

Exploring SOD1 Gene for the Detection of Fetal Down Syndrome

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Summary

It has been shown that fetal cells and circulating cell-free fetal DNA increases in the maternal circulation in women carrying trisomy 21 fetus. The current technology in non-invasive screening methods of fetal aneuploidies is focused on detecting Y-chromosomal sequences which is not practical to be used for pregnancies involving female fetuses. Hence, it is vital to develop an assay that is universal for both male and female fetus pregnancies. We attempted the use of superoxide dismutase (SOD-1) gene, which is located at the Down Syndrome Critical Region, to overcome this situation for the prenatal screening of Down syndrome. The prospective of the gene using real-time quantitative polymerase chain reaction was explored. Our results show that the level of SOD-1 sequences is significantly elevated in the third trimester normal pregnancies (mean = 11728 copies/ μ l) when compared to the second trimester (mean = 5705.6 copies/ μ l), $p < 0.005$ and non-pregnant normal women (mean = 3580.2 copies/ μ l), $p < 0.0001$. Down syndrome pregnancies have the greatest elevation compared to all the three trimesters of normal singleton pregnancies and twin pregnancies, $p < 0.05$. These data indicate that a quantitative analysis using a gene associated with a disorder could be used in screening for the prenatal diagnosis of fetal aneuploidies regardless of the sex of the fetus.

KEY WORDS: fetal Down syndrome, maternal blood, real-time quantitative pcr, SOD1 gene.

Introduction

Prenatal diagnosis currently still depends on invasive methods using cells contained in the amniotic fluid or vilus cells (1). As an alternative, non-invasive methods using fetal cells and circulating cell-free fetal DNA in the

maternal blood are currently being intensely studied.

The development of real-time quantitative PCR smooth the progress of rapid determination of trisomy 21 and extended to examine the most common fetal aneuploidies (2) such as trisomy 21. Real-time PCR quantitation for prenatal diagnosis could be performed by different laboratories, but it would probably be most practical to use a multiples of median (MoM) system similar to that which is currently used for the analysis of serum analyses (3, 4).

In this study, the development of a new assay using real-time quantitative PCR was attempted. The SOD-1 gene which is located 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. We hypothesized that significant elevation of the SOD-1 sequence in the maternal blood of the women carrying Down syndrome fetus will be seen compared to other study subjects as a result of the extra copy of this gene in the Down syndrome fetus.

Methods

Study subjects

Pregnant women above 35 years old in the first, second and third trimester attending the Department of Obstetrics and Gynecology at the Hospital Kuala Lumpur, Malaysia were recruited. Down syndrome individuals from Wisma Harapan, Kuala Lumpur and non-pregnant women were also recruited for this study. Informed consent was obtained in each instance and the study was approved by the Medical Research Ethics Committee of our institution.

Samples

Ten venous blood samples were taken from each trimester of normal pregnancy women, Down syndrome and normal individuals, respectively. In this study period, we only managed to obtain two samples from women carrying Down syndrome fetuses and both were in their second trimester. Two samples from women in their second and third trimesters carrying twin fetuses were also included in this study. About 1.5 ml of blood samples on average were collected in lithium-heparinized tubes (Meus, Italy).

DNA Extraction

The blood samples were processed within 12 hours of sampling. DNA was extracted and purified from 200 μ l

Table I - 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'

buffy coat with mixture of red blood cells using QIAamp DNA Mini Kit (Qiagen, Hilden, 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.

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). The primers and dual labeled probes for the SOD-1 gene (GenBank Accession Nr. M13267) were designed with the aid of Primer 3 Software. The size of the fragment analyzed was 88 bp. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (GenBank Accession Nr. J04038) gene was used to serve as a control to indicate the presence of DNA in the sample and the polymerase chain reaction was functional. The length of the fragment analyzed was 97 bp. The sequences for the primers and probes combination used are shown in Table I.

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, 300nM of each amplification primer and 100nM of the dual 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 MgCl₂, 200 µM each dATP, dCTP and dGTP, 400 µM dUTP, 1.25 U AmpliTaq Gold and 0.5 U AmpErase.

The DNA was analyzed 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 50°C (to permit AmpErase activity), and an initial denaturation step at 95°C for 10 minutes, followed by 55 cycles of 15 seconds at 95°C and 1 minute at 56°C. Each sample was analyzed in triplicate.

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 analyzed by the use of the software developed by Corbett Research. The concentration, expressed in copies per microliter, was calculated using the following equation (4):

$$C = Q \times \frac{VDNA}{VPCR} \times \frac{1}{Vext}$$

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

Results

In previous studies, fetal-derived Y-chromosomal sequences has been used to detect the concentration of circulating fetal DNA in the plasma of pregnant women carrying fetuses affected by trisomy 21. In this study, a successful analysis on the amount of SOD-1 sequences

Table II - Quantitative analysis of SOD-1 sequence in blood using TaqMan Assay.

Subjects	Number of subjects	Copy numbers of SOD-1 sequences
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) ^c
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.

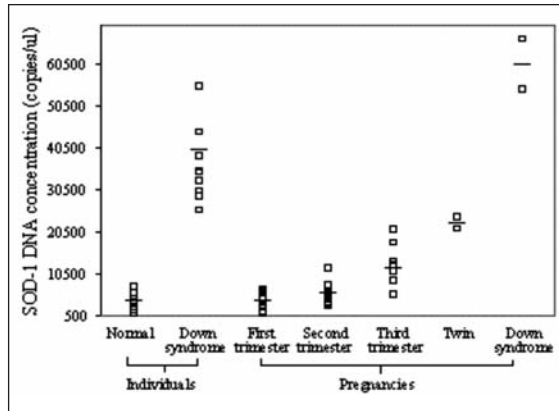


Figure 1 - Levels of SOD-1 sequences as measured by real-time quantitative PCR in pregnant and non-pregnant individuals. Horizontal bars, means.

in the maternal blood of pregnant women bearing normal and Down syndrome fetus and also in non-pregnant women and Down syndrome patients was made.

The mean concentrations of the SOD-1 sequences in the blood of the study subjects are shown in Figure 1. The concentrations are expressed in copies/μl. As hypothesized, the level of SOD-1 sequences in the Down syndrome individuals is higher than normal individuals. 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 analyzed the data between the three trimesters of the normal pregnancies and found that there is no significant increase of the SOD-1-specific DNA between the first and second trimesters ($p = 0.147$) but it is 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 fetuses, compared with mothers carrying normal fetus in all three trimesters ($p < 0.05$) and twin fetuses ($p = 0.026$).

Discussion

The TaqMan real-time quantitative PCR system has been used to determine the quantity of circulating fetal DNA in normal (4) and aneuploid pregnancies (5) 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 pregnancies involving male fetuses. For this reason, it is imperative that genes involved in a particular disorder be used in prenatal diagnosis or screening.

Prenatal screening for fetal trisomy 21 is now considered part of routine obstetric care in many parts of the world (6). In this study, we examined the SOD-1 gene

which is located on the Down Syndrome Critical Region. SOD-1 over-expression may cause premature aging and decrease function of the immune system, which can be seen in Down syndrome patients.

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 individuals relative to the normal individuals. This indicates that the extra copy of the SOD-1 gene in the Down syndrome individuals contributes to the elevation.

Next, we compared the concentrations of the SOD-1-specific DNA between the non-pregnant women and singleton pregnancy women at three 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 fetal cells into the maternal blood. More fetal cells invade the maternal circulation as the gestation week increases. Lo and co-workers (4), showed that the concentration of circulating fetal DNA constitutes 3% and 6% of the total circulating DNA in maternal plasma in the second and third trimesters and are generally low in the first trimester.

We also tested the level of the SOD-1 sequence in women carrying twin fetuses and finally analyzed the concentration of the SOD-1-specific DNA in pregnancies with Down syndrome. As hypothesized, our results showed significant elevation of the sequence in the maternal blood when the Down pregnancies were compared with the normal singleton. Even though theoretically the invasion of the fetal cells in twin pregnancy suppose to be double the singleton pregnancy and higher than Down syndrome pregnancy but in this study Down syndrome pregnancies showed higher invasion than the twin pregnancies. Our inference was that the remarkably high elevation was a result of the contribution of the extra copy of the SOD-1 gene in the Down syndrome fetus and may be because of defective placenta in the presence of Down syndrome. Bianchi and co-workers (7) and Lo and colleagues (6), demonstrated by detecting Y-chromosomal sequences, that the number and concentration of circulating fetal cells/DNA increased in pregnancies involving male fetuses affected by trisomy 21.

The advantage of the methodology used in this study is that it can be applied to pregnancies involving both the male and female fetuses for non-invasive prenatal screening. Furthermore, real-time quantitative PCR technology is an effective tool for prenatal diagnosis and screening which reduces the time and labor compared to the conventional cytogenetics method. Trialing this method with larger number of samples might turn this screening test into a diagnostic test.

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