Noninvasive Prenatal Testing for Whole Fetal Chromosomal Aneuploidies: A Multicenter Prospective Cohort Trial in Taiwan

S.W. Steven Shaw, Ching-Hua Hsiao, Chih-Yao Chen, Yuanyuan Ren, Feng Tian, Chris Tsai, Ming Chen, Po-Jen Cheng

Department of Obstetrics and Gynaecology, Chang Gung Memorial Hospital at Linkou, College of Medicine, Chang Gung University, Taoyuan, Department of Obstetrics and Gynaecology, Taipei City Hospital, Department of Obstetrics and Gynaecology, Taipei Veterans General Hospital, and Bionet Corp., Taipei, and Department of Obstetrics and Gynaecology, Changhua Christian Hospital, Changhua, Taiwan; Department of Biomedical Engineering, National Yang Ming University, Taipei, Taiwan; Berry Genomics Co. Ltd., Beijing, PR China

Key Words
Noninvasive prenatal testing · Down syndrome screening · Aneuploidies

Abstract
Objective: To evaluate the performance of noninvasive prenatal testing for all fetal chromosomal aneuploidies in an extremely high-risk group undergoing first trimester combined Down syndrome screening. Method: A multicenter cohort prospective study in Taiwan was performed between June and December 2012. Maternal plasma was collected and shotgun massive parallel sequencing was performed on each fetal chromosome. 201 Taiwanese pregnant women at >12 weeks’ gestation from 11 medical centers were enrolled in this trial. The extremely high-risk group was defined as a Down syndrome risk cutoff >1:30 or nuchal translucency >3.0 mm (n = 100), while the low-risk group was defined as a Down syndrome cutoff <1:1,500 (n = 101). Amniocentesis confirmation was performed and birth outcome was also recorded. Results: There were 11 cases of trisomy 21, 8 cases of trisomy 18, 3 cases of trisomy 13, 1 case of trisomy 16, 3 cases of 45,X, and 1 case of 47,XYY detected prenatally in 100 extremely high-risk gravidas (n = 27/100 (27%)). The overall autosomal or sex chromosome aneuploidy detection rate was 96% (27/28) because of an insufficient amount maternal plasma for one fetus with Turner syndrome. In the low-risk group, no chromosomal abnormalities were detected (specificity = 100%). There were no false-positive cases in this study. Conclusions: This first trial in Taiwan shows that noninvasive prenatal testing for whole chromosome aneuploidies can be efficiently applied in extremely high- and low-risk populations.

Introduction
Autosomal and sex chromosome aneuploidies account for the major portion of prenatal genetic problems. Down syndrome (DS) is the most common human disorder caused by a numerical chromosome defect; DS has an occurrence rate of approximately 1 in 700 live births [1]. Newborns with two other common aneuploidies, trisomy 18 and trisomy 13, rarely survive 1 month [2]. The remaining autosomal chromosome aneuploidies are extremely rare and the fetuses are not viable at birth. Sex chromosome anomalies, including Turner syndrome, Klinefelter syndrome, XYY syndrome, and triple X syndrome, are also commonly diagnosed by invasive prenatal diagnosis [3].
The traditional and current standard for the detection of fetal aneuploidies, especially trisomies 13, 18, and 21, is via screening [4, 5]. A first trimester combined test or second trimester serum quadruple test are the first-line methods to separate pregnant women into high- or low-risk categories [6, 7]. The false-positive rate in such screening programs is approximately 5%, thus resulting in unnecessary amniocenteses, which have a 0.5% pregnancy loss rate [8]. In Taiwan nearly 90% of amniocenteses are covered for gravidas of advanced maternal age (>35 years) [9, 10].

Noninvasive prenatal testing (NIPT) is a breakthrough testing tool for detecting fetal chromosome aneuploidies since Lo et al. [11] first identified cell-free fetal DNA in maternal plasma. Chiu et al. [12–14] were pioneers in NIPT and has made NIPT clinically available since 2011. We present the first prospective cohort trial involving a Taiwanese population for validation of NIPT. We also compared the NIPT results from extremely high- and low-risk gravidas undergoing DS screening for the first time.

Material and Methods

Study Design

Eleven medical centers in Taiwan which performed NIPT for whole fetal chromosomal aneuploidies were enrolled in this study. Between June and December 2012, a total of 201 Taiwanese pregnant women at >12 weeks’ gestation were recruited. The 11 medical centers were evenly distributed in Taiwan. Approval for the study was obtained from the institutional review board of Chang Gung Memorial Hospital. All of the women consented and signed an agreement to participate in the study. Five milliliters of maternal blood was collected into EDTA-K2 vacutainer tubes (Becton Dickinson, Franklin Lakes, N.J., USA) from each pregnant woman prior to amniocentesis and centrifuged as previously described [15] by Bionet Corp. in Taiwan within the first 24 h; the plasma was shipped to Berry Genomics in Beijing for DNA extraction and downstream-sequencing analysis. The final reports were delivered to the clients within 10 working days. Amniocentesis confirmation was performed for every case with a very high risk for chromosomal abnormalities. The birth outcome of every case was recorded and reported if any chromosome anomaly was found after birth. The information about the gender of fetuses was confidential to trial subjects unless any specific numerical sex chromosomal disorders such as 45,X or 47,XXY existed.

In this study, two groups of pregnant women were divided into very high-risk and low-risk groups for DS after first-trimester combined screening (fig. 1). The extremely high-risk DS group was defined as a DS risk cutoff >1:30 or a nuchal translucency (NT) >3.0 mm (n = 100), while the low-risk group was defined as a cutoff <1:1,500 (n = 101). One case from the low-risk group was excluded at 10 weeks’ gestation after sample collection. Collection was discontinued in each arm of the study when 100 cases were accrued. Four of the cases were twin pregnancies. The sequencing results were obtained independently by Berry Genomics and the karyotype information of all the samples was kept blinded until after analysis. The women who enrolled into this clinical trial did not pay any fees for the testing. Drs. Steven Shaw and Po-Jen Cheng approved the final reports before being returned to the women.

Maternal Plasma DNA Processing and Sequencing

Within 24 h of collection, the maternal blood samples were centrifuged at 1,600 g for 10 min at 4°C. The plasma was transferred to microcentrifuge tubes, and centrifuged again under the same conditions. For each sample, plasma DNA was extracted from 1 ml of the plasma using a QIAamp Circulating Nucleic Acid kit from Qiagen (Hilden, Germany). The resulting plasma DNA was used as the input DNA to make a library for sequencing using the modified ChIP Seq protocol, as published previously [13]. Plasma DNA libraries of 12 samples were indexed using 6np indexing oligos, quantified using a Kapa SYBR fast qPCR kit (Woburn, Mass., USA), pooled, and loaded into one lane in a v2 Illumina HiSeq2000 flow cell. Clustering and sequencing were conducted according to the manufacturer’s instructions using a single-ended 43-bp sequencing protocol.

Data Analysis

The sequences were blinded for each sample according to the index and mapped to the unmasked human genome sequence (hg19). The mapping algorithm used SOAP2 software for obtaining the results as previously described [16]. For all the samples, the sequences...
mapped to each chromosome were counted and the GC content was calculated. A reference set of 50 female diploid samples was generated from an independent sample set with normal 46,XX fetal karyotypes. The final calculating equation using normalized chromosome representation and CG correction to generate a Z-score was published previously [15]. The Z-scores for each pair of chromosomes was defined as increased if >3 and decreased if less than −3.

Statistics
We used predictive analytics software Statistics 18.0 to analyze the data. The mean ± SD was expressed. ANOVA was used to compare the difference between the high-and low-risk groups. The statistical significance was defined at p < 0.005.

Results

Difference between High- and Low-Risk DS Groups
Table 1 shows the basic information of the two groups. There were no significant differences in the maternal and gestational ages between the high- and low-risk DS screening groups (p > 0.05). The average maternal age was 35.1 ± 3.2 and 34.6 ± 2.6 years in the high- and low-risk pregnant women, respectively. The average gestational age was 17.3 ± 2.1 and 16.1 ± 3.0 weeks in the high- and low-risk pregnant women, respectively. Two cases of twins were included in each arm. Due to the extremely high-risk patients, the average DS screening risk, whether in the first or second trimester, was 1 in 22.8. In contrast, the average risk value in the low-risk arm was <1 in 3,000 (table 2). Twenty-eight cases of chromosome abnormalities, including trisomies 21, 18, and 13 and other autosomal or sex chromosome aneuploidies, were detected in the high-risk group by NIPT using maternal plasma. We did not detect any fetuses with an abnormal number of chromosomes in the low-risk group.

Noninvasive Detection of Whole Fetal Autosomal Chromosome Aneuploidies
As previously reported [15], we were able to show near-perfect detection of fetal aneuploidies. The Z-scores for each chromosome were obtained as previously described. Briefly, using a 50-female sample dataset as the reference and applying the standard GC correction, we calculated the Z-scores for each autosomal chromosome. Using a Z-score of 3 as the cutoff value, we defined 11 trisomy 21 candidate samples (fig. 2a) that were clearly separated from the normal samples. The 11 trisomy 21 cases were all confirmed by conventional karyotyping, yielding 100% sensitivity and 100% specificity.

We applied the same method for detection of trisomies 18 and 13. There were 8 cases of trisomy 18 and 3 cases of trisomy 13, also with 100% sensitivity and 100% specificity (fig. 2b, c). The difference in Z-scores between trisomies and normal cases was easy to identify. Interestingly, one of the trisomy 18 cases was a twin pregnancy with another co-twin having a normal karyotype diagnosed at 20 weeks’ gestation. We did selective feticide for the fetus with trisomy 18 under ultrasound guidance. The normal co-twin was then delivered without complications.

Not only can common fetal aneuploidies, such as DS, Edward’s syndrome, and Patau syndrome, be detected, but NIPT can also screen for whole fetal chromosomes, as in our previous report [15]. In this study, one fetus with trisomy 16 was detected with increased NT (4.5 mm). All of the mothers who carried fetuses with autosomal chromosome aneuploidies decided to terminate the pregnancies at the time the diagnosis was established.

Noninvasive Detection of Fetal Sex Chromosome Aneuploidies
For sex chromosome detection, 3 of 4 fetuses with Turner syndromes were diagnosed by NIPT, yielding a

<p>| Table 1. General data with comparison of DS screening in high- and low-risk groups |
|------------------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>High-risk group</th>
<th>Low-risk group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, years</td>
<td>35.1±3.2</td>
<td>34.6±2.6</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>17.3±2.1</td>
<td>16.1±3.0</td>
</tr>
<tr>
<td>Twins, number of cases</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Average screening risk*</td>
<td>1/22.8</td>
<td>1/3,179</td>
</tr>
<tr>
<td>Mean NT thickness, mm*</td>
<td>3.7±0.4</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td>Aneuploidies, n</td>
<td>28</td>
<td>0</td>
</tr>
</tbody>
</table>

Mean ± SD. * p < 0.005.

<p>| Table 2. Summary of aneuploidies detected in the study in both groups |
|------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Aneuploidies</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>False positive, %</th>
<th>False negative, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisomy 21</td>
<td>100 (11/11)</td>
<td>100 (189/189)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trisomy 18</td>
<td>100 (8/8)*</td>
<td>100 (192/192)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trisomy 13</td>
<td>100 (3/3)</td>
<td>100 (197/197)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trisomy 16</td>
<td>100 (1/1)</td>
<td>100 (199/199)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45,X</td>
<td>75 (3/4)</td>
<td>100 (196/196)</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>47,XXY</td>
<td>100 (1/1)</td>
<td>100 (199/199)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Overall</td>
<td>96 (27/28)</td>
<td>100 (172/172)</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

* Including a twin case of one co-twin with trisomy 18.
75% detection rate (table 2); 1 case was missed because the amount of maternal plasma did not meet standard requirements (<5 ml). Nevertheless, this case of Turner syndrome was included in the final results. Interestingly, 2 gravidas with 45,X fetuses decided to continue their pregnancies after thorough genetic counseling. An additional case with another sex chromosome aneuploidy (47,XYY) was also detected using the noninvasive method with final karyotyping confirmation.

Twin Pregnancies
Four gravidas had twin gestations (2 cases in each arm). According to a previous report [15], NIPT detected fetal DNA with one co-twin anomaly in twin pregnancies. We report a rare case of one co-twin with trisomy 18 and a normal co-twin. The Z-score of this case was 3.65 at 20 weeks’ gestation with an enlarged NT. Further investigation revealed major congenital heart disease based on a fetal cardiac echogram of the trisomy 18 co-twin. Intrauterine growth retardation was also noted. Selective feticide for trisomy 18 was performed after confirmation by traditional karyotyping. The other three twin pregnancies were negative based on NIPT.

Brief Summary
The overall detection rate for both groups was 96% (27/28), including the fetus with Turner syndrome that was missed due to insufficient fetal DNA collection (table 2). The overall specificity was 100% without any false-positive or false-negative results, which is in agreement with other studies worldwide. The high-risk group comprised all of the fetal chromosomal abnormalities, while no abnormal cases occurred in the low-risk group.

Discussion
This is the first prospective cohort study in a Chinese population-based country using NIPT for detection of fetal chromosomal aneuploidies. This is also the first study to compare NIPT results from gravidas at extremely high and low risk for DS. The detection rate for DS, trisomies 18 and 13, and other autosomal aneuploidies was 100%, which is in agreement with another international study [14]. The false-positive rate for NIPT was zero; thus, NIPT is a powerful testing tool. The overall detection rate for fetal chromosomal aneuploidies was 96%, with only 1 case misdiagnosed due to an insufficient amount of maternal plasma.

We defined extremely high-risk cases for DS as a cut-off >1 in 30 or NT >3.0 mm. In our study, the anomaly rate in high-risk group was 28% (28/100), which was due to the highly select population. In contrast, we did not detect aneuploidy in the low-risk cases. According to the latest opinion from the American College of Obstetricians and Gynecologists (ACOG), the indications for NIPT include advanced maternal age, abnormal ultrasound findings, and positive results from first or second trimester screening [17]. ACOG does not suggest using NIPT during routine prenatal examinations, as NIPT is expensive compared to other screening tools and has poor medical cost-effectiveness.

Our previous publications detailed the DS screening policies in Taiwan during the first and second trimester [10, 18]. The Taiwan Society of Perinatology recommends that the first-trimester combined test for DS is preferred for screening; however, if medical resources are limited and ultrasonography is not available, the quadru-
ple test in the second trimester is a suitable alternative [18]. Based on the results shown in the current study, we believe our clinical guidelines will be changed soon and NIPT will be implemented in prenatal care. Pregnant women who have concerns about the safety of invasive amniocentesis, have premature rupture of membranes, multiple pregnancies, or good economic status undergo this alternative screening method. In addition, high- or low-risk classification facilitates counseling of Taiwanese or Chinese people. Low-risk patients would feel comfortable after DS screening, while those at high risk could undergo NIPT directly to decrease the significant percentage of unnecessary amniocenteses performed in Taiwan [18].

Although the ability of NIPT to detect all fetal chromosome aneuploidies has not been verified, this is the second study from our group using the same sequencing method to analyze the fetal DNA in maternal plasma. Furthermore, Lau et al. [19] reported that secondary findings are derived from NIPT, including duplications, deletions and mosaicism among autosomal or sex chromosomes. For the detection of anomalies in twin pregnancies, we demonstrated that co-twins with Turner or Edward’s syndrome in conjunction with normal sibling twins could be reliably identified using NIPT. To utilize NIPT clinically for twin pregnancies, more data is needed. Indeed, NIPT, as a noninvasive procedure, might replace most karyotyping in the near future.

The small number of cases (only 100 cases in each arm) was a limitation in this study. Ideally, 300 cases in each arm would have achieved more powerful statistical significance. Due to the limited budget, we offered 200 mothers NIPT at no charge after having spent USD 200,000, not including the shipping and other preliminary tests. The population in Taiwan is relatively smaller than other developed countries. Nevertheless, we hope that this pilot study involving NIPT will provide useful data for all health care personnel and further contribute to the DS screening policy in Taiwan.

In summary, the next generation sequencing for NIPT can be efficiently applied in extremely high- and low-risk DS populations and provide results for autosomal and sex chromosome aneuploidies with very high sensitivity and specificity.

Acknowledgements

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