Reproductive Sciences
2018, Vol. 25(1) 131-139

DOI: 10.1177/1933719117704906
journals.sagepub.com/home/rsx
SAGE

Impact of Abnormal DNA Methylation of Imprinted Loci on Human Spontaneous Abortion

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Abstract

Currently, there is a growing concern regarding the safety of assisted reproductive technology (ART) due to increased risk of spontaneous abortion (SA) and imprinting disorders in ART-conceived offspring. Early investigations suggested that aberrant genetic imprinting may be related to pregnancy loss; however, few studies have used human tissue specimens. Here the DNA methylation patterns of 3 imprinted genes, including maternally inherited *GRB10* and the paternally inherited *IGF2* and *PEG3* genes, were evaluated in human chorionic villus samples by pyrosequencing and bisulfite sequencing polymerase chain reaction. The samples were divided into 4 groups: (1) SA of natural conception (NC; n = 84), (2) induced abortion of NC (n = 94), (3) SA after ART (n = 73), and (4) fetal reduction after ART (n = 86). The methylation levels and the percentages of abnormal methylation of the *IGF2*, *GRB10*, and *PEG3* genes between the ART group and the NC group showed no significant difference. Both *IGF2* and *GRB10* genes showed higher methylation levels in the SA group compared to the non-SA group. Additionally, determining the single-nucleotide polymorphisms of 4 loci, including *IGF2* rs3741205, rs3741206, rs3741211, and *GRB10* rs2237457, showed that the TC+CC genotype of *IGF2* rs3741211 had a 1.91-fold increased risk of SA after ART. However, there was no association between the mutant genotype of *IGF2* rs3741211 and the methylation levels of *IGF2* and *H19*, and ART might not affect the distribution of the abovementioned genotypes. It provides support for the opinion that genetic imprinting defects may be associated with SA, which might not be due to ART treatments.

Keywords

spontaneous abortion (SA), imprinted gene, DNA methylation, assisted reproduction technique (ART), single-nucleotide polymorphism (SNP)

Introduction

Currently, millions of infertile couples have successfully conceived using assisted reproductive technology (ART), and the number of births derived from ART has been growing quickly over the last decade. The ART is thought to be safe and effective. However, the delivery rate per aspiration was only 13.5% to 21.2%.[1] A major reason for the unsatisfactory live birth rate is that a number of pregnancies ended with spontaneous abortion (SA). The incidence of SA is higher (18%-30%) in ART-derived pregnancies compared to that in natural conceptions (NCs; 10%-15%).[2] Some studies have suggested that this discrepancy may be related to the infertility causes of the couples who receive ART treatments, such as older age, higher body mass index, and history of abortion.[3-5] However, Wang et al found that the risk of SA in ART-derived pregnancies was slightly increased after adjusting for maternal age and previous SA, which seemed to be associated with several variables, including the level of stimulation.[2]

The SA is defined as a pregnancy loss without outside intervention before 20 weeks of gestation, and chromosomal abnormalities are causative in approximately half of SAs, whereas other molecular mechanisms have not been fully identified.[6] Recent data show that aberrant gene imprinting may be a possible cause of pregnancy loss.[7] Genomic imprinting is an epigenetic process, and DNA methylation is one of the best

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characterized epigenetic modifications. Individual imprinted genes were first discovered in 1991, and approximately 80 imprinted genes have been recognized in humans (http://igc.otago.ac.nz).[8] Genes subject to genomic imprinting are preferentially expressed from a single parental allele in mammals. The expression of a small number of imprinted genes is crucial for normal development, as these genes are often directly involved in regulating placental development and fetal growth.[9,10]

The ART procedures, including controlled ovarian stimulation, in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and oocyte freezing, are performed during the period when the methylation patterns of germ cells and preimplantation embryos are reprogrammed genome wide.[11] The ART might influence the regulation of the imprinting process and cause genomic imprinting disorders in offspring such as Beckwith-Wiedemann syndrome (BWS) and Angelman syndrome (AS), which are caused by abnormal methylation in the differentially methylated region (DMR).[12-14] It seemed to be reassuring that Anckaert et al have demonstrated normal DNA methylation in cultured oocytes[15]; however, changes in DNA methylation following in vitro oocyte maturations were observed by Borghol and his colleages.[16] Bowdin et al failed to demonstrate an association between imprinting disorders and ART, independent of subfertility.[17] Abnormal methylation patterns of *PEG1*, *H19*, *LIT1*, and *SNRPN* which play important roles in fetal development were not found in chorionic villus from ART-derived pregnancies in our previous studies.[18,19] Nevertheless, the connection between imprinting defects and ART is still contentious. Song et al found DNA methylation differences between in vitro- and in vivo-conceived children were associated with ART procedures rather than infertility.[20] A study of human embryos showed a high frequency of imprinted methylation errors in ART preimplantation embryos.[12]

Although an abundance of animal studies from rodents to primates examining DNA methylation changes following ART have been published, the researches are heterogeneous and the data are inconsistent, and most of the studies are absent of infertility. Therefore, in the current study, we analyzed the DNA methylation patterns of 3 genes that are known to influence both fetal and placental growth, including the maternally inherited *GRB10* (in placental villous trophoblasts specifically) and the paternally inherited *IGF2* and *PEG3* genes in human chorionic villus samples (CVS) from SA after ART and NC using pyrosequencing and bisulfite sequencing PCR. Previous studies have suggested that interference of the 3 genes would lead to serious develepmental defects. A deletion and mutation of *Peg3* gene may lead to placental deficiency of nutrient transport and offspring growth retardation, frequently resulting in the death of the offspring.[21,22] Fetal and placental weights in placental-specific *Igf2* knockout mice were reduced when compared to that of wild type.[23] Mice with maternal duplication of proximal chromosome 11, where *Meg1/Grb10* is located, exhibited prenatal and postnatal growth retardation.[24] Therefore, we proposed that primary epimutations resulting in inappropriate methylation and expression patterns of the mentioned 3 imprinted genes might contribute to spontaneous pregnancy loss. Additionally, single-nucleotide polymorphisms (SNPs) of some imprinted genes, such as *IGF2*, *H19*, and *IGF2R*, were reported to be related to the methylation status of imprinted genes, thereby affecting placental growth and neonatal birth weight.[25,26] However, there are few reports about SNPs and human SA. Thus, the association between the gene genetic variants and the susceptibility to pregnancy loss was also evaluated. The objects of this study were to explore the association of aberrant DNA methylation of imprinted genes with human SA after ART and NC and provide insights into the etiology of human pregnancy loss.

Materials and Methods

Participants and Samples

Samples were collected after the patients gave their written informed consent, and the study protocol was approved by the Institutional Ethics Committee of Nanfang Hospital of Southern Medical University and was conducted in compliance with the Helsinki Declaration. The CVS were collected from women who underwent abortion procedures in Department of Gynecology and Obstetrics in Nanfang Hospital from May 2008 to November 2012. Exclusion criteria included endocrine diseases, infections, chromosomal abnormalities, immunological diseases, and anatomical abnormalities of the genital tract. Based on the source, the samples were divided into 4 groups: (1) SA after natural pregnancies ($n = 84$). (2) Induced abortion after NCs ($n = 94$). Fetal heartbeat was observed in these pregnancies. These abortions were performed due to the patient's request for personal or social reasons. (3) SA after ART ($n = 73$). In cases of SA, an intrauterine sac without a fetal heartbeat was observed. (4) Fetal reduction by transvaginal ultrasound after ART ($n = 86$). Gestational weeks ranged from 6 to 18 weeks, and the maternal age ranged from 17 to 45 years.

Gestational weeks were calculated from the last menstrual period and further determined by ultrasound. Once an intrauterine sac without a fetal heartbeat was observed, the abortion procedure would be performed in 1 to 2 weeks due to the confirmation of SA and preoperative preparations, and the CVS were collected immediately after the procedures. Multifetal reduction was performed under transvaginal ultrasound using a paracentetic needle in weeks 6 to 11. Under the guidance of the experts of prenatal diagnosis in Nanfang Hospital, the retained product of conception was removed from the uterus using a suction curette or paracentetic needle. Maternal blood was removed from the placenta, and the decidua was kept separately under a stereoscope. The CVS were cut into pieces with a diameter of about 1 mm and stored at $-80^{\circ}C$ until analyses.

Methylation Analyses

Genomic DNA was extracted using the genomic DNA purification kit (Promega, Madison, Wisconsin). The DMRs of

Table 1. Imprinted Genes and Primers Used for Bisulfite Pyrosequencing.

Genes	Gene ID	Primer Sequence	Product Length, bp	Chromosomal Localization, bp	Number of CpGs
IGF2	3481	Forward: 5′-GGGATTGGGTTAGGAGAAGTTT-3′ [a]Reverse: 5′-CCCCCAAAAATAACCAACAAT-3′ Sequencing: 5′-GGAGGTTGTAGGATGG-3′	164	Chromosome 11 (2 152 707-2 152 870)	4
GRB10	2887	Forward: 5′-GGTTTTGGAGTATAATAGGAATTT-3′ [a]Reverse: 5′-ATTACCATAAAAACCAAAAATCC-3′ Sequencing: 5′-TTAGGATTAAATTTATGTGA-3′	114	Chromosome 7 (50 818 064-50 818 177)	6
PEG3	5178	Forward: 5′-GGTGTAGAAGTTTGGGTAGTTG-3′ [a]Reverse: 5′-CTCACCTCACCTCAATACTAC-3′ Sequencing: 5′-TGTTTATTTTGGGTTGGT-3′	153	Chromosome 19 (62 043 757-62 043 909)	6

[a]The 5′-end was labeled with biotin.

Table 2. Polymorphisms of Imprinted Genes and Primers Used for Pyrosequencing.

Polymorphisms of Imprinted Genes	Primer Sequence	Amplicon Length, bp	Number of SNPs
IGF2 rs3741205, rs3741206	Forward: 5′-TTTCCTCCCGTTGCATCC-3′ [a]Reverse: 5′-TCCAATTTCTTGCTGGTGGT-3′ Sequencing: 5′-CGAGATTCTGGCGCA-3′	179	2
IGF2 rs3741211	Forward: 5′-TGCCCAGATCCTGACAAGGT-3′ [a]Reverse: 5′-CCCTGGGGAAAAACAAAA-3′ Sequencing: 5′-TGGGACAGGGCTCAG-3′	138	1
GRB10 rs2237457	[a]Forward: 5′-ATTGTCTAGTGGTTCCCCCTT-3′ Reverse: 5′-ACCTGTGGCCATCTACGTGA-3′ Sequencing: 5′-GTGCTTTCACAGAGACC-3′	206	1

Abbreviation: SNP, single-nucleotide polymorphism.
[a]The 5′-end was labeled with biotin.

IGF2, *GRB10*, and *PEG3* genes were analyzed by bisulfite pyrosequencing. Bisulfite treatment of genomic DNA was performed with the EpiTect bisulfite kit (Qiagen, Frankfurt, Germany). The TMPSQ 96MA system (Biotage, Uppsala, Sweden) along with a PyroMark Gold Q96 Reagents Kit (Qiagen) were used to take a pyrosequencing according to the manufacturer's instructions. The degree of methylation at each CpG site was determined by Allele Quantification software (Biotage). Polymerase chain reaction (PCR) and sequencing primers for bisulfite pyrosequencing (Table 1) were designed using the Pyrosequencing Assay Design Software (Biotage). All the samples were analyzed in triplicate. To confirm the pyrosequencing results, PCR products of samples were gel purified by a QIAEX II gel extraction kit (Qiagen) and cloned into the pGEM-T vector (Promega). Approximately 10 clones of each individual sample were sequenced.

Genotyping of SNPs

After extraction of genomic DNA, PCR was performed to amplify the target gene fragments. The genotypes of *IGF2* rs3741205, rs3741206, rs3741211, and *GRB10* rs2237457 were analyzed by pyrosequencing. The SNPs were selected based on previously published associations with fetal growth or birth weight-related phenotypes. The PCR and sequencing primers are shown in Table 2. The genotype was determined using SNP software. The PCR-amplified DNA samples (n = 30, random selection) were examined by Sanger sequencing to confirm the pyrosequencing results, and the results were 100% concordant.

Statistical Analyses

The methylation values of *IGF2*, *GRB10*, and *PEG3* in CVS from the 4 experimental groups were analyzed using the statistical analysis program SPSS 16.0. Data are shown as mean $\pm$ SD, or percentage if appropriate. Box plots were calculated using the program's default parameters. The bottom and the top of the box signify the 25th and 75th percentile, respectively. The T bars extend from the boxes to a maximum of 1.5 times the height of the box; samples that do not lie within the T bars are defined as outliers. Samples falling within the T bars were considered normally methylated, whereas extreme methylation values (outliers) may indicate a methylation reprogramming defect or failures to establish or maintain allelic methylation. Appropriate statistical tests were used for comparison, including independent samples *t*-test, χ^2, test and Fisher exact test. The receiver–operating characteristic (ROC) curve method was used to analyze the potential association between the methylation percentage of the 3 genes and the incidence of SA. Hardy-Weinberg equilibrium was estimated in both the groups based on allele frequencies that were calculated by counting alleles. All statistical tests were 2 tailed, and $P < .05$ was considered statistically significant.

Table 3. Clinical Characteristics in Different Groups.

Characteristics	SA Samples (Groups A and C) n = 157	Non-SA Samples (Groups B and D) n = 180	*P* Value	NC Samples (Groups A and B) n = 178	ART Samples (Groups C and D) n = 159	*P* Value
Maternal age, years	31.0 ± 4.8 (20-42)	30.0 ± 5.0 (17-45)	.109	29.4 ± 5.6 (17-45)	31.6 ± 3.8 (21-41)	<.001
Gestation, weeks	9.4 ± 2.0 (6-18)	8.1 ± 1.4 (6-13)	<.001	9.1 ± 2.0 (6-18)	8.2 ± 1.3 (6-14)	<.001

Abbreviations: SA, spontaneous abortion; NC, natural conception; ART, assisted reproductive technology.

Results

Patient Characteristics

A total of 337 particiapnts were included in this study. The clinical characteristics of all participants are summarized in Table 3. There was no significant difference in the mean maternal age between the SA group (groups A and C) and the non-SA group (groups B and D; $P = .109$), but the gestation of the SA group was significantly longer than that of the non-SA group ($P < .001$). Compared to the NC group (groups A and B), patients in the ART group (groups C and D) had higher maternal age and shorter gestation ($P < .001$).

Methylation Values of Imprinted Genes

Methylation analysis of 3 DMRs was performed on the CVS of 337 abortions. The *IGF2* DMRs are regulated in a hierarchical fashion by the sperm-derived *H19* DMR. The analyzed regions of *GRB10* and *PEG3* represent primary imprints that are established in the germline and stably maintained after fertilization. For all CVS, we achieved bisulfite conversion efficiency between 90.1% and 100%. A box plot was developed to analyze the 3 imprinted genes in all CVS (see Figure. 1A), and it showed that there were 37 cases with abnormal methylation status, and the overall incidence was 3.7% (37 of 1011). The rates of abnormal methylation of each gene were the following: *GRB10* > *IGF2* > *PEG3* (4.2% [14/337], 3.9% [13/337], and 3% [10/337], respectively).

Methylation Differences Between the SA and Non-SA Samples

The cases were grouped according to pregnancy outcomes, with 157 patients included in the SA group (groups A and C) and 180 patients in the non-SA group (groups B and D). In the SA samples, 20 (4.2%) of 471 analyzed DMR methylation values of the 3 studied genes represented outliers (Table 4). The highest number of outliers was observed for *GRB10* (6.4%, 9/157). Eighteen outliers were found among the 180 (3.3%) non-SA samples. Both *IGF2* and *GRB10* genes showed significantly higher methylation levels in the SA samples compared to the those in non-SA samples ($P = .015$, .042, respectively) but *PEG3* did not ($P = .513$). These differences persisted after adjustment for maternal age and gestation (for *IGF2*, $P = .003$; for *GRB10*, $P = .048$; for *PEG3*, $P = .988$). The ROC curve analysis showed a significant but weak positive correlation between *GRB10* methylation percentage in chorionic villus and rates of SA; higher methylation percentage was associated with a greater chance of spontaneous pregnancy loss ($P = .024$). No significant correlation was observed between the methylation percentage of the other genes and the incidence of SA ($P = .119$ and .624 for *IGF2* and *PEG3*, respectively; Figure 1B to D). Additionally, the ratio of abnormal methylation values (outliers) of *GRB10* in the SA group was significantly higher than that in the non-SA group ($P = .025$; Table 4).

DNA Methylation and ART

There were no significant differences in the mean percentage of methylation for the *IGF2*, *GRB10*, and *PEG3* genes between samples from the NC (groups A and B) and the ART (groups C and D) group ($P = .126$, .224, .396, respectively), and no significant differences were observed even after adjustment for maternal age and gestation (for *IGF2*, $P = .058$; for *GRB10*, $P = .190$; for *PEG3*, $P = .510$). The ratios of abnormal methylation of the 3 imprinted genes were further compared between the NC and the ART groups, and no significant difference was observed ($P = .380$, .872, .868, respectively; Table 4). Based on the ART procedures, the ART group was then divided into the IVF group and ICSI group (n = 102, 57, respectively). However, there were no significant differences between the 2 groups in the methylation level (supplementary Table 1).

Cloning and sequencing for the samples of interest were conducted to confirm the pyrosequencing results, and the results were in accordance with the results of pyrosequencing.

Distribution of SNPs in CVS

Considering that some SNPs might be associated with the status of methylation of imprinted genes and might be related to the placental development and fetal growth, we compared the polymorphisms of 4 loci including *IGF2* rs3741205, rs3741206, rs3741211, and *GRB10* rs2237457 in CVS from various groups. All samples were successfully genotyped for the *IGF2* polymorphisms and the SNP of *GRB10*. Hardy-Weinberg equilibrium was tested with a goodness-of-fit χ^2 test to compare the observed genotype frequencies and the expected genotype frequencies among participants ($P > .05$).

The SNPs were divided into 3 classes, namely, wild-type homozygote, variant heterozygote, and variant homozygote. The variant alleles were combined into 1 group to be compared with the wild type, due to the low frequency of variant

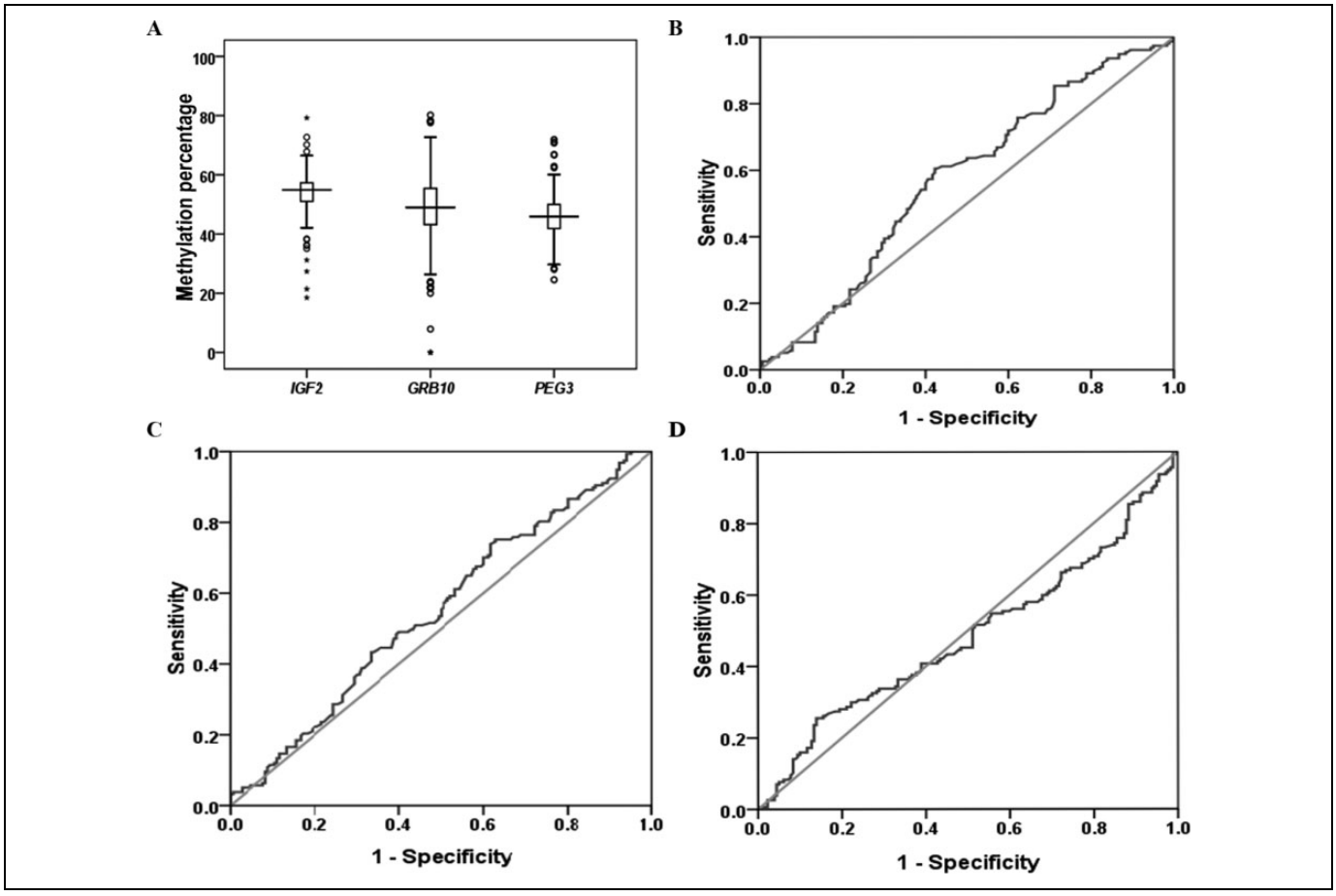

Figure 1. Methylation values in 3 imprinted genes (*IGF2*, *GRB10*, and *PEG3*) in 337 chorionic villus samples. A, Box plots show the distribution of methylation values of the 3 imprinted genes. The bottom of the box indicates the 25th percentile, and the top indicates the 75th percentile. Horizontal lines represent median values. Outliers, considered aberrant methylation values, are shown as open circles, and extreme outliers are stars. B–D, The ROC curve analysis demonstrates a positive correlation between the methylation percentage of *GRB10* (B) and spontaneous abortion incidence. The area under the curve was 0.571 (95% confidence interval, 0.510-0.632, $P = .024$). No significant correlation was observed between the incidence of SA and the methylation percentage of *IGF2* (C) or *PEG3* (D) genes ($P = .119$ and .624, respectively). ROC indicates receiver–operating characteristic.

Table 4. Human Chorionic Villus With Extreme Methylation Values.

	SA Samples (Groups A and C), n = 157		Non-SA Samples (Groups B and D), n =180		NC Samples (Groups A and B), n = 178		ART Samples (Groups C and D), n = 159	
Imprinted Genes	Methylation, mean (SD, %)	Outliers, % (n)	Methylation, mean (SD, %)	Outliers, % (n)	Methylation, mean (SD, %)	Outliers, % (n)	Methylation, mean (SD, %)	Outliers, % (n)
IGF2	55.0 (5.2)	5.1 (8/157)	53.4 (6.8)[a]	5.0 (9/180)[b]	53.6 (6.9)	5.1 (9/178)	54.7 (5.2)[c]	3.1 (5/159)[c]
GRB10	49.9 (10.8)	6.4 (10/157)	47.5 (11.1)[a]	1.7 (3/180)[a]	48.0 (12.3)	2.2 (4/178)	49.4 (9.3)[c]	2.5 (4/159)[c]
PEG3	45.7 (7.2)	1.3 (2/157)	46.2 (6.4)[b]	3.3 (6/180)[b]	46.3 (7.7)	2.8 (5/178)	45.7 (5.7)[c]	2.5 (4/159)[c]
All genes		4.2 (20/471)		3.3 (18/540)		3.4 (18/534)		2.7 (13/477)

Abbreviations: SA, spontaneous abortion; NC, natural conception; ART, assisted reproductive technology.
[a]Significantly different compared to SA samples: percentage of methylation for *IGF2* and *GRB10* genes, $P = .015$ and .042, respectively ($P = .003$ and .048, respectively after adjustment for maternal age and gestation); outliers for *GRB10*, using Chi-square test, $P = .025$.
[b]Not different compared to SA samples.
[c]Not different compared to NC samples.

heterozygote and variant homozygote. There were no significant differences in the frequencies of wild genotype and mutant genotype of *IGF2* rs3741205, rs3741206, rs3741211, and GRB10 rs2237457 in the CVS between the ART and the NC samples ($P = .801$, .429, .936, .789, respectively; Table 5). Further comparisons showed that the frequencies of genotypes

Table 5. Genotypes of *IGF2* and *GRB10* in Human Chorionic Villus.

	All Samples			NC Samples (n = 178)			ART Samples (n = 159)		
Genotypes	NC Samples (n = 178), n (%)	ART Samples (n = 159), n (%)	Odds Ratio (95% CI)	SA Samples (n = 84), n (%)	Non-SA Samples (n = 94), n (%)	Odds Ratio (95% CI)	SA Samples (n = 73), n (%)	Non-SA Samples (n = 86), n (%)	Odds Ratio (95% CI)
IGF2 rs3741205									
GG	56 (31.5)	48 (30.2)	1	22 (26.2)	34 (36.2)	1	26 (35.6)	22 (25.6)	1
GT+TT	122 (68.5)	111 (69.8)	0.94 (0.59-1.50)	62 (73.8)	60 (63.8)	1.60 (0.84-3.04)	47 (64.4)	64 (74.4)	0.62 (0.31-1.23)
IGF2 rs3741206									
AA	105 (59.0)	87 (54.7)	1	47 (56.0)	58 (61.7)	1	43 (58.9)	44 (51.2)	1
AG+GG	73 (41.0)	72 (45.3)	0.84 (0.55-1.29)	37 (44.0)	36 (38.3)	1.27 (0.70-2.31)	30 (41.1)	42 (48.8)	0.73 (0.39-1.37)
IGF2 rs3741211									
TT	106 (59.6)	94 (59.1)	1	51 (60.7)	55 (58.5)	1	37 (50.7)	57 (66.3)	1
TC+CC	72 (40.4)	65 (40.9)	0.98 (0.64-1.52)	33 (39.3)	39 (41.5)	0.91 (0.50-1.66)	36 (49.3)	29 (33.7)	1.91 (1.01-3.63)[a]
GRB10 rs2237457									
GG	58 (32.6)	54 (34.0)	1	27 (32.1)	31 (33.0)	1	23 (31.5)	31 (36.0)	1
AG+AA	120 (67.4)	105 (66.0)	1.06 (0.68-1.68)	57 (67.9)	63 (67.0)	1.04 (0.55-1.95)	50 (68.5)	55 (64.0)	1.23 (0.63-2.37)

Abbreviations: SA, spontaneous abortion; NC, natural conception; ART, assisted reproductive technology.
[a]Significantly different compared to SA samples after ART, $P = .046$.

of the 4 SNPs did not differ significantly between samples from the NC-SA (group A) and from the NC-non-SA (group B) groups ($P = .152$, .436, .765, .905, respectively). In the ART samples, *IGF2* rs3741211 SNP genotypes showed a significant association with SA ($P = .046$). Compared to the TT genotype, the TC+CC genotype had a 1.91-fold increased risk of SA after ART (95% confidence interval: 1.008-3.629). However, an association with SA was not observed in the *IGF2* rs3741205, rs3741206, or *GRB10* rs2237457 genotype ($P =$ .170, .328, 547, respectively).

Genotype Associations With the Methylation Status of Imprinted Genes

Because *IGF2* is located on chromosome region 11p15, where the *H19* gene is also present, the relationship between the methylation status of *IGF2*, *H19*, and the rs3741211 TC+CC genotype of *IGF2* was further investigated. The methylation value of *H19* was determined using the same sample set in our previous study.[18] The differences in the methylation values of *IGF2* and *H19* between the group of the TC+CC genotype and the group of the TT genotype were compared in the ART samples, but no significant differences were observed ($P =$ .584, .227, respectively; supplementary Table 2).

Discussion

In the current study, we analyzed the DNA methylation status of 3 imprinted genes in DNA samples obtained from CVS in a study population including 157 SA samples and 180 induced abortion samples derived from 178 pregnancies conceived by NC and 159 by ART. The results showed significant differences in the methylation percentage of *IGF2* and *GRB10*, but not *PEG3*, in the SA versus non-SA groups. We also presented a detailed analysis of distribution of genotypes of *IGF2* rs3741205, rs3741206, rs3741211, and *GRB10* rs2237457. It is plausible that the *IGF2* rs3741211 TC+CC genotype in CVS might be related to SA after ART. However, the mutant genotype of *IGF2* rs3741211 showed no relation with the methylation level of the *IGF2* or *H19* genes. These results suggest that the abnormal methylation pattern of imprinted genes may affect the stability of normal pregnancy and participate in the mechanisms that lead to SA. The SNPs may not be directly associated with the methylation status of imprinted genes. The occurrence of aberrant methylation and the genotype distributions of imprinted genes in ART pregnancies were comparable with that in natural pregnancies.

Genomic imprinting is a process causing the monoallelic expression of a specific subset of mammalian genes in a parent-of-origin manner. Imprinted genes are thought to play important roles in growth, development, behavior, and stem cells.[27] Defects in imprinted genes have been demonstrated to be associated with fetal growth abnormalities. Inactivation of *Mash2* and *Peg10* caused early embryonic lethality due to the abnormality of placental development.[28,29] The association between imprinted genes and SA was demonstrated in several human studies. Doria et al showed that the expression patterns of *IGF2*, *PHLDA2*, *PEG10*, and *CDKN1C* imprinting in SA or fetal deaths were deregulated during the 3 trimesters of pregnancy.[30] Pliushch and his colleagues[31] found that hypermethylation of multiple genes was displayed in 4% of SAs and 18% of stillbirths; however, no induced abortions showed extreme methylation values in multiple genes, which was consistent with our previous studies.[18,19,31] The current study showed that the methylation level of *IGF2* and *GRB10* was higher in the SA samples than that in the induced abortion samples, despite the conception methods. We proposed that inappropriate methylation and expression patterns of imprinted genes may contribute

to spontaneous pregnancy loss. Nevertheless, we cannot exclude the possibility that the observed methylation patterns represent normal variation during the progress of fetal development. We have fully taken into account the maternal age and gestational age, and the differences persisted. However, additional genetic and environmental factors might play a role in the methylation patterns.

Outliers in maternally and paternally imprinted genes including both hypermethylated and hypomethylated DMRs were observed, and 2 cases showed complete nonmethylation in the *GRB10* gene. The observed methylation abnormalities might be consistent with methylation reprogramming defects during early embryogenesis, and the hypermethylated DMRs may stem from a failure to prevent ectopic methylation of the unmethylated allele before implantation.[32] DNA hypermethylation changes at CpG islands in CVS did not appear to be direct drivers of SA progression; rather, dynamic changes in genomic imprinting deposition correlated strongly with repression of transcription. Kobayashi and his colleagues found abnormal hypomethylation at *H19* and *GTL2* in CVS and suggested that it might be transmitted directly from the father's sperm with defective imprint establishment.[33] The disturbance of DNA methyltransferase 1 (DNMT1) might account for a decreased global DNA methylation level in human villi leading to early pregnancy loss.[34]

A higher ratio of outliers of *GRB10* was observed, indicating an increased susceptibility of some genes to epigenetic alterations. *GRB10*, which is a potent growth inhibitor that encodes a cytoplasmic adapter protein, has been associated with Silver-Russell syndrome. Disruption of the maternal allele in *Grb10* resulted in overgrowth of both the embryo and the placenta in mice.[35] In contrast, maternalization of the *Meg1/Grb10* cluster in mouse proximal chromosome 11 caused by paternal deletion of *Meg1/Grb10* DMR led to severe pre- and postnatal growth retardation.[24] Further study suggested that *Grb10* might function during embryogenesis by regulating insulin/insulin-like growth factor 1 (IGF1) signaling, as these growth factors play important roles during development.[36] Thus, the abnormal methylation level of *GRB10* in CVS may result in fetal growth retardation leading to SA. Higher methylation values of IGF2 were also observed in SA-derived CVS in our study, which was reported to be related to cell survival and proliferation. After disruption of the paternal allele of *Igf2*, the weights of mutant mice were only 60% that of wild-type mice. Lopez et al found a reduction in the glycogen content of both spongiotrophoblasts and glycogen cells in the *Igf2*-null placenta, indicating that *Igf2* may play an important role in the glycogen production of placental cells, thereby affecting the placental metabolism and fetal growth.[37] *PEG3* is also abundant in placental cells and plays a pivotal role in in the p53-mediated cell death pathway and the growth of offspring.[22,38] In our study, an association between SA and aberrant methylation of *PEG3* was not observed, suggesting that *PEG3* gene imprinting may not be involved the etiology of human SA.

An association between ART and abnormal genomic imprinting in humans was not observed in our study. The methylation level of imprinted genes in CVS derived from IVF and ICSI were also comparable. Because ARTs are performed during the period of erasure and reconstruction of genomic imprinting, a series of studies have shown an increased risk of imprinted defects in ART-derived offspring.[20] Studies in humans and mice suggested that superovulation could change the methylation imprint of the oocytes without their correct primary imprint.[39] Shi et al found aberrant DNA methylation of *H19*, *PEG1*, and *KvDMR1* in human oocytes at the metaphase II stage that were rescue matured from the oocytes at the germinal vesicle/metaphase I stage.[40] Reduced methylation levels of the *H19*, *KvDMR1*, and *Snrpn* imprinting control regions in the placenta were also found after ART treatment.[41] Nevertheless, subfertility is also suggested to be associated with epigenetic instability in ART pregnancies. Epigenetic modification of sperm genes can be transmitted to the offspring.[42] Kobayashi et al demonstrated more prevalent imprinting errors and DNA sequence variants in patients with oligospermia by analyzing DNA methylation at 7 autosomal imprinted loci and the *XIST* locus in 78 paired DNA samples.[33] Although early studies have suggested an increased relative risk of BWS and AS after ART,[14,17] large epidemiological studies in Denmark, Sweden, and the United Kingdom failed to observe an increased frequency of imprinting disorders in children conceived by ART.[17,43,44] Camprubi et al also failed to find any methylation variations at imprinted domains in ART placenta biopsies.[45] Additionally, ART may not affect the frequencies of the IGF2 rs3741205, rs3741206, rs3741211, and *GRB10* rs2237457 genotypes in CVS.

Recent data demonstrated an association between polymorphisms of imprinted genes with placental and fetal development as well as the methylation percentage of the genes.[26,46] The SNPs in the *IGF2/H19* locus were associated with DNA methylation of the *IGF2* DMR, suggesting that variation in DNA methylation of the *IGF2/H19* locus is mainly determined by heritable factors and SNPs.[47] Our previous study suggested that the *DNMT3A*-448A>G polymorphism is a novel functional SNP and contributes to its genetic susceptibility to SA.[48] The current study found that the *IGF2* rs3741211 TC+CC genotype in CVS might contribute to SA after ART rather than NC, but we failed to find an association between the mutant genotype of *IGF2* rs3741211 and the methylation levels of *IGF2* and *H19* genes. It is possible that the rs3741211 SNP is in linkage disequilibrium with another variant closer to *IGF2/H19*. Murrell et al found an increase in the frequency of the CAGA haplotype of IGF2 (rs1004446, rs3741204, rs3751205, rs3741206) in patients with sporadic BWS, which was associated with the loss of maternal allele-specific methylation at *KvDMR1*.[25] The abnormal methylation level of the *KvDMR1* gene might play a role in SA.[18]

Several limitations still persist in interpreting the present findings. First, we cannot exclude the possibility that this abnormal methylation is not the cause but a consequence of the defect that leads to SA. Additional investigations of the underlying molecular mechanism of the polymorphisms and the expression of imprinted genes are warranted. Second, we merely measured minor imprinted genes with partial DMRs and SNPs. A complex,

coordinated network of imprinted genes may be responsible for the embryonic development process. Thus, further studies covering more gene loci are necessary. Finally, it cannot be neglected that the ART samples represent selected embryos with a better form suitable for transfer; however, SAs after NC do not undergo any morphological selection. Embryos of poor quality that are removed from transfer may carry more imprinting defects.[49]

In conclusion, this study first analyzed the DNA methylation status of *IGF2*, *GRB10*, and *PEG3* genes in CVS from spontaneous and induced abortions conceived by ART or naturally and further explored the functions of 4 SNP genotypes of *IGF2* and *GRB10*. Our data suggest that aberrant methylation of imprinted genes may account for the incidence of human SA, but ART may not affect DNA methylation or genotypes of imprinted genes. A variant genotype of an imprinted gene may not be directly associated with the methylation status of the gene. The small differences in methylation seen in the current study may not have clinical significance, but it provides new insights into the etiology of human SA. Further studies, involving a large sample size and covering the mechanism of DNA methylation defects and SNPs of imprinted genes contributing to SA, are needed to confirm the preliminary conclusions.

Acknowledgment

The authors gratefully acknowledge the sample collection and valuable suggestions from the staff in the Department of Gynecology and Obstetrics in Nanfang Hospital.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the National Natural Science Foundation of China (81170574) and High-level Project Matching Foundation of Nanfang Hospital, Southern Medical University (G201206).

Supplemental material

Supplementary material for this article is available online.

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