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Application of droplet digital PCR for prenatal screening of Down syndrome

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Summary

Background: This study was proposed to explore the feasibility of droplet digital PCR (ddPCR) analysis for non-invasive prenatal diagnosis of Down syndrome. *Materials and Methods:* The authors studied maternal plasma samples from 465 pregnant women carrying euploid and trisomy 21 (T21) fetuses. A methylation-sensitive restriction endonuclease, BstUI, was used to digest the hypomethylated holocarboxylase synthetase (HLCS) gene. The authors quantified digestion-resistant HLCS gene on chromosome 21 and fetal-specific rs6636 SNP allele on chromosome 14 by droplet digital PCR analysis. Maternal plasma DNA analysis was performed by comparing the ratio of hypermethylated HLCS to fetal-specific rs6636 SNP allele between two groups. *Results:* Using a rs6636 SNP allele on chromosome 14 as the reference marker, the authors analyzed 78 euploid and 28 T21 plasma samples. The ratios of the numbers of positive hypermethylated HLCS and the fetal-specific C allele in the euploid and T21 samples were significantly different ($p < 0.01$, Mann–Whitney rank sum test), all but two samples with the fetal-specific C allele were correctly classified, while the ratios of the numbers of positive hypermethylated HLCS and the fetal-specific G allele in the euploid and T21 samples were significantly different ($p < 0.01$, Mann–Whitney rank sum test); all but one sample with the fetal-specific C allele were correctly classified. *Conclusions:* The study demonstrated that ddPCR approach can be applied for prenatal screening of trisomy 21.

Key words: Down syndrome; ddPCR; HLCS; qPCR; cffDNA.

Introduction

Down syndrome, also known as trisomy 21, is one of the most common chromosome abnormalities in humans caused by the presence of all, or part of a third copy of chromosome 21[1, 2]. It occurs in about one per 1,000 babies born each year [3]. Down syndrome was present in 8.5 million individuals and resulted in 36,000 deaths in 2013 [4, 5]. Furthermore, the cause of Down syndrome is still unknown and this disease cannot be cured.

Diagnostic tests and screening tests are two categories of tests for Down syndrome. Diagnostic tests can provide a definitive diagnosis with nearly 100% accuracy. The diagnostic procedures available for prenatal diagnosis of Down syndrome are always invasive sampling including amniocentesis and chorionic villus sampling (CVS) to obtain a fetal karyotype which is performed to confirm the diagnosis[6, 7]. However, these invasive procedures carry up to a 1% risk of causing a spontaneous termination (miscarriage) [8].

In order to screen for Down syndrome, prenatal screening tests are offered to all pregnant women, including a blood test and an ultrasound (sonogram) [9, 10]. Analysis of cell-free fetal DNA (cffDNA) has being studied as a method of testing of the mother's blood which appears promising in the first trimester of pregnancy [11]. cffDNA is fetal DNA circulating freely in the maternal blood stream and can be sampled by venipuncture on the mother. Recently, the holocarboxylase synthetase (HLCS) gene (NM_000411) on chromosome 21 as a cffDNA could be detected as a fetal-specific DNA marker in maternal plasma because of the hypermethylated promoter of HLCS in placental tissues[12]. Some studies have demonstrated that cffDNA analysis can be applied to the prenatal diagnosis of Down syndrome by comparing the amount of digestion-resistant HLCS to that of a single nucleotide polymorphism (SNP) allele (rs6636, a C/G SNP) that the fetus has inherited from the father but absent in the pregnant mother [13, 14]. SNP rs6636 is located within the transmembrane emp24 protein transport domain containing 8 (TMED8) gene (AK095650) on chromosome 14. Nowadays, many different methods have been proposed to perform analysis of cffDNA. One method is quantitative real-time PCR (qPCR). qPCR is a very accessible technique and becomes the "gold standard" technology to quantify nucleic acids since the first report in 1993 [15, 16]. The other is droplet digital PCR (ddPCR), the third generation of conventional PCR technique. In ddPCR, a sample is separated into a large number of partitions where the reaction is carried out individually [17]. ddPCR provides highly sensitive measurement and absolute quantification of nucleic acid

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amounts, which is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids. It has been demonstrated as useful for the detection and quantification of rare genetic sequences, copy number variations, and relative gene expression in single cells [18-20].

In this study, the authors have recently proposed ddPCR, a novel approach for Down syndrome detection using a fetal epigenetic marker, the putative promoter of HLCS gene on chromosome 21, and a paternally-inherited fetal SNP (rs6636) allele on a reference chromosome. To demonstrate that this method can be used for Down syndrome diagnosis, the authors have explored ddPCR to analyze the absolute number of specific methylated HLCS gene fragment and rs6636 SNP in the plasma of homozygous pregnant women.

Materials and Methods

In this prospective study, the authors recruited pregnant women in Department of Obstetrics, "Shaoxing Women & Children" Hospital from January 2010 to March 2015. All pregnant women had to meet the following criteria: being in second trimester, completing the prenatal screening of Down syndrome, having no pregnancy complications, and followed up lasting one year after birth. Then they collected the samples of a total of 400 healthy volunteers and 65 patients. The patients were at high risk of Down syndrome and the invasive prenatal diagnosis was indicated on the basis of abnormal biochemical and/or ultrasound results. Amniocentesis or cordocentes was carried out in the patients to confirm Down syndrome diagnosis of the patients. All patients gave their written, informed consent to participation in the study.

Human blood samples were collected from pregnant women using the standard venipuncture procedure in EDTA tubes as previously described [21]. Blood samples were processed within two hours of collection.One or two 4-ml EDTA tubes were collected for each patient, yielding an average of around 2 ml of plasma per patient. Plasma and blood cells were separated in blood sample and preserved at -80°C.

DNA were extracted from blood cells and plasma with EDTA anticoagulation separately as followed protocol. First, add protease solution to the sample to remove proteins. Then a three-step centrifugation protocol was set up for plasma recovery, with centrifugation (i) at 8,000 g for one minute, (ii) at 14000 g for three minutes, and (iii) at 12000 g for two minutes. DNA was extracted using a QIAmp DNA blood mini kit, according to the manufacturer's instructions and resuspended in a final volume of 30 µl. Third, the ultra micro UV visible spectrophotometer nanophotometer peal was used to measure concentration and quality of the DNA. The extracted DNA was stored below -80°C until processing.

In order to measure and analyze the rs6636 genotype in maternal blood cell, real-time PCR (RT-PCR) was performed as described using two pairs of primer. Primers for rs6636 amplification were as follows: containing Forward primer (5'-TGGTAAGACTCTTAGAAATCACAGATGTT-3') and Reverse primer (5'-GTATCCCAACTAATCATTTATTATGGTCA-3') specific for rs6636. To perform the detection of rs6636 genotypes, the authors also designed the probes with labeled with FAM or VIC separately containing probe-rs6636-C: FAM-CCCCTATCTGAGAAAAT(MGB); probe-rs6636-G: VIC-CCCCTATCTGAGAAAT (MGB). Each reaction in one tube was performed in 25 µl volumes included 12.5 µl universal master mix II, 0.75 µl Forward primer, 0.75 µl Reverse primer, 0.25 µl probe-rs6636-C, 0.25 µl probe-rs6636-G, 1 µl DNA template, and 9.5 µl nuclease-free water. Real-Time PCR was performed using a plus RT-PCR System in the following conditions: pretreatment were at 50˚C for two minutes, then thermocycling conditions were 95˚C for five minutes, followed by 40 cycles of 95˚C for 15 seconds, and 60˚C for one minute. The control reactions were performed using water with no template, and in all cases, no amplification occurred.

Before ddPCR, a methylation sensitive restriction endonuclease (MSRE) enzyme BstUI was used to treat exacted DNA of peripheral blood cells to distinguish between maternal and fetal HLCS genes. Because maternal HLCS genes were not methylated, the genes could be digested by BstUI. The authors had DNA in maternal peripheral blood before or after digestion as template amplifying β-actin gene. The results showed that it could be observed only when DNA in maternal peripheral blood before digestion was the template, which demonstrated the complete digestion. First, the exacted DNA was digested by restriction enzyme BstUI [22]. DDPCR was then performed to detect the HLCS gene using a droplet digital PCR systemwhich have been described previously [23].

Reactions were performed in 20 µl volumes using 10 µl universal master mix II, 0.6 µl Forward primer (5'-CCGTGTGGCCAGAGGTG-3'), 0.6 µl Reverse primer (5'-AAAGGGCCAGGTCGGGA-3'), 0.2 µl probe [FAM-AGGATTTGGGGCTGCGC (MGB)], 6.6 µl nuclease-free water, and 2 µl template. Each sample was then loaded into the well of a droplet generator cartridge; 20 µl sample was transferred into the middle wells of the DG8 cartridge, being careful to avoid bubbles and 70 µl droplet generation oil was added to the lower wells. The sample-containing cartridge was placed into the droplet generator to generate individual droplets. Once the process was complete, 40 µl droplets were transferred into the wells of a 96-well PCR plate, sealed, and loaded into the thermal cycler. The following program was run: 50˚C for two minutes, then 95˚C for ten minutes, followed by 40 cycles of 95˚C for 15 seconds, 60˚C for one minute, and holding at 4˚C. After PCR was complete, the sealed plate was loaded into the droplet reader for the detection of complete ddPCR reactions in individual droplets. The data was analyzed using QuantaSoft software with the thresholds for detection set manually based on results from no template control wells containing water instead of DNA.

The genotype of rs6636 was detected by ddPCR as the same method as above but using 0.2 µl probe-rs6636-C, 0.2 µl probe-rs6636-G, 0.6 µl Forward primer, and 0.6 µl Reverse primer specific for rs6636.

The authors utilized SPSS software for data statistical analysis. Normality test was conducted by using one-sample Kolmogorov-Smirnov test. The data were skewed distribution. The sample number is less than 30 cases in the study. Mann-Whitney U test with two samples by randomized design was used to analyze the ration of HLCS/rs6636-C or HLCS/rs6636-G compared with control group and trisomy 21 group. *P* value was set less than 0.05, indicating a statistically significant difference.

Results

These pregnant women contained two groups: 400 healthy volunteers and 65 patients. The demographic characteristics of the patients are shown in Table 1. The median

Table 1. — *Characteristics of patient recruitment.*

Characteristic	Value	
	Healthy people	Down syndrome
Number	400	65
Range	20-34	22-34
Mean ± SD	27.56 ± 3.65	26.55 ± 2.65
Mean ± SD	54 ± 2.63	52 ± 2.03

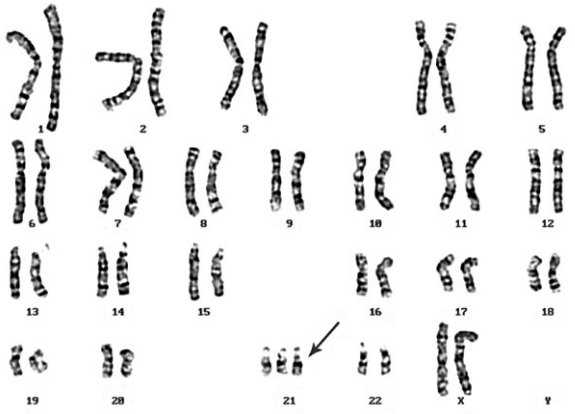

Figure 1. — Karyotype of trisomy 21.

(range) age of healthy people was 27.56 (20 to 34) years old, and that of patients was 26.55 (22 to 34). There was no statistically significant difference between the two groups in either age or weight (*t*-test, $p > 0.05$).

In the present study, 166,508 pregnant women received prenatal screening, from January 2010 to March 2015 in this hospital. In these cases, prenatal screening tests predicted that 4,330 pregnant women were a high risk of Down syndrome (positive rate 2.6%). Only 4,113 cases among these women accounted for 95% of the high risk population were willing to accept amniotic fluid or blood cell karyotype analysis with the procedure of drawing a blood sample to examine the baby's cells, photographing the chromosomes, and then grouping them by size, number, and shape, and 65 cases were diagnosed with trisomy 21. Trisomy 21 karyotype is shown in Figure 1.

The authors also performed the follow-up visit in these pregnant women from their infant's birth to one year of age to know the condition of their infant. A total of 158,182 cases had a effective and integrated record in data collection and preservation, accounting for 95% of all pregnant women. Interestingly, the authors found that a total of 15 infants were patients with Down syndrome, but their mothers were a low risk of Down syndrome. Finally, 65 cases of pregnant women of Down syndrome and 400 normal women (as control) were collected in this study.

In order to prepare a high concentration and quality of DNA for RT-PCR analysis, the authors used the ultra micro UV visible spectrophotometer nanophotometer peal to measure the ratio of A260/A280 of exacted DNA. The results showed the ratio was between 1.7-1.9, indicating a high purity of DNA, which is suitable for subsequent study.

The authors performed RT-PCR to detect genotype of rs6636 gene in peripheral blood cells by using a step one plus system and they found that 22 cases were of rs6636-CC genotype, 28 cases of rs6636-GG genotype, and 15 cases of rs6636-C/G genotype in experimental group. In addition, 135 cases were of rs6636-CC genotype, 116 cases of rs6636-GG genotype, and 149 cases of rs6636-C/G genotype in control group. The results of genotype rs6636-C/G, rs6636-G/G and rs6636-C/C are shown in Figure 2, separately .

To detect of HLCS gene and fetal specific rs6636-C, the authors used QX200 droplet digital PCR instrument to amplify the target gene from plasma of pregnant women with homozygous rs6636-G/G. They discovered that 37 cases were of fetal specific rs6636-C in plasma of pregnant women with homozygous rs6636-G/G and 12 cases in plasma of pregnant women with trisomy 21 (Figure 3). The ratio of HLCS/rs6636-C was calculated by counting the number of positive droplets. The results indicated that the ratio of HLCS/rs6636-C in trisomy 21 group was significant higher than those in control group according to the Mann-Whitney U test of two independent sample ($Z=-4.702$, $p < 0.01$) (Figure 4, Table 2).

A normal reference range of 0.75-2.12 was calculated from 37 euploid maternal plasma samples. All these samples were collected from second-trimester pregnancies. The HLCS to rs6636-C allele ratios of all these euploid samples fell within normal reference range. Thirteen trisomy 21 samples (eight male and five female fetuses) were analyzed with the EGG approach; all but two samples had a ratio greater than the upper limit (Figure 5).

To detect of HLCS gene and fetal specific rs6636-G, the authors used QX200 droplet digital PCR instrument to amplify the target gene from plasma of pregnant women with homozygous rs6636-C/C. They discovered that 41 cases were of fetal specific rs6636-G in plasma of pregnant women with homozygous rs6636-CC and 14 cases in plasma of pregnant women with trisomy 21. The ratio of HLCS/rs6636-G was calculated by counting the number of positive droplets. The results indicated that the ratio of HLCS/rs6636-C in trisomy 21 group was significant higher than those in control group according to the Mann-Whitney U test of two independent sample ($Z=-5.056$, $p < 0.01$)(Table 3).

A normal reference range of 0.86-2.0 was calculated from 41 euploidy maternal plasma samples. All these samples were collected from second-trimester pregnancies. The HLCS to rs6636-G allele ratios of all these euploid samples fell within normal reference range. Fifteen trisomy 21

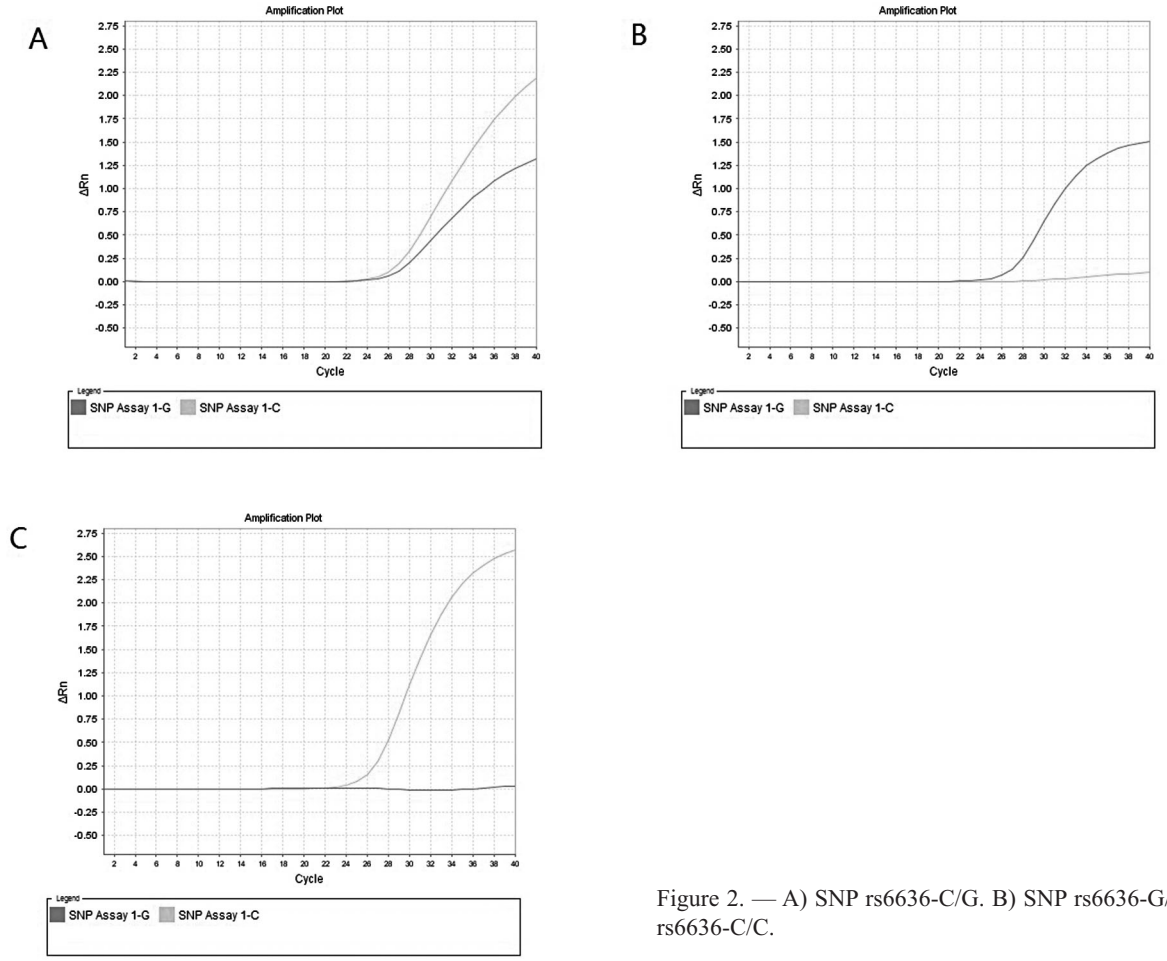

Figure 2. — A) SNP rs6636-C/G. B) SNP rs6636-G/G. C) SNP rs6636-C/C.

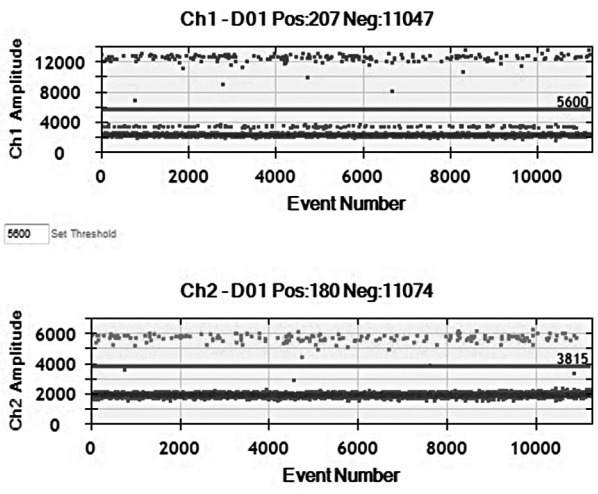

Figure 3. — ddPCR analysis of rs6636-C/G in plasma, Ch1 indicates the detection of rs6636-C by the FAM channel; Ch2 indicates the detection of rs6636-G by the VIC channel. The intermediate pink line is the threshold line. The blue line and green dots are positive droplets; the grey line indicates negative droplets. The number of positive droplets detected by QX200 system is the number of templates for 2 ul DNA extraction.

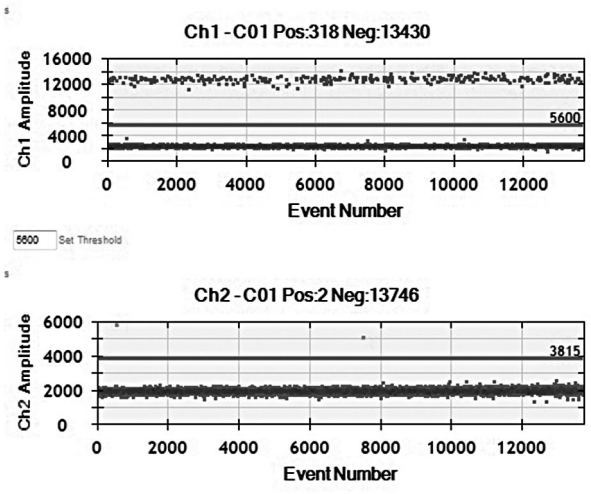

Figure 4. — ddPCR analysis of HLCS in plasma, Ch1 indicates the detection of HLCS gene by the FAM channel; Ch2 indicates the VIC channel. The intermediate pink line is the threshold line. The blue line and green dots are positive droplets; the grey line indicated negative droplets. The number of positive droplets detected by QX200 system is the number of templates for 2 ul DNA extraction.

Table 2. — *Statistical data for HLCS to rs6636-C ratio in maternal plasma DNA samples.*

	Euploidy pregnancy groups (median)	Trisomy 21 pregnancy groups (median)	Z value	*p* value
HLCS to rs6636-G ratio	1.73	3.12	-4.702	*<0.01*

Table 3. — *Statistical data for HLCS to rs6636-G ratio in maternal plasma DNA samples.*

	Euploidy pregnancy groups(median)	Trisomy 21 pregnancy groups (median)	Z value	*p* value
HLCS to rs6636-G ratio	1.72	3.05	-5.056	*<0.001*

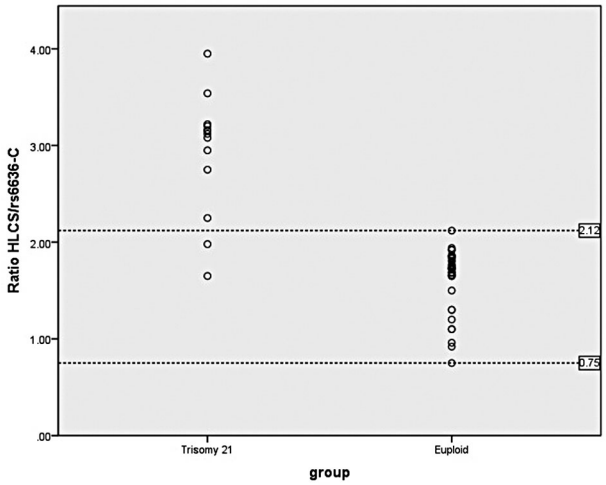

Figure 5. — Ratio of hypermethylated HLCS to rs6636-C allele in Bst UI-digested, euploidys, and trisomy 21 maternal plasma DNA samples. Normal reference range of 0.75-2.12 is depicted by dotted lines.

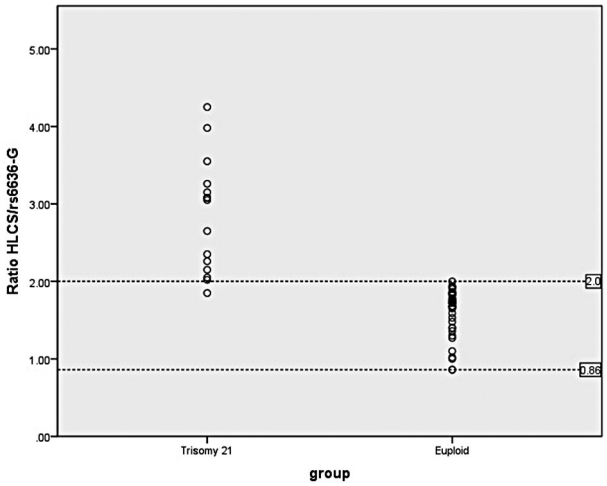

Figure 6. — Ratio of hypermethylated HLCS to rs6636-G allele in Bst UI-digested, euploidy, and trisomy 21 maternal plasma DNA samples. Normal reference range of 0.86-2.0 is depicted by dotted lines.

samples (nine male and six female fetuses) were analyzed with the EGG approach, all but one sample had a ratio greater than the upper limit (Figure 6).

Discussion

In this study, the authors had 166,508 pregnant women that underwent prenatal screening from January 2010 to March 2015, and had a total of 95% of pregnant women with a follow up visit, and collected data of 65 cases of children with Down's syndrome as well as specimens. However, 5% of the pregnant women had lack of data due to absence of follow-up visits. On one hand, some pregnant women refused their visit. On the other hand, some could not be contacted because of moving away or changed phone number, which are all very common in Zhejiang province, China.

At present, invasive sampling (amniocentesis or CSV to obtain a fetal karyotype) could provide a definitive diagnosis of Down syndrome [7]. However, on one hand, these procedures of the karyotype analysis would bring a 1% risk of causing miscarriage, which some pregnant women worried about [8]. On the other hand, the karyotype analysis was performed in a certain period of limited time because amniocentesis is usually performed in the second trimester, between 15 and 20 weeks of gestation, CVS in the first trimester between 9 and 14 weeks [24]. It is the most common way to carry out prenatal screening in second trimester for Down syndrome diagnosis. It is popular in most of the hospitals because of its advantages including economic, rapid, and simple operation. Nevertheless, prenatal screening in second trimester was low in detection rate, and high in omission rate [25]. The detection indicators in prenatal screening are not a direct fetal index. There were 15 cases with low risk of Down syndrome that were missed in this study. Therefore, it is essential to find a new way with a high detection rate, while with a low false negative rate for Down syndrome diagnosis. qPCR has been seen as the gold standard technology to quantify nucleic acid and has shown a wide range of possible applications, such as clinical di-

agnosis, molecular research, and forensic studies [17-20]. Nowadays, it is a very accessible technique. The present study also included qPCR to analyse the rs6636 genotype in maternal blood cell. However, in general, some pitfalls of qPCR detection should be overcome because of using intercalating dye defined as non-specific.

ddPCR should even be considered a modified qPCR, showing high sensitivity that allows an absolute quantitation. The present study demonstrated that ddPCR could distinguish between normal diploid and trisomy 21 by HLCS/rs6636-G ratio using QX200 droplet digital PCR instrument to detect of HLCS gene and fetal rs6636 SNP. However the authors discovered one false positive case. Tong *et al.* have also reported a false negative case according to the ratio of HLCS/rs6636-G to distinguish between normal diploid and trisomy 21 [14], which showed the same results as in the present study. Moreover, because there are many factors to consider cffDNA, much more work clearly needed.

In conclusion, the authors have shown that ddPCR displayed a high sensitivity and specificity in prenatal screening of Down syndrome compared to qPCR and the karyotype analysis. The present study also indicated that this approach could be applied to the prenatal diagnosis of Down syndrome.

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