Prenatal Diagnosis of Fetal Trisomy 21 from Maternal Peripheral Blood

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This study was undertaken to establish a noninvasive prenatal genetic diagnostic method for trisomy 21 using the fetal nRBCs that is rarely present in maternal circulation. Peripheral venous blood samples were collected from 30 women with an advanced maternal age, abnormal triple marker test results, or abnormal ultrasound findings such as an increased nuchal translucency. The blood samples were treated with heparin. The triple density gradient centrifugation, and MACS using CD45 and CD71 were used to isolate the fetal cells. FISH analysis using probe 21 was performed with GPA-immunostaining. The study population consisted of 30 patients from 13 to 25 weeks of gestation, and nRBCs were separated in all cases. In GPA-immuno FISH analysis using probe 21, 3 cases of trisomy 21 were diagnosed and these results were confirmed by the amniocentesis. In conclusion, a prenatal diagnosis of trisomy 21 through GPA-immuno fluorescence in situ hybridization (FISH) analysis using separated fetal nRBCs is a useful, innovative, accurate, rapid and noninvasive diagnostic method.

Key Words: Prenatal diagnosis, fetal nRBCs, trisomy 21, MACS, GPA-immuno FISH

INTRODUCTION

Most chromosomal anomalies with congenital defects at birth are trisomy 21, 18, 13 or mono-

MATERIALS AND METHODS

Twenty milliliter samples of the peripheral venous blood were collected from 30 pregnant women between gestational ages of 13 and 25 weeks for whom prenatal genetic counseling and procedures were indicated. Informed consent was obtained in all cases prior to performing the invasive prenatal techniques.

Isolation of fetal cells

A modified Ganshirt-Ahler method was ap-
plied to isolate the fetal cells. The blood samples were treated with heparin. The maternal venous blood samples were diluted 1:2 with phosphate buffered saline (PBS) and preserved at room temperature. Subsequently, 6 ml of the diluted blood samples were underlain with Ficoll-Histo-paque (Sigma, St. Louis, Montana, USA) 1077, 1107 and 1119 in 50 ml tubes and centrifuged for 30 min at 3000 rpm (4°C). The mononuclear cells, in the second layer from the top, were isolated. These mononuclear cells were washed with 1 x PBS three to four times in 15 ml polyethylene tubes. The washed cells were resuspended with 20 μl/10^5 cells using anti-CD45 magnetic microbeads (DAKO, GmbH, Bergisch Gladbach, Germany) and incubated in a refrigerator for 20 min. For the CD45-negative cell separation, separation columns were designed for the optimal negative selection and a MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) system was used. The cells adherent to the beads were removed and the negative-selected cells were washed two to three times with 1 x PBS. After washing, the cells were centrifuged for 10 minutes at 1200 rpm at 4°C, the pellets were treated with 30 μl/10^6 cells of anti-CD71 magnetic microbeads (DAKO) and then incubated for 20 min at 8°C. After incubation, the CD71-positive cells in the MACS separation column were isolated using a pressure syringe in a MACS kit. The isolated fetal cells were washed 2 to 3 times with 1 x PBS and centrifuged for 5 minutes at 1,000 rpm. After the top layer was removed, the remaining layers were resuspended with PBS and again centrifuged. The cell solution, which was centrifuged using cytosine (Sandon, Germany), was taken (about 40 μl) and made into a slide. The slide was incubated for 20 minutes at 37°C and treated with 50 mM of warm KCl. The KCl was removed after 20 minutes. The slide was treated with 30% of fresh fixative (3:1 v/v methanol acetic acid) and 70% 75 mM KCl. After 5 minutes, the slide was treated with a new cooled fixative for 1 minute and dried for 2 minutes using a slide warmer.

**Immunocytochemistry**

The slide was refixed in cold Carnoy’s solution and air-dried. After dehydrating with 1 x tris-buffer saline (TBS), the slide was air-dried, treated with glycophorin A (GPA) monoclonal antibodies, and incubated in a moisture chamber. The slide was subsequently washed with 1 x TBS and combined with secondary antibodies (rabbit anti-mouse IgG (DAKO)). The slides were again washed with 1 x TBS, and the anti-rabbit APAAP complexes (DAKO) were then added.

In the moisture chamber, the slide was reacted and washed with 1 x TBS. The next step involved staining with the alkaline phosphatase and a fast red substrate (DAKO) using the DAKO protocol. After counter-staining with hematoxyline, the slide was washed and examined by optical microscopy. When the signal was observed, the image was captured using a CCD camera, the special site and the morphology of the cells were captured and digitized using Immuno-FISH software (Cytovision chromosome Auto Analyzer, Santa Clara, California, USA). The GPA-immuno-stained slide was washed 3 times with 2 x SSC/ Tween 20 and dried. The slide was then treated twice with 70% ethanol, and 90% ethanol twice, and then dried. After being prepared in a 73°C denaturation solution, the slide was treated with ethanol at concentrations of 70%, 85%, and 100%, hybridized using a probe mixture (2 μl of probe 21 was mixed with 8 μl hybridization buffer) on a hot plate, and then sealed with rubber cement in a humidity chamber overnight. The cover slip was removed the next day and the slide was washed 3 times with 50% formamide/2 x SSC, with 2 x SCC for 10 minutes, and 2 x SCC/0.1 NP-40 for 5 minutes. The slide was then air-dried in a dark room. Ten milliliters of the counter-stain was laid on the target area and examined using fluorescent microscopy.

**RESULTS**

In 30 women with an advanced maternal age, abnormal triple marker test results, or abnormal ultrasound findings such as an increased nuchal translucency, were identified using a GPA-immunostain of fetal RBCs and a subsequent FISH using a chromosome 21 specific DNA probe (Fig. 1). In 3 cases, 3 signals were identified, which were consistent with the amniocentesis results.
Fig. 1. FISH of probe 21 (WCP) to the fetal nRBCs purified by MACS and GPA-immunostaining. Note the three chromosome-21 signals.

Out of 20 cells (case 8), 1 signal was identified in 2 cells and 2 signals were identified in 3 cells. In case 14, 1 signal among 18 nucleated cells was identified in 5 cells and 2 signals were identified in 3 cells. In case 22, 1 signal was identified in 2 cells and 3 signals were identified in 3 cells from a total of 15 nucleated cells identified. In these 3 cases, the accuracy of FISH was 75%, 56%, and 60%, respectively. Due to a technical error, the accuracy in the 30 cases was estimated to be 83.7%. However, given that this did not affect the diagnosis of fetal trisomy 21, the analysis of the fetal nRBCs retrieved from the maternal peripheral blood may be successfully applied clinically to diagnosing Down’s syndrome (Table 1).

DISCUSSION

Amniocentesis, chorionic villi sampling and cordocentesis are all invasive cytogenetic methods used for a prenatal diagnosis of trisomy 21, 18, 13 or monosomy X. They all encompass a potential danger of fetal injury, infection and pregnancy failure. Furthermore, more than two weeks are required for a cell culture. The maternal serum triple marker test is a safe non-invasive method, but its accuracy drops to 60%. For these reasons, there is a need for a noninvasive, accurate, and fast method for a prenatal diagnosis, and the importance of a prenatal diagnosis using fetal cells in the maternal peripheral blood has attracted a great deal of attention.

After Schmorl first discovered trophoblasts in the lung tissue of a mother who died of eclampsia, many studies aimed at proving the existence of fetal lymphocytes, trophoblasts, and nucleated red blood cells (nRBCs) in the maternal peripheral blood have been conducted. The research has mostly focused on fetal nRBCs. The life expectancy of fetal nRBCs is relatively short at 25-35 days. Therefore, nRBCs from any previous pregnancy can be ruled out, adults normally do not have nRBCs, and all of the fetus’ genome is represented in the fetal nRBCs. To prove the fetal origin of the detected nRBCs, PCR on the Y-specific DNA sequence was performed on the fetal nRBCs retrieved from the 12-18 gestational week period. This study demonstrated that the most accurate testing time is during early or mid pregnancy.

After 20 weeks gestation, hematopoiesis takes place in the bone marrow and the main products are lymphocytes and leukocytes, with the nRBCs representing only a small portion. Even during the early or mid pregnancy period, only one out of $10^2-10^3$ maternal blood cells are nRBCs from a fetal origin. Therefore, a complicated series of purification and condensation processes are required.

Centrifugation using the triple density gradient (Ficoll-Histopaque 1077, 1107, 1119) originally introduced by Bhat et al. (1993) can condense the content by 40 times and remove most of the trophoblasts and lymphocytes. Adding FACS or MACS to this process enhances the purification. MACS is considered to be superior to FACS since the process is simple and fast, and its operation does not require sophisticated training. It is relatively cheap, and can lower the false positive rate by decreasing the cell contamination. The condensed product can be directly used for further PCR or FISH. However, the combination of triple density gradient centrifugation and MACS cannot fulfill the requirement for a purification and condensation process.

CD 71 is an antibody to the transferrin receptor that is found universally on nRBCs. It is known to be present on all cells that produce iron as well as nRBCs. CD 36 is an antibody to the thrombospondin receptor that is present on immature RBCs, monocytes and lymphocytes, but not on
mature RBCs. Glycophorin A (GPA) is a glycoprotein present on the surface of a RBC but not on lymphocytes. CD 45 is present only on the surface of leukocytes, and CD 32 is found only on the surface of mature granulocytes. Neither of these are present on the surface of nRBCs. Zheng et al. used a combination of MACS with CD 45 and CD 32 to eliminate the unnecessary lymphocytes and leukocytes. In other studies, CD 71, CD 36 and GPA have been used.

In this study, a modification of the Gänshirt-Ahlerdt method was used. Triples density gradient centrifugation, CD 45 applied negative MACS and CD 71 applied positive MACS were sequentially used. From this combination, pure fetal nRBCs could be obtained from maternal peripheral blood in all cases.

There are some studies that prenatally diagnosed chromosomal anomalies using PCR with a specific DNA sequence and FISH with specific DNA probes, which were applied to the purified nRBCs. Lo et al. and Yang et al. succeeded in determining the fetal sex using fetal DNA from the maternal peripheral blood without a condensation process. Prince et al. determined the fetal sex with a 67% sensitivity and 64% specificity.

Table 1. Details of the 30 Blood Samples of Trisomy 21 by MACS/GPA-immuno FISH

<table>
<thead>
<tr>
<th>Case</th>
<th>Gest.age (weeks)</th>
<th>Indication</th>
<th>GPA(+) cell</th>
<th>FISH 21 signal</th>
<th>Karotyping by amniocentesis</th>
<th>Accuracy (%)</th>
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<tr>
<td>1</td>
<td>13</td>
<td>U/S</td>
<td>16</td>
<td>0 1 15 0</td>
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<td>100</td>
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<td>21</td>
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<td>6</td>
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</table>

AMA, Advanced maternal age; U/S, Increased fetal nuchal translucency; Triple, Triple marker positive for Down's syndrome.
when the cell separating process was omitted, using flow cytometry to separate the fetal cells and then applying PCR to them.\textsuperscript{22} Elias et al.\textsuperscript{3} and Ganshirt-Ahlerl et al.\textsuperscript{8} applied FISH to the sorted nRBCs using either FACS or MACS to prenatally diagnose trisomy 18 and 21. Such findings have supported the use of fetal nRBCs isolation as a means of making a prenatal genetic diagnosis.

In this study, GPA immuno-FISH was used. The cells retrieved from CD 45 and CD 71 applied MACS were further delineated by staining with the cell surface protein GPA. In order to clarify the use of isolated fetal cells in a prenatal diagnosis of trisomy 21, 30 high-risk pregnant women were recruited to test the GPA-immuno-FISH analysis. All cases including the 3 cases of trisomy 21 were diagnosed with an accuracy of 83.7\%, a result superior to that reported by Al-Mufli R et al. in which only 61\% was correctly diagnosed, with a cutoff value of 5\%.\textsuperscript{31} At the time of the diagnosis of trisomy 21, 64\% of the sorted cells exhibited 3 signals, also being decidedly higher than that of Al-Mufli R’s report. This suggests that the application of GPA-immunos­tain may fortify the ability to diagnose accurately while decreasing the number of cells retrieved.

In conclusion, a prenatal diagnosis of trisomy 21 from fetal cells in the maternal peripheral blood shows clinical promise in terms of speed, accuracy and noninvasiveness. However, further studies involving many cases are warranted to enable the widespread use of this method.

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