Effect of abnormal GpG methylation in the second trimester of pregnancy on adverse health risk of offspring

YU SHI[1,2*], MINGXING LIU[2*], XIAOLIN LONG[2], DUNJIN CHEN[2], HAIYAN ZHENG[2] and SHILING CHEN[1]

[1]Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515; [2]Key Laboratory for Major Obstetric Diseases of Guangdong Province, Key Laboratory for Reproduction and Genetics of Guangdong Higher-Education Institutes, Guangzhou Institute of Obstetrics and Gynecology, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510150, P.R. China

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Abstract. Effect of abnormal GpG methylation in amniotic fluid cells during the second trimester of pregnancy on adverse health risk of offspring was investigated. In total, 237 sets of amniotic fluid cells were collected from patients who received prenatal diagnosis in the Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) from April 2010 to October 2011. Among them, 156 sets were from singleton and 81 sets were from twins. *H19* gene was amplified by PCR, and the product was purified and pyrosequencing was used to detect the DNA methylation level of GapG. Follow-up records of the birth outcomes of pregnant women's offspring were collected. Positive rate of DNA amplification in 200 cases of amniotic fluid cells was 84.4% (200/237). Average age of singleton pregnancies was higher than that of twins (P<0.05), and no significant differences were found in gestational age and PCR amplification rate (P>0.05). There was no difference in the methylation level of GapG between singleton and twins (P>0.05), but the abnormal methylation rate of GapG1 in twin fetuses was significantly higher than that of singleton (20.3 vs. 3.6%, χ^2=8.364, P=0.004). Offspring sex, singleton or twins, mode of delivery, time of pregnancy, and low birth weight showed no significant effect on GapG methylation level of *H19* in the second trimester of pregnancy. No offspring deformities were found regardless of the increased or decreased degree of methylation (P>0.05). The number of fetuses born may cause abnormal GapG1 methylation, but no effect of GapG methylation on the adverse health risk of offspring was found.

Correspondence to: Dr Shiling Chen, Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, 1838 Guangzhou Avenue, Guangzhou, Guangdong 510515, P.R. China
E-mail: esh3rw@163.com

*Contributed equally

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Introduction

Human fertility is a natural phase-out process, and development of the embryos or fetuses may be terminated by implantation failure or abortion early in the most sensitive period of fetal development due to abnormal imprinting (1,2). Human genome imprinting is a complex process of molecular biology, and modification, regulation, and expression happen throughout the entire process of fetal development, in which intrauterine development stage is an important epigenetic modification stage of the fetus (3). *H19* gene, as a non-coding RNA, ultimately functions as mRNA. *H19* is an imprinted gene that has been extensively studied and plays an important role in the formation, implantation and growth of gametes and embryos. Abnormal imprinting of *H19* is one of the causes of human Beckwith-Wiedemann Syndrome (BWS) and Silver-Russell Syndrome (SRS) (4,5). Methylation is one of the important mechanisms in gene imprinting process. The target of the methylation is in the DMR region of the differentially methylated region, and more often in the CpG island in the promoter region (6). In the present study, amniotic fluid cells in the second trimester of pregnancy were used to investigate the effect of DMR methy lation of *H19* gene on adverse health risk of offspring with an expectation of providing evidence for clinical evaluation of the safety of offspring.

Patients and methods

Patients. A total of 237 sets of amniotic fluid cells were collected from patients who received prenatal diagnosis in the Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) from April 2010 to October 2011. All the amniotic fluid samples were collected during 16-21 weeks of gestation. SPSS 16.0 (SPSS Inc., Chicago, IL,USA) was used for Boxplot statistical analysis.; karyotypes of 33 fetuses were 46, XX and karyotypes of 48 fetuses were 46, XY. *H19* gene was amplified by PCR, and the product was purified, and pyrosequencing was used to detect the DNA methylation level of GapG. Follow-up records of the birth outcomes of pregnant women's offspring were collected. Inclusion criteria: Normal fetal karyotype; fetal ultrasound examination showed

no abnormalities; amniotic fluid without blood pollution; pregnant women without adverse pregnancy history, no anatomical abnormalities, no family history of genetic disease or any pregnancy complications. The study was approved by the Ethics Committee of Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China), and all patients signed informed consent.

Research methods. Amniotic fluid puncture was performed by an experienced gynecologist under amniocentesis to collect amniotic fluid and sent to the laboratory. The fluid was centrifuged at 2,500 x g for 10 min; the supernatant was discarded, and the amniotic fluid cells were pelleted and stored at -80˚C for subsequent experiments.

DNA of amniotic fluid was extracted by using Qiagen Blood DNA extraction KTI kit (Qiagen GmbH, Hilden, Germany). Nucleic acid analysis software was used with 2 μl of DNA rehydration solution (Qiagen GmbH) as a blank calibration to measure DNA concentration and detect purity.

Samples were removed from the -80˚C freezer, thawed naturally at room temperature, and bisulfite conversions were performed by using low melting point agarose in combination with the CpGenome DNA modification kit (Chemicon International Inc., Temecula, CA, USA).

H19 fragment (Genbank AF125183; starting and ending positions: 7870-8100) was amplified by semi-nested PCR using PCR Hot Start premix buffer (Promega Corporation, Madison, WI, USA) and Taq™ Hot Start Version (Takara Bio, Inc., Otsu, Japan). Primer synthesis was performed by Invitrogen (Invitrogen; Thermo Fisher Scientific, Inc. Shanghai, China). The first round of PCR reaction conditions (F1 and R primers were used): 95˚C for 5 min, followed by 35 cycles of 94, 58 and 72˚C for 1 min, followed by 72˚C for 5 min. The second round PCR reaction conditions (F2 and R primers were used): 95˚C for 5 min, followed by 40 cycles, of 94˚C for 1 min, 60˚C for 30 sec and 72˚C for 30 sec, followed by 72˚C for 5 min. β-actin was the endogenous control. Primers were: F: 5'-AGGTGT TTTAGTTTTATGGATGATGG-3' and F: 5'-TGTATAGTATATATATGGGTATTTTTGGAGGT TT-3', R: 5'-TCCTATAAATATCCTATTCCCAAATAACC-3'. Primers of β-actin were: F: 5'-TCACCCACACAATAA-3'; and R: 5'-CTAATCATATCCCCTAAACA-3'.

PCR products were purified and subjected to pyrosequencing to detect DNA methylation by using PyroMark Gold Q96 reagent (Qiagen GmbH). Sequencing primer was designed by using the Pyrosequencing Assay Design software 1.0.6 version (Biotage AB, Uppsala, Sweden). Sequence of the primer 5'-TGTAGGTTTATATATTATAG-3' was used to analyze the sequence (5'-TTCTGAGTTCTGTTTAAAT TGGGGTTCTGTTCTGTGGAAACTGTTTCTGGTTA-3') containing 6 CpG sites for potential methylation. Primers F2 and R were used for *H19*.

Observation indicators. The length of amplified *H19* fragment by PCR was 230 bp. Endogenous control was set to calculate amplification success rate. Analysis of pyrosequencing results: percentage of 'C' to 'T' was measured for each locus. 'C%' was the degree of methylation of the locus. The third locus is the bisulfite conversion assay site. The average 'C%' of the remaining five loci was the average degree of methylation of the *H19* imprinting gene DMR. If more than two loci failed to be detected, pyrosequencing was determined to be unsuccessful. Success rate of pyrosequencing was recorded, and methylation degree and average methylation degree of sites 1-6 were recorded. Follow-up records of the birth outcomes of pregnant women's offspring were collected. Follow-up data collection principles: For twins with same sex, higher *H19* methylation level corresponded to the one with bigger birth weight. If one of the twins was successfully tested for the degree of methylation of *H19* in amniotic fluid cells, the average growth index of the offspring was taken.

Table I. Results of bisulfite-PCR amplification of *H19* DMR region from amniotic fluid cell DNA.

Variables	Singleton	Twins	Statistical value	P-value
Pregnant women (age)	36.0±3.2	34.4±3.6	10.666	0.001
Gestational week (week)	18.0±0.9	18.2±0.9	0.363	0.547
Amniotic fluid specimens (cases)	156	81		
Positive amplification samples (cases)	127	73		
Positive amplification rate (%)	81.41	90.12	5.482	0.140

Table II. Pyrosequencing success rate of *H19* gene DMR methylation status in amniotic fluid cells.

Variables	Singleton	Twins	Statistical value	P-value
PCR positive samples (cases)	127	73		
Successfully sequenced samples (cases)	98	64		
Sequencing success rate (%)	77.2	87.7	3.629	0.304

Statistical analysis. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used. Count data were processed by Chi-square test. Measurement data were expressed as mean ± SD and comparisons between two groups were performed by t-test. $P<0.05$ was considered to indicate a statistically significant difference. Mean ± 2SD standard deviation was used as normal range of test results in Boxplot statistical analysis, otherwise regarded as outliers. SPSS 16.0 (SPSS Inc., Chicago, IL,USA) was used for Boxplot statistical analysis.

Results

Results of bisulfite-PCR amplification of H19 DMR region from amniotic fluid cell DNA. A total of 237 cases of amniotic

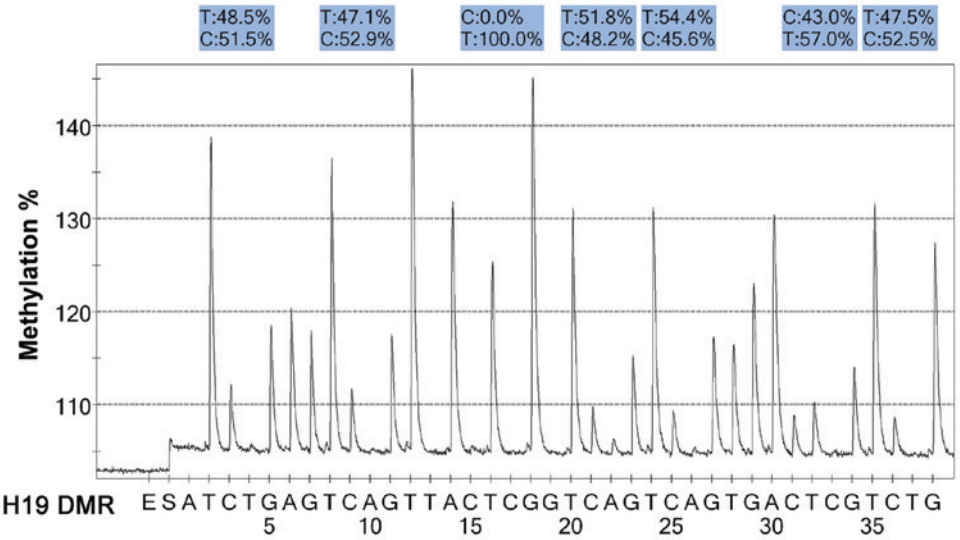

Figure 1. Sequencing results of *H19* gene DMR methylation. Pyrosequencing showing C to T conversion rate of 100%, and the average methylation rate was 49.0%.

fluid cells were treated by sodium bisulfite and subjected to PCR amplification reaction, and 200 cases of positive amplifications were obtained. Overall successful amplification rate was 84.4%. Non-specific bands were not observed. Average age of singleton pregnant women was higher than those with twins (P<0.05). No significant difference in success rate and gestational age was found between singleton pregnancy and twin pregnancy (P>0.05; Table I).

Pyrosequencing success rate of H19 gene DMR methylation status in amniotic fluid cells. Two hundred cases of amniotic fluid cells with positive bisulfite-PCR amplification of *H19* DMR were subjected to pyrosequencing, and 162 cases were sequenced successfully, and the overall sequencing success rate was 81.0%. There was no difference in success rate of pyrosequencing between singleton and twin groups (P=0.304; Table II).

Methylation status of H19 gene DMR in the second trimester of pregnancy. All amniotic fluid cell DNA was effectively converted to be phosphorylated with a conversion rate of 90.1-100%. In pyrosequencing 162 cases of amniotic fluid cells were successful, including 98 cases of singleton pregnancy and 64 cases of twin pregnancy. In singleton group, sequencing failed in loci 1 in 16 cases. In twins group, sequencing failed in loci 1 in 5 cases. Sequencing results are shown in Fig. 1. There was no difference in the methylation rate of loci 1-6 and the average methylation rate between singleton and twins group (P>0.05; Table III).

Abnormal methylation status of DMR of H19 gene in the second trimester of pregnancy. Abnormal *H19* gene DMR methylation determination: Mean ± 2SD standard deviation was used as normal range of test results in Boxplot statistical analysis, otherwise regarded as outliers. Abnormal methylation rate of DMR of *H19* gene was higher in twins than in singleton; significant difference was found in loci 1 (P=0.004), but no significant difference was found in other loci (P>0.05; Table IV).

Table III. *H19* DMR methylation rate in amniotic fluid cells during the second trimester of pregnancy (%).

Items	Singleton (n=98)	Twins (n=64)	Statistical value	P-value
Loci 1	48.380±10.246[a]	49.450±24.071[b]	0.285	0.594
Loci 2	48.705±11.336	49.098±19.768	0.059	0.809
Loci 3	44.505±10.849	44.680±18.746	0.021	0.885
Loci 4	40.741±10.423	41.590±19.575	0.204	0.652
Loci 5	32.473±11.975	33.116±16.957	0.072	0.789
Loci 6	50.682±13.222	49.133±20.139	2.335	0.129
Average methylation rate	44.271±9.944	44.383±18.969	0.047	0.829

Loci 1 [a]n=82, [b]n=59.

Table IV. Abnormal methylation status of DMR of *H19* gene in the second trimester of pregnancy (n, %).

Items	Singleton (n=98)	Twins (n=64)	Statistical value	P-value
Loci 1	3 (3.6)[a]	12 (20.3)[b]	8.364	0.004
Loci 2	5 (5.1)	7 (10.9)	1.085	0.298
Loci 3	4 (4.1)	8 (12.5)	2.867	0.090
Loci 4	6 (6.1)	10 (15.6)	2.933	0.087
Loci 5	8 (8.2)	9 (14.1)	0.875	0.350
Loci 6	3 (3.1)	7 (10.9)	2.898	0.089
Average methylation rate	5 (5.1)	7 (10.9)	1.165	0.280

Loci 1 [a]n=82, [b]n=59.

Table V. Comparison of offspring's birth outcomes between normal and abnormal *H19* gene methylation level.

Variables	DMR methyl % normal	DMR methyl % abnormal	Statistical value	P-value
Cases	141	12		
Male (%)	70 (49.6)	4 (33.3)	1.178	0.278
Twin (%)	49 (34.8)	8 (66.7)	4.891	0.028
Cesarean section (%)	101 (71.6)	10 (83.3)	0.341	0.559
Birth gestational week (w)	37.1±1.9	36.2±1.3	1.555	0.122
Height (cm)	47.1±3.3	46.3±3.5	0.760	0.461
Weight (kg)	2.82±0.59	2.63±0.59	1.050	0.295
Premature delivery (%)	54 (38.3)	7 (58.3)	1.852	0.754
Low birth weight (%)	52 (36.9)	7 (58.3)	2.148	0.143
Congenital malformations (%)	1 (0.7)	0 (0)	0.086	0.770

Comparison of offspring's birth outcomes between normal and abnormal H19 gene methylation level. Nine cases in 162 newborns were lost during follow-up. Significant differences were found between DMR methyl % normal and abnormal group. Proportion of twins in *H19* abnormal methylation group was higher than that in normal group (66.7 vs. 34.8%, χ^2=4.891, P=0.028). Offspring sex, singleton or twins, mode of delivery, time of pregnancy, and low birth weight showed no significant effect on GapG methylation level of *H19* in the second trimester of pregnancy (P>0.05; Table V).

Discussion

As an important part of epigenetics, DNA methylation can inhibit gene expression at the transcriptional level, which is closely related to tumorigenesis, gene imprinting, inactivation of X chromosome and maintenance of chromosome structure (7,8). Therefore, DNA methylation is still a hot research field in epigenetics.

Pyrosequencing was used in this study. With the advantages of high throughput, high precision, high stability, real-time quantification and automated sequencing capabilities, pyrosequencing technology has been widely used in sequence, methylation analysis and forensic identification and other fields (9). It has been reported that the pyrosequencing technology has a sensitivity and specificity of 100% for disease diagnosis, and the results are completely consistent with methylation-specific PCR results (10). Compared with bisulfite sequencing PCR (BSP), pyrosequencing has the advantages of high repeatability and consistency (11,12).

Results of this study showed that average methylation level of DMR in *H19* of singleton pregnancies showed no difference to that of twins in the second trimester of pregnancy. The difference between this and previous studies is that the offspring are in intrauterine growth and development stages. Results showed no significant differences in DMR methylation level of *H19* gene between singleton and twin, but abnormal DMR methylation rate of twins is higher than that of singleton pregnancy, suggesting that fetal development of twins may have been disturbed by external factors, and the sensitivity of DMR CpG1 to *H19* gene is stronger than that of other CpG sites. Although the level of methylation of DMR CpG1 in amniotic fluid cells of twin pregnancies during the second trimester pregnancy was abnormal, the average methylation level did not change. On one hand, DMR CpG1 of *H19* gene may have limited influence on *H19* gene function; on the other hand, besides methylation, expression of imprinted genes may also be affected in many other epigenetic regulatory mechanisms. Intrauterine growth stage is a key period of fetal epigenetic modification (3). With the increase of gestational age, maternal peripheral blood estrogen levels increased significantly in early pregnancy. Use of certain medications exposes the fetus to a high estrogen environment, which interferes with the establishment of fetal epigenetic modification (13). In addition, it has been speculated that abnormalities in epigenetic modification may also be related to the dysfunction of DNMT-1 in maintaining methylation of the imprinted gene before embryo implantation (14,15). Fetal acid intake during pregnancy, nutritional status, and smoking may affect the methylation pattern of *H19* DMR in the offspring (16). The relative lack of fetal nutrition due to twin pregnancies may activate the function of the pituitary-adrenal axis to stimulate excessive fetal adrenal gland growth and upregulate IGF2 expression, thus affecting the fetal *H19* DMR methylation status (17).

In the present study, correlation analysis between *H19* gene methylation and the birth outcomes showed that neonatal sex, mode of delivery and birth weight showed no significant correlation with *H19* methylation level in the second trimester of pregnancy (P>0.05). Proportion of twins in the abnormal methylation group was higher than that in the normal methylation group (P<0.05). In addition, changes of the average methylation rate of DMR in amniotic fluid cells did not have significant effect on birth outcome. Increased or decreased abnormal methylation level showed no significant effects on deformity of offspring. Normal and abnormal methylation showed no significant correlation with neonatal sex, gestational age of birth, average body weight and height. The data show that *H19* methylation level in amniotic fluid cells during the second trimester of pregnancy may not be correlated with birth outcomes. Degree of methylation of *H19* in amniotic fluid cells has no predictive value on the

birth weight of offspring. Number of the fetuses born may affect abnormal methylation of *H19*. The formation of human individual and intrauterine growth and development is a relatively long and complex process. Growth and development of the fetus are regulated by many factors of the placenta or maternal body. On one hand, aberrant methylation of *H19* gene in the second trimester of pregnancy may be corrected; on the other hand, the birth outcome is associated with multiple factors, and the effect of abnormal methylation of *H19* gene may be neutralized (18,19). Due to the lack of a comparative study of the methylation status of *H19* in amniotic fluid cells during late pregnancy, solid conclusion on the effects of *H19* methylation on birth outcomes of offspring cannot yet be obtained, which is also a deficiency of this study.

All subjects were normal fetuses tested by amniotic fluid screening in the second trimester of pregnancy. Only the appearance of newborns at birth was grossly judged. One congenital malformation was observed, and the incidence of deformity was significantly lower than that reported in previous studies. Due to the short follow-up period, it is still not possible to rule out abnormalities of internal organs or occurrence of imprinted gene-related diseases during the subsequent growth and development. In addition, the sample size is small, so the conclusion is unilateral. Long-term, systematic follow-up and experimental studies are still needed.

In conclusion, abnormal methylation of *H19* gene in the second trimester of pregnancy mainly occurs in CapG1, and the number of fetuses born may affect abnormal GapG1 methylation, but CapG1 does not affect the average methylation of *H19* gene, which may have a limited impact on *H19* gene function. Level of methylation of *H19* gene in amniotic fluid cells during the second trimester of pregnancy was not related to birth outcome of offspring, so the predictive value was limited.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YS and XL conceived and designed the study. ML and DC collected the patient data. HZ and SC were responsible for analysis and interpretation of the data. YS drafted the manuscript. ML revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). Signed informed consents were obtained from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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