[BIO15] A non-invasive prenatal DNA screening test for Down Syndrome

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Introduction

Prenatal diagnosis still depends on invasive methods using cells contained in the amniotic fluid or villus cells (Suzumori, 1999). These procedures carry a small but important rate of miscarriage (Vyas, 1994). The discovery on the presence of foetal cells in maternal circulation was a turning point for the development of a safe, accurate and reliable prenatal diagnosis, which could be offered to as a routine test to any pregnant women regardless their age.

Genetic analysis of the identified foetal cells or foetal DNA has relied primarily on fluorescence in situ hybridisation (FISH) and polymerase chain reaction (PCR) to amplify unique foetal gene sequences (Goldberg, 1997). PCR is known to be a powerful tool in amplifying small amounts of DNA thus, it is very useful in detecting genetic diseases from foetal DNA isolated from maternal blood.

In this study, the ability to detect foetal DNA and foetal cells in a minimal amount of maternal blood taken during the first, second and third trimester of pregnancy was first investigated. The presence of foetal DNA in the maternal blood taken from the forearm and fingertip was detected using PCR. Foetal haemoglobin (HbF) staining was also performed using maternal blood smears in order to isolate the foetal cells and subjected it to PCR for confirmation. Next, the development of a new assay using real-time quantitative PCR was attempted. The SOD-1 gene, which is at the Down Syndrome Critical Region was chosen for the reason that the triplication of the genes in that region could give the effect seen in Down syndrome. As this approach is focused on the gene involved in the disorder, it can be used for all pregnancies regardless of the sex of the foetus.

Materials and Methods

Study subjects

Pregnant women attending the antenatal clinic of Department of Obstetrics and Gynaecology at the Hospital Kuala Lumpur were recruited. Blood samples (200 µl) from the tip of the finger were taken using a lancing device (Roche Diagnostic, Germany) and were collected in sterile microcentrifuge tubes. For foetal haemoglobin staining and real-time quantitative analysis the blood samples were drawn from the forearm and were collected in lithium-heparinised tubes. In addition, a male blood sample was also obtained to be used as control. For non-invasive prenatal DNA screening test of Down syndrome, the blood samples of two pregnant women carrying Down syndrome foetus were obtained from the Fetal Medicine and Health Clinic, Cheras. The mean gestational age of the subjects carrying euploid normal foetuses at first, second and third trimesters were 9.2, 18.7 and 32.1 weeks, respectively. The mean gestational age of the subjects carrying normal twin foetuses and Down syndrome foetuses were 23.5 and 19.5 weeks, respectively. Down syndrome patients from Wisma Harapan, Kuala Lumpur, a school for the mentally challenged children and normal individuals were recruited for this study as controls. Informed consent was obtained in each instance, as was ethical approval by the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, ethics committee.

Genomic DNA extraction

DNA extraction and purification were performed from 200 µl of whole blood using QIAamp DNA Mini Kit (Qiagen, Germany) according to the "blood and body fluid spin protocol" as recommended by the manufacturer. The DNA preparations were eluted in 100 µl of nuclease free water (Eppendorf, Germany)

Foetal haemoglobin staining

Ten maternal blood samples of the first, second and third trimester male pregnancies were used for foetal haemoglobin staining. One microliter of the fresh maternal blood, which was collected in the lithium-heparinised tubes (Meus, Italy), was used to make a thin

blood film on a clean glass slide (Corning, Canada). Foetal cells were identified using a foetal cell stain kit (Sigma, USA) according to the manufacturer's instructions.

Microscopy and cell scraping

The stained slides were examined using a light microscope (Olympus, CHK2-F-GS, Japan) with x100, x400 and x1000 magnifications. The positively stained foetal nucleated cells were identified and counted. These cells were individually scraped with a sterile needle. Approximately 8-10 cells were transferred to a 0.5 ml eppendorf tube containing 5 µl sterile distilled free water.

PCR of foetal DNA

All reactions were set up in a PCR workstation with precautions to avoid contaminations. DNA from a male and a non-pregnant female were used as controls. A negative control without template was also performed to ensure no contamination took place during the experiment. PCR amplification of venous blood drawn from the forearm was performed in a total volume of 20 µl containing 100 ng genomic DNA, 200 µM dNTPs, 5 pmole of SRY-109F and SRY-245R primers (Lo et al., 1998) (Genbank sequence accession number: L08063), 1.5 mM $MgCl_2$, 1X Taq Polymerase buffer and 2.5 U Taq Polymerase (Promega, USA). Thermal cycling was initiated with 10 minutes at 95ºC, followed by 40 cycles of 95ºC for 15 seconds, 58ºC for 1 minute and 72ºC for 1 minute, then a final extension step of 72ºC for 10 minutes using a PCR machine (Eppendorf, Mastercycler Gradient, Germany).

PCR of foetal cells

The scraped foetal cells in 5 µl sterile distilled water were subjected to the PCR conditions described above (as for venous blood) but with the addition of 30 cycles of the denaturing, annealing and extension steps.

Electrophoresis

The PCR products (5 µl) were analysed by electrophoresis in a 1.5% ethidium bromide (Bio-Rad, USA) stained agarose (Amresco, USA) gel. The gel was photographed using a documentation and analysis system (Eastman Kodak Company, USA).

TaqMan real-time quantitative PCR

In essence, SOD-1 gene sequence was used as a molecular marker to detect Down syndrome pregnancies. Real-time quantitative PCR analysis was performed using the Rotor-Gene 2000 (Corbett Research, Australia), which is essentially a combined thermal cycler and a fluorescence detector with the ability to monitor the progress of individual PCR reactions. The primers and dual labelled probes for the SOD-1 gene (GenBank Acession Nr. M13267) were designed with the aid of Primer 3 Software. The size of the fragment analysed was 88 bp. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (GenBank Acession Nr. J04038) gene was used to serve as a control that DNA was indeed present in the sample analysed and the polymerase chain reaction was functional. The length of the fragment analysed was 97 bp. The sequences for the primers and probes combination used are shown in TABLE 1.

For the TaqMan PCR analysis, the amplification reactions were set up in a reaction volume of 20 µl containing 100 ng of the extracted DNA, 300 nM of each amplification primer and 100 nM of the deal labelled TaqMan probe and the necessary components provided in the Taqman PCR Core reaction Kit (Perkin Elmer, Branchburg, New Jersey, USA). This corresponded to 2 µl of 10x Buffer A, 4 mM $MgCl_2$, 200 µM each dATP, dCTP and dGTP, 400 µM dUTP, 1.25 U AmpliTaq Gold and 0.5 U AmpErase. The uracil N-glycolase activity of latter, in combination with dUTP, was used to prevent contamination by carry-over of PCR products.

The DNA were analysed for these two markers in the same analytic run since the thermal profiles for both the SOD-1 and GAPDH TaqMan assay were identical. Thermal cycling was carried using a 2 minute incubation at 50ºC (to permit AmpErase activity), followed by an initial denaturation step at 95ºC for 10 minutes, which facilitates activation of the AmpliTaq Gold polymerase activity, followed by 55 cycles of 15 seconds at 95ºC and 1 minute at 56ºC.

TABLE 1   Sequences of real-time quantitative PCR primers and probes.

	SOD-1	GAPDH
Forward	5' gtg gta gtc tcc tgc agg tct 3'	5' ccc cac aca cat gca ctt a 3'
Reverse	5' gcc ttc gtc gcc ata act 3'	5' cta gtc cca ggg ctt tga tt 3'
Probe	5' (FAM) ttc cgt tgc agt cct cgg aac (TAMRA) 3'	5' (JOE) caa gtt gcc tgt cct tcc tag ctc tt (TAMRA) 3'

The results were expressed as copy numbers, whereby the conversion factor of 6.6 pg of DNA per cell was used. The number of copies of SOD-1 DNA present in the sample was determined using a known concentration of SOD-1 genomic DNA calibration curve. Amplification data were analysed by the use of the software developed by Corbett Research.   The mean quantity of each triplicate was used for further calculation. The concentration, expressed in copies per microliter, was calculated using the following equation (Lo *et al.*, 1998).

$$C = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}},$$

Where C = target concentration in sample (copies per microliter); Q = target quantity (copies) determined by sequence detector in PCR; V_{DNA} = total volume of DNA obtained after extraction, typically 100 µl per Qiagen extraction; V_{PCR} = volume of DNA solution used for PCR; and V_{ext} = volume of sample extracted, typically 200 µl.

Statistics

The Student t-test was performed using Microsoft Excel.

Results

PCR of foetal DNA

FIGURE 1 shows the presence of foetal *SRY* gene in the maternal blood taken from the tip of the finger. Lanes 2-6 show the presence of male foetal DNA at gestational age from 6-10 weeks. However, no bands were seen in female pregnancies from 6-10 weeks gestation (lanes 8-12). The intensity of the band at 137 bp increases as the gestational age increases. Confirmation of the sex of the foetus was obtained by ultrasound at 24th week of gestation and again at birth.
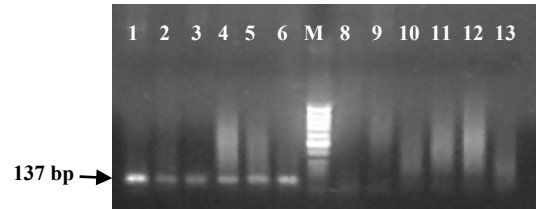

FIGURE 1 Polymerase chain reaction amplification of SRY gene from maternal blood. Lane 1 = male positive control; 2-6 = DNA from pregnant women with male foetus at 6, 7, 8, 9, 10 weeks gestation, respectively; M = 50 bp DNA marker; 8-12 = DNA from pregnant women with female foetus at 6-10 weeks gestation, respectively; 13 = non-template control.

Foetal haemoglobin staining and PCR of foetal cell

Foetal haemoglobin is resistant to acid elution, therefore when the blood smears were immersed in acid buffer, adult haemoglobin was eluted from the erythrocytes, whereas foetal haemoglobin remained intact.   When the blood smears were subsequently stained, erythrocytes having foetal haemoglobin take up stain, while those containing only adult haemoglobin appear as "ghosts" (FIGURE 2). The ratio of nucleated foetal to maternal cell was found to be about 5 in 10^6.   FIGURE 3 shows the results of the Y-specific region amplified giving a 137 bp band from the stained foetal cells using the *SRY* primers.
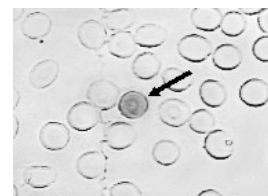

FIGURE 2 Foetal haemoglobin staining of maternal blood smear. Arrow indicates the stained foetal cell while maternal cells appear as 'ghost' cells; (oil immersion, 1000x).
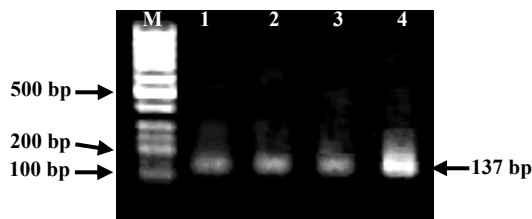
FIGURE 3 Polymerase chain reaction amplification of Y specific region of foetal cells scraped from foetal haemoglobin stained slides. Lane M = 50 bp marker; 1-3 = foetal cells from women with male foetus at 38, 38, 39 weeks gestation, respectively; 4 = male positive control.

TaqMan real-time quantitative PCR

The mean concentrations of the SOD-1 sequences in the blood of the study subjects are shown in TABLE 2. The concentrations are expressed in copies/µl. As hypothesized, the level of SOD-1 sequences in the Down syndrome patients is significantly high when compared to normal individuals ($p = 9.35 \times 10^{-07}$).

Our data show that the number of SOD-1 sequences in maternal blood is not significantly increased when the first ($p = 0.575$) and second trimester ($p = 0.056$) pregnancies were compared with the non-pregnant women, however the concentration is significantly elevated in the third trimester from the non-pregnant women ($p = 9.23 \times 10^{-05}$). We analysed the data between the three trimesters of the normal pregnancies and found that there is no significant increases of the SOD-1-specific DNA between the first and second trimesters ($p = 0.147$) but significantly elevated in the third trimester when compared with the second trimester ($p = 0.002$).

The analysis of twin pregnancies showed that SOD-1-specific DNA level is increased significantly when compared with the third trimester normal pregnancy ($p = 0.003$). Significantly more SOD-1 sequences were detected in blood samples from mothers carrying Down syndrome foetuses, compared with mothers carrying normal foetus in all three trimesters ($p<0.05$) and twin foetuses ($p = 0.026$).

Discussion

Currently, invasive prenatal diagnostic procedures such as chorionic villus sampling and amniocentesis are usually offered to women 35 years of age and older, in the absence of specific foetal abnormalities, positive family history, or other indications of elevated risk (Bianchi *et al.*, 1997). A technique which is suitable to be applied to women at all age for non-invasive prenatal diagnosis by means of foetal cells and DNA in maternal blood is being developed at present. The current technology using this approach is focused on detecting Y-chromosomal sequences, which is impossible to be used for pregnancies involving female foetuses. Therefore, this study was carried out to investigate the presence of foetal cells and DNA in maternal blood and finally to make use of this source of foetal genetic material for prenatal screening of Down syndrome for women at all age regardless of the sex of the foetus.

An experiment was conducted using 200 microliters of maternal blood obtained from the tip of the finger at 6-10 weeks of gestation to detect the presence of foetal DNA. This is the first ever report showing the ability to detect foetal DNA as early as 6 weeks of gestation in maternal blood obtained from the fingertip. Many researchers (Biscoff *et al.*, 1998; Cheung *et al.*, 1996 and Bianchi *et al.*, 1994) needed approximately 18 ml or more of maternal blood to carry out prenatal diagnosis or foetal cell detection. These findings suggest that a small amount of maternal blood from the fingertip would be sufficient to perform prenatal diagnosis using maternal blood. The foetal cell detection by HbF staining method showed increased foetal cells in the maternal blood in each trimester. The ratio of nucleated foetal to maternal cells in non-enriched samples in this study was 2 in 10^6 in the first trimester, 3 in 10^6 and 5 in 10^6 in the second and third trimester of pregnancies, respectively. Hamada and colleagues (1993) found that the concentration of foetal cells in non-enriched samples to be <1/100000 in the first trimester. These findings suggest that a first trimester non-invasive prenatal diagnosis approach might be feasible.

The TaqMan real-time quantitative PCR system has been used to determine the quantity of circulating foetal DNA in normal (Lo *et al.*, 1998) and aneuploid pregnancies (Zhong *et al.*, 2000) by analysis of the Y chromosomal sequences. The main limitation of this quantitative analysis in maternal blood is that this approach can only be used in

TABLE 2   Quantitative analysis of SOD-1 sequence in blood using TaqMan Assay

Subjects	Number of samples	Levels of SOD-1 sequences per µl blood
Non-pregnant women	10	3580.2 (982-7510)
Down syndrome patients	10	40422 (25772-78603)[a]
First trimester	10	4100.4 (1156-6618)[c]
Second trimester	10	5705.6 (3077-11942)[bc]
Third trimester	10	11728.5 (5486-20942)[ac]
Twin pregnancies	2	22846.5 (21488-24205)[bc]
Down syndrome pregnancies	2	60685 (54669-66701)

Values represent mean (range).
[a] Significantly different from Non-pregnant women, $p < 0.0001$
[b] Significantly different from third trimester, $p < 0.005$
[c] Significantly different from Down syndrome pregnancies, $p < 0.05$

pregnancies involving male foetuses. For this reason, it is imperative that genes involved in a particular disorder be used in prenatal diagnosis or screening. In this study, a successful analysis on the amount of SOD-1 sequences (located on the Down Syndrome Critical Region, chromosome 21) in the maternal blood of pregnant women bearing normal and Down syndrome foetus and also in non-pregnant women and Down syndrome patients were made

Firstly, we looked at the levels of SOD-1 sequences in the non-pregnant normal women and Down syndrome individuals. The level is significantly elevated in the Down syndrome patients relative to the normal individuals. Next, we compared the concentrations of the

SOD-1-specific DNA between the non-pregnant women and singleton pregnancy women at 3 different trimesters. We found that the concentrations increased 1.6 and 3.3 fold in the second and third trimester, respectively. Comparison between the second and third trimester pregnancies also showed a 2-fold increase of the sequence in the maternal blood. These results suggest that the increment occurs due to the invasion of foetal cells into the maternal blood. Our data correlate well with the observation by Lo and co-workers (Lo *et al.*, 1998), which showed that the concentration of circulating foetal DNA constitutes 3% and 6% of the total circulating DNA in maternal plasma in the first and third trimesters and are generally low in the first trimester.

We also tested the level of the SOD-1 sequence in twin pregnancies and finally analysed the concentration of the SOD-1-

specific DNA in pregnancies with Down syndrome. Our results showed significant elevation of the sequence in the maternal blood when the Down pregnancies were compared with the normal singleton and twin pregnancies. This indicated that more foetal cells invade the maternal circulation in the incidence of Down syndrome. Our analysis balanced with the findings by Bianchi and co-workers (1997) and Lo and colleagues (1999), in which they demonstrated that the number and concentration of circulating foetal cells/DNA increased in pregnancies involving foetuses affected by trisomy 21.

Real-time PCR quantitation for prenatal screening could be performed by different laboratories, but it would probably be most practical to use a multiples of median (MoM) system akin to that which is currently used for the analysis of serum analytes (Zhong *et al.*, 2000).

Real-time quantitative PCR technology is an effective tool for prenatal diagnosis and screening which reduces the time and labour compared to the conventional cytogenetics method. Even though our finding demonstrated that a gene involved in the particular disorder could be used in prenatal screening, a larger number of samples will be needed to determine the practicability of this method and to convert the screening test to diagnostic test.

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