Noninvasive Prenatal Diagnosis of Down Syndrome in Samples from Southwest Chinese Gravidas Using Pregnant Plasma Placental RNA Allelic Ratio

Youcheng Zhang,^{1–3} Hongqian Liu,^{1,2} Xinlian Chen,^{1,2} Xiaoyan Xie,^{1,2} Shanling Liu,^{1,2,*} and He Wang^{1,2,*}

Objective: The purpose of this research was to attempt a preliminary study of noninvasive prenatal diagnosis of Down syndrome in Southwest Chinese gravidas by using the plasma placental RNA allelic ratio. **Methods:** The genotypes of the single-nucleotide polymorphisms (SNPs) located in the transcribed regions of the gene *PLAC4* were detected in population samples collected in Southwest China by using polymerase chain reaction–restriction fragment length polymorphism, and SNPs with a higher heterozygosity were selected. Mass spectrometer analysis was adopted, and cases with the heterozygous SNPs on *PLAC4* mRNA in maternal plasma were selected from 29 pregnancies with a euploid fetus and from 21 pregnancies with a trisomy-21 fetus, and then their RNA-SNP allelic ratios were further determined for noninvasive prenatal diagnosis of Down syndrome. **Results:** Of all 50 singleton pregnancies, 37 gravidas were found with at least one heterozygous SNP on *PLAC4* mRNA in maternal plasma. Among them, 13 pregnancies with a trisomy-21 fetus were detected by the analysis of the RNA-SNP allelic ratio. **Conclusion:** The plasma placental RNA allelic ratio can be used for noninvasive prenatal diagnosis of Down syndrome, if SNPs on *PLAC4* mRNA in maternal plasma are heterozygous.

Introduction

DOWN SYNDROME (DS) is the most commonly found congenital mental retardation, with an incidence of 1 in every 800 live births (Lau *et al.*, 1998). Karyotyping of fetal cells is the gold standard for prenatal diagnosis of DS. However, it will take at least 14 days from the obtaining of fetal cells to the available result, in which a gravida has to be suffering an uneasy episode. In addition, the procedures to obtain fetal cells such as chorionic villus sampling and amniocentesis are invasive, and may impose potential risks to the mother or the fetus (ACOG Practice Bulletin No. 88., 2007). Therefore, it is desirable to develop a rapid and noninvasive prenatal diagnostic method to diagnose DS.

The discovery of cell-free fetal nucleic acids in maternal circulation opens a new window for noninvasive prenatal diagnosis, which has been moved into the application of testing fetal genetic disorders (Lo *et al.*, 1997; Lo and Chiu, 2007). Cell-free fetal nucleic acids in maternal blood include the fragments of cell-free fetal DNA (cff DNA) and cell-free fetal RNA (cff RNA), which can be both detected in all three trimesters of pregnancy (Puszyk *et al.*, 2008). Recently, several methods for noninvasive prenatal diagnosis of chromosomal

aneuploidy have been reported that made use of cell-free fetal nucleic acids in maternal plasma (Lo *et al.*, 2007a, 2007b; Fan *et al.*, 2008; Tsui *et al.*, 2010). They can bring out results as quick as in 1 or 2 days, and fulfill the expectation of rapidness and safety. Plasma placental RNA allelic ratio is a method that relies on cff RNA for chromosomal aneuploidy detection (Lo *et al.*, 2007b).

Plasma placental RNA allelic ratio is a method of using allelic ratios of placental-specific mRNA in maternal plasma to detect fetal chromosomal aneuploidy, which makes use of the genetic single-nucleotide polymorphism (SNP) in certain populations. Such as in detecting fetal trisomy 21, a specific placental-derived mRNA transcribed from chromosome 21, *PLAC4* mRNA (Kido *et al.*, 1993), which can be detected in the plasma of women in all three trimesters of pregnancy, but will be cleared from maternal plasma within 24 h after delivery, was identified as deriving from fetus (Lo *et al.*, 2007b). Then, for a fetus with a transcribed heterozygous SNP locus of *PLAC4*, the relative dosage of chromosome 21 can be found out by determining the ratio between two alleles of this heterozygous SNP on *PLAC4* mRNA in maternal plasma. The allelic ratio was expected to be 1:1 in a euploid fetus, but 2:1 or 1:2 in a trisomy-21 fetus. It has been reported that the

¹Prenatal Diagnosis Center, Key Laboratory of Ob/Gyn and Pediatric Diseases and Birth Defects of Ministry of Education and ²Lab of Genetics, Department of Ob/Gyn, West China Second University Hospital, Sichuan University, Chengdu, P.R. China.

³Department of Obstetrics, People's Hospital of GuiZhou Province, GuiYang, P.R. China.

*These two authors contributed equally to this work.

diagnostic sensitivity and specificity can reach 100% and 89.7%, respectively (Tsui *et al.*, 2010).

Since different SNPs existed possibly in different nationalities with the same gene, we set our goal to find the SNPs' heterozygous condition of *PLAC4* in a Southwest Chinese population, and to explore the possibility of noninvasive prenatal diagnosis of DS for gravidas in this area by the method of plasma placental RNA allelic ratio. So, we first detected the genotypes of 10 SNPs located in the transcribed regions of the gene *PLAC4* in Southwest Chinese populations and selected three SNPs with a higher heterozygosity. Then, by performing genotyping and ratio determination of these three SNPs on maternal plasma mRNA, we explored noninvasive prenatal detection of fetal trisomy 21.

Materials and Methods

Subject enrollment

This study was approved by the Medical Ethics Committees of the West China Second University Hospital, Sichuan University. All patients recruited for this study were born in Southwest China. Informed consent was obtained from each patient before the blood draw. About 5 mL of peripheral blood was collected, respectively, from every one of 100 unrelated donors for genotyping SNPs of the transcribed regions of the gene *PLAC4*; these donors included 60% of women and 40% of men. Fifty singleton pregnancies at 22–24 weeks of gestation were recruited for our study, including 21 pregnancies that carried a trisomy fetus and 29 pregnancies carried a euploid fetus. About 12 mL of maternal peripheral blood was collected after a fetal karyotype was confirmed. Five maternal peripheral blood samples after 24 h of delivery were also collected to detect the clearance of *PLAC4* mRNA in maternal plasma.

Sample processing

Genomic DNA was extracted from peripheral blood cells of 100 unrelated donors by using the RelaxGene Blood DNA System (TIANGEN), according to the manufacturer's instructions.

The collected maternal blood sample was immediately centrifuged at 1600 g for 10 min at 4°C. Plasma was transferred to clean microcentrifuge tubes and recentrifuged at 16,000 g for 10 min at 4°C to remove residual cells. Then, the supernatant was, respectively, distributed into microcentrifuges of 1.5 mL. Every microcentrifuge included 0.3 mL of plasma and 0.9 mL of the TRIzol LS reagent (Invitrogen), and was stored at –80°C. When maternal plasma RNA was being extracted, 240 µL of chloroform was added into the 1.2 mL of the plasma and TRIzol LS mixture, and centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was transferred to a new tube, and 345 µL of 100% ethanol was added too. Then, the mixture was processed with the RNeasy mini kit (Qiagen), according to the manufacturer's protocol. DNase 1 treatment was performed before the first wash step. The total RNA was eluted with 40 µL of RNase-free water.

SNP genotyping

Ten SNPs were selected from the transcribed regions of the *PLAC4* gene according to their heterozygosity-sorting order, and then genotyped by using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. The details are shown in Table 1.

PCRs were performed in a 25-µL volume containing 2.5 µL of 10× PCR buffer, 1 mM of MgCl₂, 10 pmol of forward and reverse primer, 0.2 mM of dNTPs, 2.5 U of Taq DNA polymerase (TaKaRa), and 100 ng of genomic DNA template. PCR conditions were initially denatured for 5 min at 94°C;

TABLE 1. TEN SINGLE-NUCLEOTIDE POLYMORPHISMS LOCATED IN THE TRANSCRIBED REGIONS OF THE GENE *PLAC4* AND THEIR POLYMERASE CHAIN REACTION–RESTRICTION FRAGMENT LENGTH POLYMORPHISM STRATEGIES

dbSNP accession numbers	Sizes of selected sequence (bp)	Primer pairs	Restriction enzyme	Enzyme recognition sequence	Sizes of digestion products (bp)	Allele
rs7844	180	Foward: CAGGGCCACAAGGTGTAAAT Reverse: TGGGCAACAAGGTAACCACT	Eam1105I	GACNNN'NNGTC	None 107-73	C G
rs9977003	367	Foward: TGTGCAGAAAGGCTGTGTTC Reverse: AAACCGTGGGACCAGTGTAG	BspLI	GGN'NCC	None 232-135	A G
rs11909439	207	Foward: CAAGCCCACAGGAGATGTTT Reverse: AGGGCACTACAACCACAACC	DpnI	GA'TC	None 122-85	T C
plac4-41471145	382	Foward: TCCCATGAATGAGACAGTGG Reverse: CTGTGGGGCAAGACTAGGAG	TaqI	T'CGA	240-142 240-111-31	A G
plac4-41476236	200	Foward: TCCCTGTCTTTACCCTGTCTTT Reverse: GTTGAGCTGCTGTGGTCTTG	HapII	C'CGG	None 144-56	T C
rs73902945	219	Foward: AAAGGAGTTCAAGGGCCAAT Reverse: TTGTTTGTTTTGCAAGGAAGG	TscAI	CASTGNN'	None 167-52	A G
rs73902941	247	Foward: GCGTTTATGGTATTGCGAGA Reverse: TGCAGTCAGAGGACAGGATG	BstUI	CG'CG	None 190-57	A G
rs73364412	247	Foward: CCTGGCACTGGAGGAGTAAA Reverse: CCTTCCCAAGTCTCACCTGT	Hin1II	CATG'	None 171-76	G A
rs57965115	213	Foward: ACAGCAAGCGGATGACAATA Reverse: AGGTCAAGCCTGTGTCCACT	HincII	GTY'RAC	None 160-53	T C
rs1802815	219	Foward: CAAGCCAGTGGTGTTAGCAA Reverse: CCCAGAAGTTACCACCTCA	AccI	GT'ATAC	None 144-75	C A

SNP, single-nucleotide polymorphism; dbSNP, database of SNP.

TABLE 2A. PRIMER SEQUENCES FOR REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION AMPLIFICATION OF THE mRNA OF THE THREE HIGHER HETEROZYGOUS SINGLE-NUCLEOTIDE POLYMORPHISMS

SNP	Forward PCR primer	Reverse PCR primer
rs7844	ACGTTGGATGCATACAAAATACAAAGTGTG	ACGTTGGATGAAACCGTGGACCAAGTGTAG
PLAC4-41471145	ACGTTGGATGAAAATCGACAGAAAGGC	ACGTTGGATGTAGCACAGATTTTCTGA
PLAC4-41476236	ACGTTGGATGAAAATACTGCTTCCAC	ACGTTGGATGTAGCAAGACTAAGTCTCTG

The bold fonts indicate the 10-mer tags. The tags can distinguish the unused PCR primers from the extension products in the mass spectrometer analysis.

TABLE 2B. SEQUENCES AND MOLECULAR WEIGHTS OF THE EXTENSION PRIMER AND EXTENSION PRODUCTS FOR TWO ALLELES OF EACH OF THE THREE HIGHER HETEROZYGOUS SINGLE-NUCLEOTIDE POLYMORPHISMS

SNP	Extension primer	Molecular weight (Da)	Extension product 1	Molecular weight (Da) 1	Extension product 2	Molecular weight (Da) 2
rs7844	CCAGTGTAGAAAGAAATGGA	5596.7	CCAGTGTAGAAAGAAATGGAC	5843.9	CCAGTGTAGAAAGAAATGGAG	5883.9
PLAC4-41471145	TGAATATCTATTTTGACAGGTT	6754.4	TGAATATCTATTTTGACAGGTTC	7001.6	TGAATATCTATTTTGACAGGTTT	7081.5
PLAC4-41476236	AAGTCTCCTGGTTGAAC	5185.4	AAGTCTCCTGGTTGAACC	5432.6	AAGTCTCCTGGTTGAACT	5512.5

The bold fonts indicate the extended dNTPs. According to the extension direction of these primers, for rs7844, the base matched to C or G located in sense strand was extended, so the allele genotypes of the extension products should be G/G or G/C; for PLAC4-41471145, the base matched to G or A located in the sense strand was extended, so the allele genotypes of the extension products should be C/C or C/T; for PLAC4-41476236, the extended base was located in the sense strand, so the allele genotypes of the extension products should still be C/C or C/T.

followed by 35 cycles of 45 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and with a final extension for 7 min at 72°C. Amplification was verified by running 3 µL of PCR product on a 2% agarose gel containing gelview and UV visualization. Then, each PCR product was digested for 16 h, respectively, according to every restriction enzyme's recommended protocol for digestion. About 10 µL of the digested reaction was run on a 3% agarose gel containing gelview, and gels were photographed. Genotypes were assigned by band patterns. Three SNPs with a higher heterozygosity were chosen.

Plasma placental RNA allelic ratio analysis for noninvasive prenatal diagnosis of DS

After analyzing the results of PCR-RFLP, we chose three SNPs with a higher heterozygosity, which were rs7844, PLAC4-41471145, and PLAC4-41476236. Then, we performed genotyping and ratio determination of these three SNPs on maternal plasma mRNA of those 50 pregnant women by matrix-assisted laser desorption and an ionization time-of-flight (MALDI-TOF) mass spectrometer. The steps consisted of reverse transcription (RT)-PCR amplification and base extension as well as analysis of the extension products by a mass spectrometer. Sequences of the oligonucleotides used in these reactions are shown in Tables 2a and b.

RT-PCR amplification: PLAC4 mRNA of those 50 pregnant maternal plasma was amplified by one-step RT-PCR using the respective primers of the three SNPs. The reaction was set up using the Quant One Step RT-PCR Kit (TIANGEN) in a reaction volume of 100 µL, which contained 10 µL of 10× RT-PCR buffer, 400 µM of dNTPs, 20 µL of 5× RT-PCR enhancer, 1 µL of RNasin, 0.125 U/µL of Hotmaster Taq polymerase, 1.0 µL of Quant RTase, 600 nM of forward and reverse primer, 40 µL of maternal plasma RNA template, and a sufficient quantum of RNase-free ddH₂O. The reaction was initiated at 50°C for 2 min, followed by reverse transcription at 50°C for 30 min, denaturation at 94°C for 5 min, and 45 cycles of PCR at 94°C for 45 s, 55°C for 30 s, and 65°C for 1 min, and a final extension at 65°C for 10 min. The reaction without reverse transcription was also set up to eliminate the false-positive result by DNA contamination. The reaction of samples after 24 h of delivery was also set up to confirm the clearance of PLAC4 mRNA in maternal plasma.

Base extension and analysis: Base extension and analysis of RNA-SNP of all samples were performed in a 384-well reaction plate. About 5 µL of the RT-PCR product was subjected to shrimp alkaline phosphatase (SAP) treatment to prevent the influences of remaining dNTPs in the subsequent primer extension. About 0.17 µL of 10× SAP buffer (Sequenom), 0.3 U of SAP enzyme (Sequenom), and 1.53 µL of ddH₂O were added in every well of the RT-PCR product. The reaction was incubated at 37°C for 40 min, followed by inactivation at 85°C for 5 min.

For primer extension, to every well was added 2 µL of iPLEX reaction containing 0.2 µL of 10× iPLEX buffer (Sequenom), 0.2 µL of iPLEX Termination mix (Sequenom), 0.804 µL of primers, 0.041 µL of iPLEX enzyme (Sequenom), and 0.755 µL of ddH₂O. Extension conditions were 94°C for 35 s; followed by 5 cycles of 52°C for 5 s and 80°C for 5 s; then followed by 40 cycles of 94°C for 5 s, 52°C for 5 s, and 80°C for 5 s; and with a final extension at 72°C for 3 min. The extension products were cleaned up with Clean Resin (Sequenom) to

remove salts, and then a MALDI-TOF mass spectrometer analysis was performed on a MassARRAY compact System (Sequenom). When analyzed, a homozygous SNP showed only one peak, but a heterozygous SNP showed two different peaks (Fig. 1). The peak area of the base extension product corresponding to the allele was taken as the relative amount of the SNP allele.

Statistical analysis and GenBank accession

Statistical analysis was performed with SPSS 16 software (SPSS, Inc.). All our assays were designed from the reference sequence of PLAC4, according to the GenBank Accession, NM_182832.

Results

The analysis of the results of PCR-RFLP revealed three SNPs with a higher heterozygosity in the transcribed regions of the gene *PLAC4*, and every SNP had only two kinds of allelic genotypes, including one kind of heterozygote and one kind of homozygote. The heterozygosity of every SNP, respectively, was rs7844, 25%; PLAC4-41471145, 48%; and PLAC4-41476236, 21%. An expected population coverage of 69% by the heterozygotes of these three SNPs was calculated $[1-(1-25\%)(1-48\%)(1-21\%) \approx 69\%]$. Then, we detected the genotype distribution of these three SNPs on *PLAC4* mRNA in maternal plasma. Every SNP also had two kinds of allelic genotypes, and the type of heterozygote or homozygote was also, respectively, similar to that in DNA. The heterozygosity of every mRNA-SNP, respectively, was rs7844, 28%; PLAC4-41471145, 36%; and PLAC4-41476236, 10%. In 50 singleton pregnancies, the total amount of heterozygotes of these three mRNA-SNPs was 37, so an exact population coverage of 74% can be achieved in our experimental design (37/50=74%).

The allelic ratio analysis of the 37 heterozygous RNA-SNPs was performed, and the detail results are shown in Figures 1 and 2. For rs7844 samples, the RNA-SNP ratios were calculated using the relative amount of the higher-mass allele dividing the relative amount of the lower-mass allele. All five trisomy-21 samples showed a lower allelic ratio and deviated from the euploid group (Fig. 2). For PLAC4-41471145 samples, the RNA-SNP ratios were calculated by using the relative amount of the lower-mass allele dividing the relative amount of the higher-mass allele. All eight trisomy-21 samples showed a lower allelic ratio and deviated from the euploid group (Fig. 2). For PLAC4-41476236, since samples with heterozygous SNP were only detected in trisomy-21 cases, the comparative analysis cannot be conducted. Since all five trisomy-21 samples with a heterozygous rs7844 SNP and all eight trisomy-21 samples with a heterozygous PLAC4-41471145 SNP were diagnosed to be T21 pregnancies, but not euploid pregnancies, the data from the analysis of these two SNPs offered a diagnostic sensitivity of 100%.

Discussion

It has become an important research goal to discover markers that are specific to the fetus in maternal blood for noninvasive prenatal diagnosis of DS. Discovering epigenetic markers of cfDNA is a preferential choice (Old *et al.*, 2007), but these markers may differ in different individuals and change during gestation (Puszyk *et al.*, 2008). cfRNA could

FIG. 1. Mass spectrometer tracing the RNA-SNP allelic ratio determination of the maternal plasma samples with the mRNA heterozygote. The molecular weights of the base extension products (shown as sharp peaks) were depicted by x-axis, whereas the intensity in arbitrary units was depicted by y-axis. (1) and (2) were samples with heterozygotic rs7844 SNP. (1) depicted one sample from a pregnant woman carrying a euploid fetus; (2) depicted one sample from a pregnant woman carrying a trisomy-21 fetus. (2) showed a lower C/G ratio than (1). (3) and (4) were samples with heterozygotic PLAC4-41471145 SNP. (3) depicted one sample from a pregnant woman carrying a euploid fetus; (4) depicted one sample from a pregnant woman carrying a trisomy-21 fetus. (4) showed a higher C/T ratio than (3). (5) was one sample with heterozygotic PLAC4-41476236 SNP from a pregnant woman carrying a trisomy 21-fetus. SNP, single-nucleotide polymorphism.

serve as another resource for choice of markers. PLAC4 mRNA, a chromosome-21 transcript, which rapidly disappears from maternal circulation after delivery and cannot be detected in the plasma of nonpregnant women, should be a virtual preference for fetal-specific makers (Lo *et al.*, 2007b). Lo *et al.* (2007b) definitively confirmed the fetal origin of

PLAC4 mRNA molecules in maternal plasma and applied it to investigation of noninvasive prenatal detection of DS for detection of the PLAC4 RNA-SNP ratio in maternal plasma. In their article, only one SNP rs8130833 was used to determine the RNA-SNP allelic ratio. We adopted their method of RNA-SNP allelic ratio determination, but chose other three

FIG. 2. RNA-SNP allelic ratios of maternal plasma samples from euploid and trisomy-21 pregnancies. Δ , Trisomy 21; $\circ$, Euploid. **(a)** was the analysis of rs7844 heterozygote samples. The y-axis depicted the allelic ratios of C/G. Number of euploid samples=9, and number of trisomy-21 samples=5. All trisomy-21 samples showed a lower C/G ratio and deviated from the euploid group. **(b)** was the analysis of PLAC4-41471145 heterozygote samples. The y-axis depicted the allelic ratios of T/C. Number of euploid samples=10, and number of trisomy 21 samples=8. All trisomy-21 samples showed a lower T/C ratio and deviated from the euploid group.

SNPs (rs7844, PLAC4-41471145, and PLAC4-41476236) in the transcribed regions of the gene *PLAC4*, to assess the accessible application of this technique to noninvasive prenatal diagnosis of DS in Southwest China.

Since only the heterozygous fetus with *PLAC4* mRNA SNPs can be detected using this method, it is indispensable for RNA-SNP allelic ratio testing that there should be surely enough samples with heterozygous SNPs. The reason we investigated the genotypes of SNPs located in the transcribed regions of the gene *PLAC4* was to find out as many as possible heterozygous SNPs and SNPs with a higher heterozygosity, so as to offer as many as possible samples for testing.

We found three SNPs with a higher heterozygosity from our detection of the transcribed regions of the gene *PLAC4* for populations in Southwest China. The heterozygosity of PLAC4-41476236 was similar to that in GenBank, but the heterozygosities of rs7844 and PLAC4-41471145 were detected as different from those in GenBank. This suggested that there may be a specific distribution characteristic for SNPs located in the transcribed regions of the gene *PLAC4* in Southwest Chinese populations. Since our sample size was not big enough, we should be cautious in interpreting the results as a generalized result for populations in Southwest China.

We then detected the genotype distribution of these three SNPs on maternal plasma mRNA for those 50 pregnant women. Since no case with a euploid fetus was detected to have a heterozygous SNP of PLAC4-41476236, we carried out only the mRNA-SNP allelic ratio analysis of maternal plasma

samples with heterozygous SNPs of rs7844 and PLAC4-41471145. The analysis results from two SNPs indicated a diagnostic sensitivity of 100%, offering a significant accuracy for noninvasive prenatal diagnosis of DS.

The heterozygous genotyping results of three mRNA-SNPs in maternal plasma reached population coverage of 74%. We believed that higher population coverage can be reached if more SNPs of the transcribed regions of the gene *PLAC4* were detected. In these 37 heterozygous samples, there were 18 pregnancies carrying a trisomy-21 fetus, but we could not perform the mRNA-SNP allelic ratio analysis of all these 18 cases. In 18 cases with DS, five T21 pregnancies of rs7844 and eight T21 pregnancies of PLAC4-41471145 can be diagnosed from their respective euploid reference range, but for the cases with heterozygous SNP of PLAC4-41476236, since no pregnancies with a euploid fetus were detected, a reference range used in group comparison was nonexistent, and therefore the allelic ratio analysis cannot be performed even though five pregnancies with a trisomy-21 fetus were detected. So, only 13 samples were analyzed (5 T21 pregnancies of rs7844 and 8 T21 pregnancies of PLAC4-41471145). In reality, the reference range of euploid pregnancies is essential for the application of this method. For every heterozygous SNP, trisomy-21 pregnancies cannot be diagnosed until the reference range of euploid pregnancies is provided, and there must be more samples of euploid pregnancies than those of trisomy-21 pregnancies. For the foreseeable future, the establishment of the reference range of euploid pregnancies may be a promising aspect for

noninvasive prenatal diagnosis of DS using the plasma placental RNA allelic ratio.

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Author Disclosure Statement

The authors have no conflicts of interest to disclose.

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Address correspondence to:

Shanling Liu, Ph.D.

Prenatal Diagnosis Center

Key Laboratory of Ob/Gyn and Pediatric Diseases

and Birth Defects of Ministry of Education

Sichuan University

Chengdu 610041

P.R. China

E-mail: sunny630@126.com

He Wang, M.D.

Prenatal Diagnosis Center

Key Laboratory of Ob/Gyn and Pediatric Diseases

and Birth Defects of Ministry of Education

Sichuan University

Chengdu 610041

P.R. China

E-mail: wanghe2008_cd@126.com