Abstract: Loss of imprinting (LOI) of insulin-like growth factor II gene (IGF2) is an epigenetic abnormality associated with human diseases. However, little is known about the characteristics of IGF2 imprinting in newborn cord blood cells. METHODS: A total of 923 cord blood samples from term singletons and related clinical data were collected; IGF2 imprinting status in 273 specimens were successfully analyzed using RT-PCR and restriction fragment length polymorphism. RESULTS: LOI of IGF2 was detected in 20.9% of informative samples. The mean birth weights (BW) in the LOI and the normal imprinting groups were 3462.7 ± 460.2 g and 3363.7 ± 427.7 g, respectively. The abdominal perimeters in the LOI group tended to be larger than that in the normal imprinting group. Pregnancy complications, delivery modes, newborn diseases, occurrences of malignant tumors in grandparents, and other maternal factors were not associated with LOI of IGF2. 22.2% of the infants with IGF2 LOI also showed LOI in their father’s lymphocytes while 21.4% in their mother’s lymphocytes. CONCLUSIONS: About 20% of Han Chinese newborns indicated LOI of IGF2 in their cord blood lymphocytes that may represent the epigenetic characteristics in this ethnic group. While IGF2 LOI tends to be weakly inherited between...
parents and offspring, abnormal imprinting seems to be statistically unrelated with phenotypes of newborns, although it might have an association with later phenotypes of infants.

**Keywords:** IGF2, genomic imprinting, cord blood, birth weight, Han Chinese

1. **Introduction**

Genomic imprinting is an important biological phenomenon defined by allelic-specific epigenetic modifications, brought about by the parent-of-origin specific gene expression. In other words, the expression of the imprinted genes depends on whether they are inherited maternally or paternally [1, 2]. Genomic imprinting is tissue-specific, phase-specific and probably race-specific in some cases [3]. The IGF2 gene encodes for insulin-like growth factor II, which is structurally homologous to insulin and displays growth promoting and metabolic effects on various cell types. IGF2 is the first gene discovered to be imprinted and expressed exclusively from the paternal allele in human. It is also the first imprinted gene found to display loss of imprinting (LOI) or aberrant imprinting in human diseases. Evidently, LOI or reactivation of the normally silent maternal allele of IGF2 is associated with an increase of IGF2 expression that may subsequently play an important role in human diseases [4]. Research shows that embryos were less likely to survive when both the paternal and the maternal alleles of IGF2 were not expressed. In another case, if the silent maternal allele was activated while the normally expressed paternal allele remained silent, abortion seemed unavoidable as result of the trophoblast cells’ failure to grow [5]. Research also exhibits that about 25% Beckwith-Wiedemann syndrome (BWS) were related to LOI of IGF2 [6] while IGF2 LOI were also associated with various tumors [1]. This study focuses mainly on investigating the imprinting status of IGF2 in cord blood lymphocytes from Han Chinese newborns, to figure out the epigenetic features this ethnic group and evaluate the possible associations between IGF2 LOI and infant phenotypes as well as other related factors.

2. **Materials and Methods**

2.1 **Collection of cord blood samples and clinical data**

A total of 4 ml of cord blood samples were collected from 923 Han Chinese term singleton newborns at Nanjing Drum Tower Hospital, affiliated with Nanjing University Medical School, in China from August 2005 to March 2006. This study was approved by the Scientific Research Ethics Committee of Drum Tower Hospital and informed consents were obtained from all participants.

Clinical data were obtained from involved parents and newborns, which included parents family history, heights of mothers and their weight gain during pregnancy, obstetrical history, levels of hemoglobin, hematokrit, and other biochemical profiles in second and third-trimester including albumin, total protein, fasting plasma glucose, triglyceride, cholesterol, L-cholesterol, H-cholesterol, apoprotein A1, apoprotein B in serum. Complications of pregnancy and childbirth events were also noted. In addition, a physical examination was performed for all newborns and their birth weights,
heights, and head, chest, and abdominal circumferences, and shoulders radical lines were recorded. Moreover, blood glucose levels in cord blood and heels 2 hours after birth, and bilirubin percutaneously were measured. The neonatal screening program of congenital hypothyrosis, using dissociation-enhanced lanthanide fluroimmunoassay (DELFIA) and phenylketonuria through the fluorescent assay of all normal neonates, were then carried out. Each newborn was followed up for 30 days after birth; after the 30-day period, ultrasound scanning was performed to check the infants’ livers and kidneys.

2.2 Nucleic acid preparation

Nucleated cells from cord blood and peripheral blood samples were separated using gradient centrifugation with Ficoll-Hypaque. DNA was extracted with the proteinase K/phenol chloroform protocol as described previously [7], while RNA was extracted with Trizol (invitrogen), following the manufacturer’s instruction.

2.3 Screening for informative samples

The IGF2 exon 9 Apal polymorphism site was detected using PCR. The primer sequences are as follows: forward primer: 5’-CTTGGAATTGGATCGAGCTCAATTTGG-3’; reverse primer: 5’-GGTCGTGCCAA TTACATTCTCA-3’. DNA was denatured at 94 °C for 3 min, amplified by 36 cycles of 94 °C for 1 min, 55 °C for 40 sec, and 72 °C for 40 sec. PCR products were digested for at least 4 hours with endonuclease Apal at 30 °C (10 unites of enzyme for 8 µl PCR product and 1.5 µl buffer in a final volume of 15 µl); ran through a 2% agarose gel and visualized with ethidium bromide. The A (not digested by Apal) and B alleles (digested by Apal) are 292 bp and 229 bp, respectively. Samples of heterozygous A/B at the Apal polymorphism were selected to synthesize cDNA.

2.4 RNA reverse transcription and IGF2 imprinting assay

In order to avoid any possible gDNA contamination, the RNA was treated with RNase-free DNase I (Invitrogen) and RNase inhibitor (Promega) for 25 min at 25 °C, followed by heating for 15 min at 65 °C, to inactivate the DNase I, and then the treated RNA was extracted with Trizol once. cDNA was synthesized by SuperScriptIII (invitrogen) with 1 µl of random hexamer, 1 µl of 10 mmol/L dNTP, 4 µl of 5 X buffer, 2 µl of DTT in a total volume of 15 µl at 25 °C for 25 min, and at 42 °C for 50 min. Reverse transcriptase was inactivated through heating for 10 min at 70 °C. cDNA was amplified by PCR using the primers for IGF2, under the same conditions used for gDNA with the only exception of increasing the amount of cDNA to two µl. A total of 12.5 µl of RT-PCR product was over digested with Apal for at least 4 hours at 30 °C, under the same conditions as above, ran on a 2% agarose gel, and visualized with ethidium bromide using Labwork 4.5 software (UVP). Loss of imprinting (LOI) was assigned when the ratio of the more-abundant to less-abundant allele was less than 3:1[7].

To ensure the absence of gDNA contamination, all RT-PCR analysis were performed in duplicate, that is, in both the presence and absence of reverse transcriptase.

2.5 Statistical analysis
The various groups were compared using the $\chi^2$ test. Differences of means of continuous parametric data were analyzed using the $t$ test. All statistical data were analyzed by means of SPSS version 13.0. All $p$ values are two-sided; a $p$ value smaller than 0.05 indicated significant difference.

3. Results

3.1 IGF2 imprinting status in cord blood nucleated cells

392 out of the 923 cord blood samples collected (42.5%) were heterozygous (A/B) and informative of the IGF2 exon 9 Apal polymorphism site while the other 345 (37.3%) were homozygous A/A and 186 (20.2%) were homozygous B/B. Out of the 392 informative samples, 310 RNA samples were available and 273 samples were successfully amplified by RT-PCR. LOI was shown in 57 (20.9%) of the informative specimens examined whereas the monoallelic expression was detected in 107 specimens with allele A (39.2%) and 109 with allele B (39.9%).

3.2 Characteristics of parents of infants with LOI

No statistical differences were observed in maternal ages, gestational ages, pre-pregnancy body mass index (BMI), weight gain during pregnancy and parity between normal imprinting and LOI groups (see Table 1); none of the pregnant women engaged in drug abuse, smoking, or alcohol assumption. The delivery ways were similar between the two groups. There were also no significant differences observed in the level of hemoglobin, hematokrit, and other biochemistry profile in the second- and third-trimester. Incidences of pregnant complications between LOI and normal imprinting groups were as followings: preeclampsia was 9.72% (21/216) vs. 5.26% (3/57), diabetes mellitus 3.24% (7/216) vs. 1.75% (1/57), oligoamnios 5.09% (11/216) vs. 1.75% (1/57), polyhydramnios 4.62% (10/216) vs. 8.87% (5/57), and malignant tumor in maternal and paternal grandparents 1.16% (10/864) vs. 2.63% (6/228), respectively, all of which indicated no significant differences. Nevertheless, frequency of LOI tended to be higher in polyhydramnios than in oligoamnios.

Analysis of parents’ age distribution unexpectedly revealed that frequency of LOI tended to increase when mothers were over 35 years old, but no significant differences were noted in comparison to other age groups ($p = 0.240$). Although the mean paternal age of the LOI group was not significantly higher than that of the normal imprinting group (31.5 ± 5.07 yrs vs. 30.4 ± 4.47 yrs, $p = 0.111$), fathers over the age of 35 had a higher chance of having LOI babies (29.8% vs. 17.6, $p < 0.05$) (see Table 1).

3.3 Characteristics of infants with LOI

All of the 273 neonates with LOI were live birth. Seven of the newborns had congenital defects, including inguinal/umbilical/ diaphragmatic hernia, congenital heart diseases, sacrococcygeal teratoma, and congenital adrenal cortical hyperplasia; one newborn with diaphragmatic hernia died 3 days after birth. In general, we did not find any specific defects in the two groups (see Table 2). Incidences of fetal distress, asphyxia and jaundice in the LOI and normal imprinting groups were 12.5% (27/216) vs. 15.8% (9/57), 2.3% (5/216) vs. 1.75% (1/57) and 14.4% (31/216) vs. 17.5% (10/57), respectively, with
no significant differences (see Table 3). No case of phenylketonuria or congenital hypothyrosis was found in these individuals.

**Table 1.** Clinical information in newborn singletons with normal imprinting and LOI of IGF2.

|                        | Normal imprinting singletons (%) | LOI singletons (%) | p value |
|------------------------|----------------------------------|-------------------|---------|
| **Total**              | 216                              | 57                |         |
| Pre-pregnancy BMI      | 20.7 ± 2.54                      | 20.8 ± 2.57       | 0.792   |
| (kg/m², mean ± s.e.m.) |                                  |                   |         |
| Parity                 |                                  |                   |         |
| 0                      | 111(51.4)                        | 27(47.4)          |         |
| ≥ 1                    | 105(48.6)                        | 30(52.6)          |         |
| Mean gestational age   | 39.5 ± 1.20                      | 39.6 ± 0.91       | 0.111   |
| (week) (mean ± s.e.m.) |                                  |                   |         |
| Maternal ages (years)  |                                  |                   |         |
| < 25                   | 44(20.4)                         | 10(17.5)          |         |
| 25 - 29                | 121(56.0)                        | 29(50.9)          |         |
| 30 - 34                | 46(21.3)                         | 15(26.3)          |         |
| ≥ 35                   | 5(2.3)                           | 3(5.3)            |         |
| Mean ± s.e.m.          | 27.3 ± 3.55                      | 27.9 ± 4.00       | 0.260   |
| Paternal ages (years)  |                                  |                   |         |
| < 25                   | 11(5.1)                          | 4(7.0)            |         |
| 25 - 29                | 88(40.7)                         | 22(38.6)          |         |
| 30 - 34                | 79(36.6)                         | 14(24.6)          |         |
| ≥ 35                   | 38(17.6)                         | 17(29.8)          | 0.041a  |
| Mean ± s.e.m.          | 30.4 ± 4.47                      | 31.5 ± 5.07       | 0.111   |
| Pregnancy complications|                                  |                   |         |
| Hypertensive disorders | 21(9.7)                          | 3(5.3)            | 0.290   |
| Diabetes mellitus      | 7(3.2)                           | 1(1.8)            | 0.554   |
| Oligoamnios            | 11(5.1)                          | 1(1.8)            | 0.270   |
| Polyhydramnios         | 10(4.6)                          | 5(2.3)            | 0.222   |

aOR = 1.99 95%CI [1.02, 3.88]

We also compared the physical constitution of the infants. The mean BW was 3462.7 ± 460.2 g vs. 3363.7 ± 427.7 g in the LOI and the normal imprinting group, respectively (p = 0.127). Other body indexes did not show any significant differences as well, including BMI, sitting height, head circumference, chest circumference and shoulders radical lines. However, abdominal circumference in the LOI group tended to be larger than that of the normal imprinting group (p = 0.053), although the difference is not significant. In addition, in comparison to the 15.3% (33/216) in the normal imprinting group, the frequency of 2500 - 2999 g BW in LOI group is only 5.3% (3/57) (p < 0.05). The occurrence of BW over 4000 g in the LOI group, on the other hand, was 12.3% (7/57), which is not
significantly higher than the 6.5% (14/216) in the normal imprinting group ($p = 0.144$), while the incidence of low BW under 2500 g was 3.5% (2/57) in LOI group, in comparison to the 1.5% (3/216) in the normal imprinting group ($p = 0.288$) (see Table 3). In comparing the rate of LOI of $IGF2$ among the different BW groups, the graph revealed a U shaped distribution (see Figure 1), with the LOI rate of BW under 2500 g to be surprisingly as high as 40% (2/5), which is the highest rate among the BW groups compared. Due to the limited number of cases, we were unable to note any statistical differences among the other groups. We also evaluated and compared the relationship between BW and the decrease in the level of plasma glucose 2 hours after birth and found that the plasma glucose values for infants with BW under 2500 g and over 4000 g with LOI were all 2.8 mmol/L, which is larger than the 1.5 mmol/L and 1.2 mmol/L found in normal imprinting infants ($p < 0.01$). We followed up the newborns with LOI for 30 days and, after the 30-day period, scanned the infants’ livers and kidneys using ultrasound but failed to find any neoplasm among them. We also measured the weight gain of all of the LOI infants as well as 60 of the 216 normal imprinting infants during the 30-day period. The mean weight gains in the LOI and normal imprinting groups were 1173 ± 258.6 g and 1246.0 ± 243.3 g, respectively ($p = 0.118$).

**Figure 1.** The birth weight distribution of singletons with LOI of $IGF2$. The percentage of LOI in singletons with 2500-3000g birth weight is significantly lower than that in singletons over 4000g birth weight (8.33% vs. 33.4%, $p < 0.05$). Although the percentage of LOI in singletons under 2500g birth weight is up to 40% (2/5), there was no statistical differences compared to other groups due to the limited cases.
Table 2. IGF2 imprinting status and congenital defects

| Congenital defects                  | Normal imprinting | LOI | Notes                           |
|------------------------------------|-------------------|-----|---------------------------------|
| Inguinal/umbilical hernia           | 2                 | 1   |                                 |
| Diaphragmatic hernia               | 1                 | 0   | Died in 3 days after birth      |
| Congenital heart diseases           | 1                 | 0   | Ventricular septal defect 3mm   |
| Sacrococcygeal teratoma            | 1                 | 0   | Benign                          |
| Congenital adrenal cortical hyperplasia | 0               | 1   | Familial                        |

3.4 Inheritance assay of IGF2 LOI

To investigate the possible hereditary nature of IGF2 LOI, we obtained peripheral blood samples from parents of newborns with LOI of IGF2 in their cord blood to examine their IGF2 imprinting status. 34 paternal and 37 maternal samples were examined; of which, 27 specimens (10 paternal and 17 maternal) were informative at the IGF2 Apal site. Of the 27 samples, the IGF2 Apal sites of 23 specimens could be successfully detected using RT-PCR. The result indicated that for 2 out of 9 infants (22.2%) with LOI, their father’s lymphocytes also showed LOI while 3 out of 14 (21.4%) had LOI in their mother’s lymphocytes. All of these parents appeared normal without any familial diseases or congenital malformations. Moreover, there were no LOI newborns found to have both parents showing LOI.

Table 3. Characteristics of term singletons according to IGF2 imprinting status in cord blood cells

| Items                          | Normal imprinting | LOI | p value |
|-------------------------------|-------------------|-----|---------|
| Total                         | 216               | 57  |         |
| Sex                           |                   |     |         |
| Male (%)                      | 99(45.8)          | 32(56.1) |       |
| Female (%)                    | 117(54.2)         | 25(43.9) |       |
| Birth weight distribution (%) |                   |     |         |
| < 2500g                       | 3(1.4)            | 2(3.5) | 0.288  |
| 2500 - 2999g                  | 33(15.3)          | 3(5.3) | 0.046a |
| 3000 - 3499g                  | 97(44.9)          | 26(45.6) | 0.836  |
| 3500 - 3999g                  | 69(31.9)          | 19(33.3) | 0.839  |
| ≥ 4000g                       | 14(6.5)           | 7(12.3) | 0.159  |
| Physical constitution (mean ± s.e.m.) |           |     |         |
Mean Birth weight 3363.7 ± 427.7 3462.7 ± 460.2 0.127
BMI (kg/m^2) 13.56 ± 1.34 13.61 ± 1.32 0.542
Sitting height (cm) 32.1 ± 1.93 32.2 ± 1.74 0.892
Head circumference (cm) 33.7 ± 1.42 33.7 ± 1.55 0.963
Chest circumference (cm) 32.8 ± 2.3 33.0 ± 1.63 0.450
Shoulders radical lines (cm) 12.4 ± 0.97 12.5 ± 0.96 0.608
Abdominal perimeter (cm) 33.2 ± 2.02 33.8 ± 2.27 0.053
Other newborn diseases and defects (%)
Fetal distress 27(47.4) 9(15.8) 0.514
Asphyxia 5(8.8) 1(1.8) 0.797
Jaundice 31(14.4) 10(17.5) 0.548
Phenylketonuria 0 0
Congenital hypothyrosis 0 0
*OR=0.31,95%CI [0.09,1.04]

4. Discussion

*IGF2*, a growth factor indispensable during fetal development, plays an important role in the regulation of placental nutrient supply and subsequently, the modulation of fetal growth [8, 9]. Many researches have shown an association between LOI of *IGF2* and BWS, a congenital overgrowth, malformation, and embryonic tumor predisposition syndrome [10]. LOI of *IGF2* might be an early stage event in some tumors and can be found in over 20 sorts of malignancy [1, 11]. LOI of *IGF2* in peripheral blood lymphocytes (PBL) occurs in about 10% of normal people [12, 13]. This particular population might be at high risk for developing cancers and diseases. In response to the growing speculation, suggested by researchers, that LOI of *IGF2* might be a potential heritable biomarker for neoplasm predisposition [12, 14], we felt the need to survey *IGF2* imprinting status in various kinds of ethnicity and developmental stages. In some small sample studies, *IGF2* showed biallelic expression in the cord blood of 20% - 30% infants, which is higher than that of adults [4, 13]. However, owing to the limited number of samples investigated and a lack of clinical background on related individuals, these studies were unable to identify the traits of newborns with *IGF2* LOI in their cord blood. Under strict quality control, we set up a large bank of cord blood samples for both the experimental conditions and clinical data collections. In addition, donations of blood from some parents allowed us to survey the possible hereditary nature of abnormal imprinting. By examining a relatively large number of specimens for the first time, we found that ~20% of Han Chinese newborns showed LOI of *IGF2* in cord blood nucleated cells, suggesting that it may represent an epigenetic characteristic for this ethnicity, which has the largest population in the world.

Although *IGF2* LOI has been linked to many human diseases, the mechanism remains unclear. Theoretically, the total amount of mRNA encoding *IGF2* increases one-fold when a silenced maternal allele is expressed. Experimentally, many quantitative research also demonstrated that, on average, the mean level of *IGF2* expression increases one-fold [15] and LOI in local tissue can induce tumor growth by promoting cell proliferation. It was reported that a high expression of *IGF2*, induced by LOI, is characteristic of undifferentiated, immature and hyperplasia cells, which promotes the proliferation.
of not only cancer but also normal cells [16, 17]. Moreover, epidemiological data consistently prove the existence of a positive relationship between increased body size and colorectal malignancy as well as the IGF2 peptide level in serum, and BMI tends to increase in patients with colorectal cancer [18, 19].

Our study indicated no noticeable increase in the mean BW of newborns with LOI of IGF2. However, it did show the incidence of significant LOI decreases in low BW groups. These results highlighted the notion that epigenetic regulation does indeed have some effects on BW modulation. It is noteworthy to mention that while there were no significant statistical differences in the abdominal perimeters between the LOI group and the normal imprinting group, it tended to increase in the LOI group. Neonates in these two groups also showed a rapid decrease in glycemia after birth. Hypoglycemia could be seen in 30% - 50% of BWS without any symptoms, and could be naturally recovered within the first 3 days, for which hyperinsulinism may be accounted [20, 21].

Environmental exposures such as tobacco smoking, alcohol and nutrient ingestion including calcium, folic acid, selenium and fat did not show any correlations to LOI of IGF2 [14]; we showed that many maternal environmental factors from second and third trimester mothers, including concentration of albumin and lipid in serum, pre-pregnancy BMI, and weight gain during pregnancy, also did not appear to have an impact on imprinting. The latest mouse study demonstrated that maternal nutrient deficiency during development could induce LOI, thus confirming the idea that environmental factors can give rise to the epigenetic alterations [22]. The fact that the biallelic expression of IGF2 originates from the loss of maternal imprinting suggest that abnormal maternal factors might be the primary cause of LOI [23].

This study showed that LOI of IGF2 tended to increase with mother’s age although no significant differences were observed. On the other hand, newborns with LOI also increased significantly with fathers over 35 years old, suggesting possible paternal influences on aberrant imprinting; father’s age may alter offspring imprinting through contributions of abnormal sperms including spermatozoal RNA as well as the epigenetic alterations as previously described [24].

Research relating to LOI inheritance in humans is very lacking. Although we have observed a weak association between parental and newborn LOI, it is still too early to draw any conclusions. Additional investigations based on a larger specimen size are needed for further confirmation. In our studies, many newborns with LOI of IGF2 did not indicate significant phenotypes. Thus, we cannot exclude the possibility that epigenetic abnormality induced phenotypes may occur gradually throughout life. One way to account for this problem is to continue to follow these infants to observe any phenotype changes in future. In addition, the relationship between IGF2 imprinting status in lymphocytes and IGF2 peptide concentration in serum remains to be investigated.

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