothers in the initial study. Subsequently, a total of 276 infants including 134 infants in initial study were finally investigated for the association between polymorphism of the paternal allele of *IGF2* gene and feto-placental growth by haplotype analysis. All infants were born at ≥ 35 weeks of gestational age in The Center for

Infant and Maternal Health at the Asahikawa Medical Collage or the Mori Obstetrics and Gynecology Hospital in Asahikawa between October 2004 and September 2007. Their mothers did not have any complications including pregnancy-induced hypertension. The birth weight, length and head circumference and its SDS according to Japanese standards (15) was normally distributed. The gestational age was corrected with a crown-rump length in the early gestation by fetus echography. Small for date (SFD) infant was defined as the infant that SDS of birth weight and birth length were below -1.5 SD.

The study was approved by the local institutional review board (the ethics committees in Asahikawa Medical College), and an informed parental consent was obtained prior studying all cases.

Identification of IGF2 gene polymorphism from the paternal allele

Genomic DNA was extracted from cord and maternal whole blood using standard protocols. The samples from 134 infants in the initial study were analyzed for single nucleotide polymorphisms (SNPs) from a database of Japanese Single Nucleotide Polymorphisms (JSNP: http://snp.ims.u-tokyo.ac.jp/index_ja.html) by PCR followed by whole length direct sequencing.

Thereafter, three tag-SNPs were extracted to identify haplotypes with linkage disequilibrium test by SNPAlyse software (Dynacom, Yokohama, Japan) (figure1): C-1176T (rs1003484), T-21865C (rs3741211) and A-21111G (rs3741206). The selection criteria for tag-SNPs were pairwise correlation with r-square and D' >0.8. Subsequently, total of 276 samples were analyzed for these 3 tag-SNPs by similar methods.

Genomic DNA was amplified using the following primer sets: For C-1176T, L-GAGGCACATGGATTGGAGTC and R- GGCCTTTCTCATTCCCATTT; for T-21865C, L-CCTCACTGGCCTCGTCAAG and R- CACAAGCTCG GTGGTGACTC; for A-21111G, L-GAACACACAGCTGTCTTCACAAG and R- GACACACAGC TCTGCTTGACG. Target sequences were amplified in a PCR with Takara PCR Amplification Kit (TAKARA BIO INC. Japan). PCR amplification was performed using 100 nag of genomic DNA in a 12.5μl volume with 1μl of 2.5mM dNTPs, 0.625μl of 25mM MgCl2, 1μl of 10pmol/l each primer, and 0.5U Taq DNA polymerase. For all primer pairs, after heating to 94 °C for 4 minutes, 35 cycles were performed at 94 °C for 30 second, 55 °C for 30 second, and 72 °C for 30 second before a final step of 72 °C for 7 minutes. PCR products were electrophoreses by the ABI Prism 310 Genetic

Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed using the GeneScan and customized Genotyper software packages (Applied Biosystem).

Haplotype analysis (estimation of maximum likelihood haplotype frequencies and differences in haplotypic distribution between SFD infants and AFD (appropriate for date)/HFD (heavy for date) infants) was carried out using Alrequin software (http://anthropologie.unige.ch/arlequin/methods.html). The haplotype counts were estimated based on EM estimates of haplotype frequency. The permutation test was conducted to confirm differences in haplotypic distribution using Analyze software (DYNACOM, Yokohama, Japan, http://www.dynacom.co.jp). Furthermore, because the *IGF2* gene is an imprinted gene expressed from the paternal allele, we deduced the haplotype of the paternal allele as a result of a combination of the infant and maternal diplotypes according to table 1.

Quantification of CTCF site 6 and H19 DMR methylation by MassARRAY

Quantitative methylation analysis of the *IGF2/H19* was performed using the MassARRAY Compact system (Sequenom, San Diego, CA). This system utilizes mass spectrometry (MS) for the detection and quantitative analysis of DNA methylation using homogenous MassCLEAVE

base-specific cleavage and matrix-assisted laser depsorption/ionization time-of-flight MS (16). Genomic DNA was converted with sodium bisulfite using EZ DNA methylation kit (Zymo Reseach). Biasulfite-treated DNA was subjected to *IGF2/H19* specific PCR to CpG regions CTCF6 and *H19* DMR. The primers were (underlined nucleotides are gene-specific sequences): CTCF6, L-aggaagagagGGTGTTTTAGTTTTATGGATGATGG, R-cagtaatacgactcactatagggagaaggctAAATATCCTATTCCCAAATAACCCC; *H19* DMR, L-aggaagagagTTTTTATTAAAGGTTAAGGTGGTGAT, R-cagtaatacgactcactatagggagaaggctAATAACACAAAAAACCCCTTCCTAC). Each reverse primer has a T7-promotor tag for *in vitro* transcription (5'-cagtaatacgactcactatagggagaaggct-3'), and the forward primer is tagged with a 10-mer to balance PCR (5'-aggaagagag-3'). Amplification of 1 μl of bisulfite-treated DNA was performed using HotStar Taq polymerase (Qiagen) in a 5-μl reaction volume using PCR primers at a 200 nM final concentration. PCR amplification was performed with the following parameters: 94 °C for 15 min hot start, followed by denaturing at 94 °C for 20 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min for 45 cycles, and final incubation at 72 °C for 3 min. After shrimp alkaline phosphatase treatment, 2 μl of the PCR products were used as a template for in vitro transcription and RNase

A cleavage for the T-reverse reaction as per the manufacture's instructions (Sequenom). The samples were conditioned and spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser (Samsung, Irvine, CA), followed by spectral acquisition on a MassARRAY Analyzer Compact matrix-assisted laser desorption/ionization time-of-flight MS (Sequenom). The resultant methylation was analyzed by the EpiTyper software v1.0 (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites.

Statistical analysis

Student's t test was used to estimate the significance of differences between two groups. Comparisons of the genotype or allele frequencies between groups were performed with a $\chi 2$ test. The level of significance was set at $p=0.05$.

Results

In the initial study, the genotype frequencies of *IGF2* gene polymorphisms from the paternal allele, as summarized in table 2, were in Hardy-Weinberg equilibrium. Overall, 48 infants

(35.8%) were haplotype TTA, and haplotype CCA and CTG were 29 (21.6%) and 26 (19.4%), respectively. A total of 76.9% was occupied in these 3 haplotypes. Further, there was statistically significant difference in individual paternal *IGF2* genotype frequencies observed between SFD infants and AFD/HFD infants evaluated by chi-square analysis. Paternal haplotype CTG in SFD infants was significantly higher than that in HFD/AFD infants. There was similar distribution for total of 276 infants in subsequent study including 134 infants in initial study. Thus, we investigated feto-placental growth by haplotypes between *IGF2* paternal allele haplotype CTG and non-CTG in the subsequent study.

In this study, the characteristics of infants were not significantly difference in the two groups (CTG and non-CTG infants). However birth weight, birth length and placental weight in CTG infants were lower than that in non-CTG infants (table 3). Although SDS of birth weight and length were not related to the CTG haplotype, the number of infants less than -1.5SD birth weight in CTG haplotype was higher than those in non-CTG infants. Moreover, the placental weight corrected for birth head circumference, which was almost maintained stability as gestational age, was significantly lower in case of the CTG haplotype. There were no significant differences in birth head circumference or placenta/birth weight ratio.

The expression of *IGF2* genes is imprinted. Although the *IGF2* gene shares an enhancer with the *H19* gene, *H19* is expressed only from the maternal allele, and *IGF2* only from the paternally inherited allele (17-19). An imprinted control region (ICR) of paternal specific methylation upstream of *H19* appears to be the site of epigenetic imprinting of these genes. The methylation status of ICR acts as a CTCF boundary that blocks the interaction of *IGF2* with the enhancer. This enhancer-blocking activity is lost when ICR is methylated, thereby allowing expression of *IGF2* paternally. Because the previous report of SRS demonstrated the hypomethylation of CTCF site 6 and *H19* DMR in *H19/IGF2* (12), we assessed the methylation status of CTCF site 6 and *H19* DMR, located upstream of *H19*, by MassARRAY. However, there are no significant differences in CTCF site 6 and *IGF2* DMR region between haplotype CTG and non-CTG infants (figure2). Thus, there were no relationship between the methylation status of *H19/IGF2* and paternal haplotypes in this study.

Discussion

The major finding of this study is that the *IGF2* haplotype CTG from the paternal allele (tag SNPs: C-1176T, T-21865C and A-21111G) is associated with a reduction in placental weight,

birth weight and birth length. However this haplotype was not associated with the methylation status of CTCF site 6 and *H19* DMR in *H19/IGF2*.

The ratio of IGF-II to the soluble form of IGF2R is related to birth weight (20). The *H19* RNA (ribonucleic acid) has growth suppressing function through inhibiting translation of *IGF2* RNA (21). Moreover, recent studies have reported that genetic variation in the *IGF2 receptor* gene (22) and *H19* gene (23) are associated with size at birth in human. These results suggest that IGF-II may also have a role in regulating fetal growth in human. In the rodent, IGF-II is associated with fetal growth, especially placental growth (8). In mice with homozygous deletion of the *IGF2* gene, fetal growth is impaired, and pups have birth weight approximately 60 % those of wild type animals in both instances (24). Moreover, the mice with homozygous deletion of the *IGF2* gene are accompanied by small placental size (25). Our findings also support the notion by demonstrating the relation between *IGF2* polymorphism from paternal allele and feto-placental growth in human.

SRS was characterized by severe pre- and postnatal growth retardation, congenital hemihypertrophy and characteristic facial features, including triangular shaped face with a broad forehead and pointed, small chin with a wide, thin mouth (12). Several individuals with

clinically typical SRS were identified by an epimutation (demethylation) in the telomeric imprinting center region ICR1 of the 11p15 region. The epigenetic defect was associated with relaxation of imprinting and biallelic expression of *H19* and downregulation of *IGF2*. These findings provided new insights into the pathogenesis of SRS by strongly suggesting importance of the 11p15 imprinted region. In contrast, we could not demonstrate any relationship between the methylation status of CTCF site 6 and *H19* DMR in *H19/IGF2* and paternal haplotypes. Thus, our findings could not reveal why *IGF2* haplotype CTG from paternal alleles is associated with feto-placental growth. This may suggest that different mechanisms may operate in fetal growth for small normal babies compared to SRS baby.

There are other differentially methylated regions in *IGF2/H19* (H19 promoter, *IGF2* DMR2, CTCF site1-5) and human chromosome 11p15.5 cluster contains other imprinted gene (*KCNQ1OT1, CDKN1C*) that control growth and development (26,27). Further, three systems, including DNA methylation, RNA-associated silencing and histone modification, are used to initiate and sustain epigenetic silencing. They interact and stabilize each other. Disruption of one or other of these interacting systems can lead to inappropriate expression or silencing of genes, resulting in 'epigenetic diseases' (28). Other modifications of the *IGF2* gene except for

DNA methylation may operate. Therefore, further research is needed to confirm whether paternal *IGF2* haplotype CTG associate with epigenetic modifications.

We measured IGF-I, IGF-II and IGF binding protein (IGFBP)-3 levels in cord blood in only 142 of 276 infants in both groups (29 of CTG infants and 113 of non-CTG infants). Although these data was only partial, there was no difference in IGF-I, IGF-II and IGFBP-3 levels in cord blood between haplotypes (data was not shown). However, although we could not examine the expression of genes in placentas, the expression of *IGF-2* in placenta may decrease in CTG infants. Recently, Constancia et al. demonstrated that a deletion from the *IGF2* gene of a transcript from the P0 promoter specifically expressed in the labyrinthine trophoblast of the murine placenta leads to reduced growth of the placenta, followed several days later by fetal growth restriction (8). Monk et al. identified a human equivalent to the mouse P0 transcript that is paternally expressed in all fetal and placental material except pancreas. Further, they have found the human *IGF2-P0* transcript start site that has promoter activity (29). Similar to the mouse, the human *IGF2-P0* transcript is paternally expressed. However, its expression is not limited to placenta. Similar to the mouse, if placental-specific transcripts in human were identified as the mouse *IGF2-P0* transcript, the dysfunction or down-regulation of these

transcripts may explain our findings that *IGF2* haplotype CTG from paternal alleles associate with feto-placental growth with no difference in cord blood IGF-II. Although further research must be investigated the expression of *IGF2* in placenta, we could not prepare for placental examination in current study.

In previous human studies, IGF-II levels at birth had no significant relationships with size at birth (20). These associations with growth factor levels at birth in term deliveries may be weakened by the inability to consider gestational stage-dependent effects on fetal growth. The ratio of IGF-II to the inhibitory soluble IGF2R was significantly related to birth weight, ponderal index, and placental weight in one study (20). We could not show these associations because we did not measure soluble IGF2R level in cord blood.

For preliminary postnatal growth assessment, we investigated by questionnaires the parents of 215 infants who were 18 month of age. In Japan, the skilled public health nurses performed accurately the routine examinations for infants' growth and development at key ages (4, 18 and 36 months) in local communities. We assessed postnatal growth of infants at 18 month of life at the routine examinations. We received replies from only 87 (15 of CTG infants and 72 of non-CTG infants) parents to the questionnaires. There were no significant differences in body

weight in both groups. However height at 18 months in CTG infants was significantly shorter than that in non-CTG infants (78.4 ± 2.7 .vs 80.3 ± 2.8, p=0.02). Moreover, SDS of height and body mass index (BMI) in CTG infants were significantly lower than those in non-CTG infants at 18 months of age (-1.1 ± 0.9 .vs -0.4 ± 0.9, p=0.01, 9.2 ± 2.7 .vs 10.4 ± 1.1, 0.004, respectively). The infants with *IGF2* haplotype CTG did not appear to catch up by 18 month of age. Although our follow-up data was only partial, it suggested that paternal *IGF2* haplotype CTG might be associated with postnatal growth by 18 month of age. This result is incompatible with the postnatal growth of *IGF2* knockout mice (7). However, it could not be concluded that this haplotype associated postnatal growth in human because of low follow-up rate and duration.

Recent research has linked low birth weight with an increased prevalence of hypertension, hypercholesterolemia, and cardiovascular disease later in life (30). Common variants in *IGF2* gene could contribute to fetal growth restraint and compensatory postnatal catch-up growth. O'Dell et al. reported that *IGF2 Apal* AA genotype was associated with lower mean body weight and body mass index but higher serum IGF-II levels than GG genotype in middle-aged males (31). Furthermore, relatively higher IGF-II levels were also associated with a reduced risk

of gaining weight (32). Sandhu et al revealed that individuals who developed obesity had lower baseline IGF-II levels. Thus, because it is suggested that IGF-II is associated body size in adulthood, it is important to clarify the association between *IGF2* gene haplotype CTG from paternal allele and metabolic disease in later life in future.

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Figure legends

Figure1） Tag SNPs on *IGF2* sellected after Linkage Disequilibrium Study of 48 contols

Figure2） Schematic diagram showing the position of CpG islands (CTCF6, DMR) at *IGF2/H19* gene and methylation status of these sites on paternal haplotypes.

The numbers on the X-axis are the following methylation sites in CTCF6 and H19 DMR resion. The black boxes are CTG infants and the grey boxes are non-CTG infans.

CTCF6

GGTGTCCCAGTTCCATGGATGATGGGGATCT**CG**GCCCTAGTGTGAAACCCTTCT

CG C A G G G T C T C T G G C A G G C A C A G A G C C **CG**

GGGGCTCTTGCATAGCACATGGGTATTTCTGGAGGCTTCTCCTT**CG**GTCTCAC

CGCCTGGATGGCA**CG**GAATTGGTTGTAGTTGTGGAAT**CG**GAAGTGGC**CG****CG**

CG G **CG** G C A G T G C A G G C T C A C A C A T C A C A G C C **CG** A G C C **CG**

CCCCAACTGGGGTT**CG**CC**CG**TGGAAA**CG**TCC**CG**GGTCACCCAAGCCA**CG****CG**T

CG C A G G G T T C A **CG** G G G G T C A T C T G G G A A T A G G A C A C T C

DMR

CCCTCACCAAAGGCCAAGGTGGTGAC**CG**A**CG**GACCCACAG**CG**GGGTGGCTGGGGGAGT**CG**

AAACT **CG** CCAGTCTCCACTCCACTCCCAAC **CG** TGGTGCCCCA **CG** **CG**

GGCCTGGGAGAGTCTGTGAGGC**CG**CCCAC**CG**CTTGTCAGTAGAGTG**CG**CC**CG** **CG**AGC**CG**

TAAGCACAGCC **CG** GCAACATG **CG** GTCTTCAGACAGGAAAGTGGC **CG** **CG** AATGGGAC **CG**

GGGTGCCCAG**CG**GCTGTGGGGACTCTGTCCTG**CG**GAAAC **CG** **CG**GTGA **CG** AGCACAAGCT

CGGTCAACTGGATGGGAAT **CG** GCCTGGGGGGCTGGCAC **CG** **CG**CCCACCAGGGGGTTTG **CG**

GCACTTCCCTCTGCCCCTCAGCACCCCACCCCTACTCTCCAGGAA **CG** TGAGTTCTGAGC **CG**

TGATGGTGGCAGGAAGGGGCCCTCTGTGCCATC

Table1) The paterns guessed as offspring IGF2 paternal haplotype

offspring diplotype	maternal diplotype	paternal haplotype
AA	AA	**A**
AA	AB	**A**
A**B**	AA	**B**
AB	AB	unknown
AB	BB	**A**
BB	AB	**B**
BB	BB	**B**

diplotype: combination of haplotypes

Table2) Frequency of offspring *IGF2* haplotypes from paternal allele

haplotypes	overall	HFD/AFD	SFD	*p*
	(n=134)	(n=105)	(n=29)	
TTA	48(35.8)	38(36.2)	10(34.5)	ns
CCA	29(21.6)	25(23.8)	4(13.8)	ns
CTG	26(19.4)	15(14.3)	11(37.9)	0.03
CTA	3(2.2)	2(1.9)	1(3.5)	-
TCA	1(1.5)	2(1.9)	0(0)	-
others	3(2.1)	3(2.9)	0(0)	-
unknown	23(17.2)	20(19.0)	3(10.3)	ns

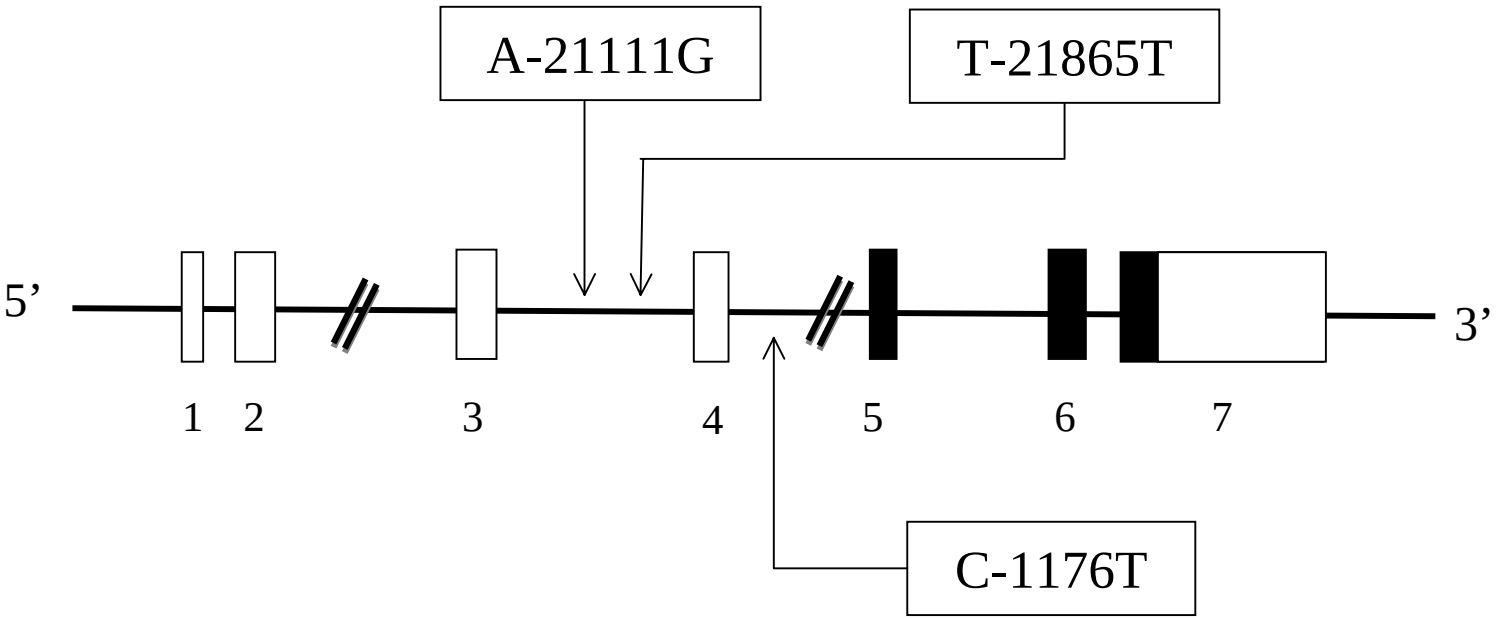
n (%)

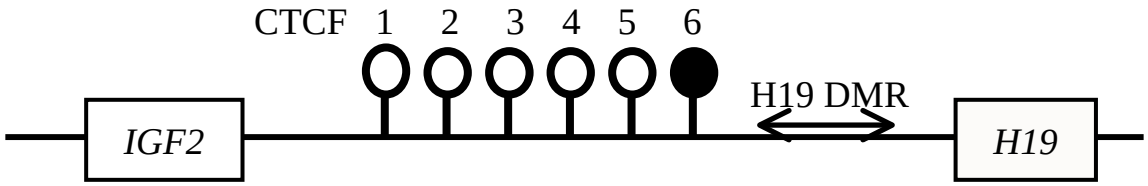
AFD: appropriate for gestational age, HFD: heavy for gestational age, SFD: small for gestational age

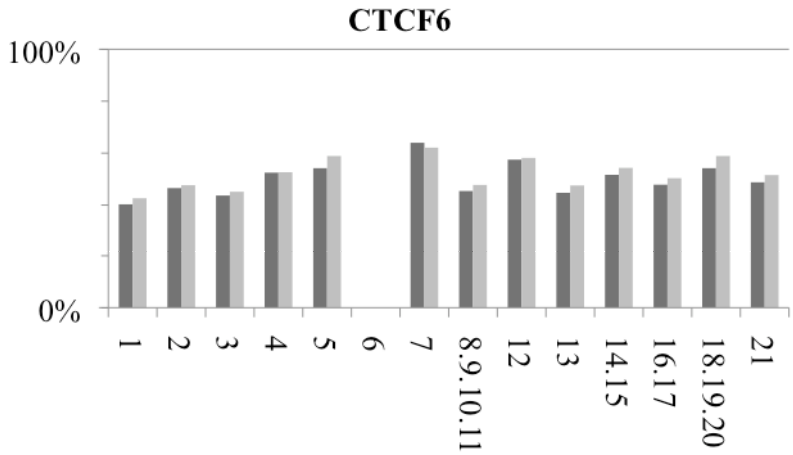
% : haplotype frequency in each groups

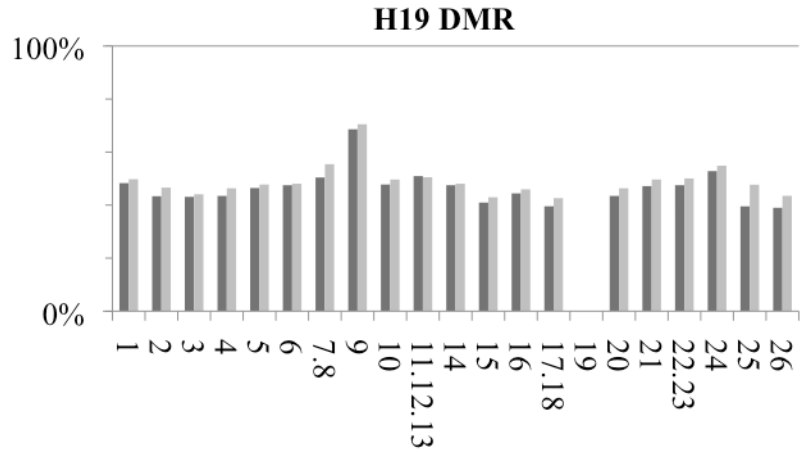
Table3) Characteristics of the final study population by offspring IGF2 paternal allele haplotypes (mean ± SD)

	non-CTG (221)	CTG (55)	*p*
Gestational age (wks)	39.4 ± 2.9	39.0 ± 1.3	ns
Sex (male)	100	23	
Mother			
height (cm)	158.5 ± 5.2	157.6 ± 5.3	ns
birth weight (g)	3124.9 ± 437.2	3032.4 ± 387.5	ns
primigravida	116	35	ns
smoking	103	27	ns
Father			
height (cm)	171.9 ± 5.0	172.3 ± 5.1	ns
birth weight (g)	3230.5 ± 500.9	3156.3 ± 440.5	ns
Birth weight (g)	3009.5 ± 414.4	2850.1 ± 509.3	0.02
SD score	-0.2 ± 1.0	-0.4 ± 1.0	ns
No of SDS ≤ -1.5	23 (10.4%)	11 (20.0%)	0.048
Birth length (cm)	48.3 ± 2.0	47.6 ± 3.0	0.04
SD score	-0.6 ± 0.9	-0.7 ± 1.0	ns
No of SDS ≤ -1.5	36 (16.3%)	11 (20.0%)	ns
Birth head circumference (cm)	33.6 ± 1.3	33.5 ± 1.4	ns
SD score	0.0 ± 0.8	0.0 ± 0.6	ns
Placental weight (g)	582.7 ± 109.0	542.7 ± 126.1	0.02
Placenta/head circumference (g/cm)	17.2 ± 2.9	16.1 ± 3.4	0.03


A-21111G
T-21865T
5’
3’
1
2
3
4
5
6
7
C-1176T


CTCF 1 2 3 4 5 6
H19 DMR
IGF2
H19


CTCF6
100%
0%
1
2
3
4
5
6
7
8.9.10.11
12
13
14.15
16.17
18.19.20
21


H19 DMR
100%
0%
1
2
3
4
5
6
7.8
9
10
11.12.13
14
15
16
17.18
19
20
21
22.23
24
25
26