Original Article

JGM Journal of Genetic Medicine

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First trimester screening for trisomy 18 by a combination of nuchal translucency thickness and epigenetic marker level

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Purpose: The aim of this study was to assess the diagnostic efficacy of noninvasive prenatal screening for trisomy 18 by assessing the levels of unmethylated-*maspin* (U-*maspin*) and fetal nuchal translucency (NT) thickness during the first trimester of pregnancy.

Materials and Methods: A nested case-control study was conducted using maternal plasma samples collected from 65 pregnant women carrying 11 fetuses with trisomy 18 and 54 normal fetuses. We compared the U-*maspin* levels, NT thicknesses, or a combination of both in the first trimester between the case and control groups.

Results: U-*maspin* levels and NT thickness were significantly elevated in the first trimester in pregnant women carrying fetuses with trisomy 18 when compared to those carrying normal fetuses (27.2 vs. 6.6 copies/mL, $P<0.001$ for U-*maspin*; 5.9 vs. 2.0 mm, $P<0.001$ for NT). The sensitivities of the U-*maspin* levels and NT thickness in prenatal screening for fetal trisomy 18 were 90.9% and 90.9%, respectively, with a specificity of 98.1%. The combined U-*maspin* levels and NT thickness had a sensitivity of 100% in prenatal screening for fetal trisomy 18, with a specificity of 98.1%.

Conclusion: A combination of U-*maspin* levels and NT thickness is highly efficacious for noninvasive prenatal screening of fetal trisomy 18 in the first trimester of pregnancy.

Key words: Trisomy 18, First trimester combined screening, Noninvasive prenatal testing, Epigenetic marker, Nuchal translucency.

Introduction

Trisomy 18 is caused by the presence of an extra copy of all or part of chromosome 18, and is the second most common autosomal trisomy with a prevalence of 1 in 6,000 pregnancies. It represents one-third of the chromosomal trisomies identified at 10 to 14 weeks of gestation [1]. Trisomy 18 is associated with severe disability and compromised survival, with at most 10% of the patients surviving to one year of age. Conventional screening for trisomy 18 has a detection rate of 92% with a false positive rate (FPR) of 2.1% [2]. However, a delay in screening until the second trimester means that decisions about invasive testing or therapeutic abortion of pregnancy must be deferred accordingly.

In prenatal screening, the fetal nuchal translucency (NT) thickness is the single most effective marker of trisomy 18 and other major chromosomal abnormalities. It appears as a subcutaneous accumulation of fluid behind the fetal neck in a sonogram. NT increases with gestational age, and the incidence of chromosomal abnormalities is related to increasing NT [3]. Increased NT is caused by cardiac failure secondary to structural malformations, abnormalities in the extracellular matrix, abnormal or delayed development of the lymphatic system, and fetal anemia or hypoproteinemia [4-7]. During the first trimester of pregnancy, NT has been widely used as a screening test for fetal aneuploidy either alone or in combination with serum markers [8]. The sensitivity of fetal NT measurements in detecting trisomy 18 is reported to range from 75% to 91% [9,10].

Recently, the quantification of fetal DNA by methylation-based DNA discrimination in maternal plasma has been used for noninvasive detection of fetal chromosomal aneuploidies, and promising results have been reported [11-13]. The gene *maspin* (serpin peptidase inhibitor, clade B [ovalbumin], member 5; *SERPINB5*) was first identified as a fetal-specific epigenetic marker in maternal plasma that allowed for the measurement of cell-free fetal DNA concentrations in pregnancy-associated disorders [14]. Furthermore, *maspin* is located on chromosome 18q21.33 and is hypomethylated in the placental (so-called, fetal) cell-free DNA, but completely methylated in the maternal component in cell-free DNA. The quantitative assessment of the placental unmethylated form, unmethylated-*maspin* (U-*maspin*) was applied to detect the overrepresentation of chromosome 18 in maternal plasma contributed by a trisomy 18 fetus [15].

The objective of this study was to evaluate the feasibility of prenatal screening for trisomy 18 using a combination of U-*maspin* levels and fetal NT thickness in the first trimester of pregnancy.

Materials and Methods

1. Ethics statement
This study was conducted in accordance with the Declaration of Helsinki. Appropriate institutional review board approval was obtained for this study from the Ethics Committee at Cheil General Hospital (CGH-IRB-2008-07). Written informed consent was obtained from each participant before the collection of blood samples and subsequent analysis.

2. Participant recruitment and sample collection
We performed a nested case-control study of women who enrolled in the Cheil General Hospital Noninvasive Prenatal Testing program. Participants were women who received prenatal care at Cheil General Hospital. For this study, 65 women were selected from a larger sample population of 793 enrolled women, according to the criteria described below. The case group consisted of 11 women who were subsequently assessed via chorionic villus sampling for fetal karyotyping to be carrying a fetus with trisomy 18. The control group contained 54 women who delivered normal healthy neonates at term (37 weeks of gestation or more) without medical or obstetric complications. The data were collected from pregnant women with singleton pregnancies in whom fetal ultrasound was performed and the fetal NT thickness was measured as part of routine antenatal care at 11 to 14 weeks of gestation. The gestational age was calculated from the first day of the last menstrual period and was used to age-match the trisomy 18 and normal groups. Maternal peripheral blood samples were collected just before chorionic villus sampling in the first trimester.

3. Sample processing
Maternal peripheral blood samples (10 mL) were collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 1,600 ×g for 10 minutes at 4℃. The supernatant plasma was re-centrifuged at 16,000 ×g for 10 minutes to minimize the additional release of maternal DNA and then stored at –80℃ until examined. Circulating fetal DNA was extracted from 1 mL of maternal plasma using a QIAamp DSP Virus Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Each DNA sample was eluted into 30 μL of sterile DNase-free water and then coded for subsequent blinded analysis.

4. Real-time quantitative methylation-specific PCR (qMSP) analysis of U-*maspin*
Concentrations of U-*maspin* sequences (GenBank accession no. NT_025028.14) were measured by qMSP assays using a CFX96 Real Time System (Bio-Rad, Hercules, CA, USA). DNA extracted from 1 mL of maternal plasma was bisulfite-converted using the EZ DNA Methylation Conversion Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The following U-*maspin* primers and hydrolysis probe were used for the qMSP assay: forward primer, 5'-TGGTTTTGT-GTGGGTTGAGAG-3'; reverse primer, 5'-GGCCGGACTATAAAT-TACATACATACA-3'; probe, 5'-(VIC) ATTGTTGTATGTATGTTTG (MGBNFQ)-3'. The 5' end of the reverse primer was modified by the addition of GGCCGG to enhance the accuracy of the qMSP assays. The PCR reaction mixture contained 12.5 L of iQ Super-

mix (Bio-Rad), 200 nM primers, 400 nM hydrolysis probe, and 5 L of converted DNA in a total volume of 25 L. Initial denaturation cycling conditions were 95℃ for 10 minutes, followed by 50 cycles of 95℃ for 15 secocns, 58℃ for 30 seconds, and 72℃ for 30 seconds. Calibration curves were prepared for each assay using serial dilutions of single-stranded synthetic DNA oligonucleotides specific to the U-*maspin* amplicon (Applied Biosystems, Foster City, CA, USA). Each standard was amplified in triplicate and was included on every PCR plate. For U-*maspin*, the slope and r^2 values from the standard curves were −3.367 and 0.994, respectively. The PCR efficiency was calculated from the slope of the curve using the following formula: Efficiency=$10^{(-1/slope)}$−1. The U-*maspin* assay amplified with almost optimal efficiency of 98%.

The U-*maspin* levels were expressed as copies/mL, and a conversion factor of 6.6 pg of DNA per cell was used in order to express the results as a copy number [16]. Strict precautions were taken against contamination, and multiple negative-control water blanks were included in every analysis. To reduce interexperimental variations, each sample was analyzed in triplicate. The final data reflect the average of the results. The average intra-assay coefficient of variability was 2.1% for U-*maspin*.

5. Fetal NT measurement

Fetal NT thickness was measured according to the Fetal Medicine Foundation guidelines [17]. The fetus was imaged in the midsagittal plane at a neutral position. The image was magnified so that only the fetal head and upper thorax were included. NT was defined as the normal subcutaneous fluid-filled space between the back of the fetal neck and the overlying skin. The widest thickness of translucency was measured, and the measurements were taken using the inner horizontal line borders of the calipers that were placed on the line that defines NT thickness. Increased NT was defined as the presence of a zone with at least 2.8 mm of translucency.

6. Statistical analysis

The clinical characteristics of the study population were compared between cases and controls using the Student's *t*-test for continuous variables and the chi-square test or Fisher's exact test for categorical variables. A comparison of U-*maspin* levels and NT between the two groups was performed using the Mann-Whitney *U* test. The combined analysis of U-*maspin* and NT was scored positive if U-*maspin* or NT was greater than a cutoff value, and was scored negative if U-*maspin* and NT were less than a cutoff value. The accuracy for detecting fetal trisomy 18 using the U-*maspin* levels and NT was determined using the results of fetal karyotyping. A receiver operating characteristic (ROC) curve analysis was performed to determine the optimal cutoff value of the factors used for detecting fetal trisomy 18. The area under the ROC curve (AUC) represents the overall discriminatory ability of a test. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), FPR, false negative rate (FNR), and accuracy were calculated in accordance with standard formulas. A *P*-value less than 0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package for Social Sciences version 12.0 (SPSS, Chicago, IL, USA).

Results

The clinical characteristics of the study groups are summarized in Table 1. At the time of maternal blood sampling, the maternal age was significantly higher in the case group than in the control group (*P*<0.01). Gravidity, nulliparity, body mass index, and gestational age did not differ significantly between the study groups (*P*>0.05 for all).

The median concentrations of U-*maspin* were 27.2 (interquartile range [IR], 23.0-40.6) and 6.6 (IR, 5.3-10.8) copies/mL in the case and normal groups, respectively. The concentrations of U-*maspin* were significantly higher in the case group than in the control group (*P*<0.001) (Fig. 1). The median NT was 5.9 (IR, 5.0-6.7) and 2.0 (IR, 1.8-2.2) mm in the case and control groups, respectively. NT was significantly elevated in women carrying fetuses with trisomy 18 when compared to those carrying normal fetuses (*P*<0.001).

The accuracy of each factor for noninvasive detection of fetal trisomy 18 during the first trimester is shown in Table 2. The optimal cutoff value (19.19) for U-*maspin* and NT was determined at 90.9% sensitivity and 98.1% specificity. The ROC curves of each factor are presented in Fig. 2. The AUCs of U-*maspin* levels, NT thickness, and a combination of both were 0.985 (95% con-

Table 1. Clinical characteristics of the case and control groups

Characteristic	Trisomy 18 (n=11)	Normal (n=54)
Maternal age (yr)	37 (34-40)*	32 (30-34)
≥35 yr	8 (72.7)	11 (20.4)
Gestational age (wk)	12.3 (12.2-12.4)	12.4 (12.1-12.5)
Body mass index (kg/m²)	21.4 (19.2-21.8)	20.4 (18.9-22.2)
Nulliparity	4 (36.4)	19 (35.2)
Gravidity	2.0 (1.0-3.0)	2.0 (1.0-3.0)

Values are presented as median (interquartile range) or number (%).
P<0.01.

fidence interval [CI], 0.918-1.000), 0.909 (95% CI, 0.812–0.966), and 0.991 (95% CI, 0.928-1.000), respectively (P<0.001 for all). The sensitivities of U-*maspin* levels and NT thickness in prenatal screening for fetal trisomy 18 were 90.9% and 90.9%, respectively, with a specificity of 98.1% (Table 2). A combined analysis of U-*maspin* and NT thickness had a sensitivity of 100% in prenatal screening for fetal trisomy 18 with a specificity of 98.1%, indicating a higher sensitivity for a combined analysis of U-*maspin* and NT thickness than either alone. The FPR and FNR of the combined analysis were 1.9% and 0%, respectively, whereas those for U-*maspin* levels alone were 1.9% and 0%, respectively, and those for NT thickness alone were 1.9% and 9.1%, respectively. Moreover, the PPV and NPV of a combined analysis with U-*maspin* levels and NT thickness for the detection of trisomy

18 were 91.7% and 100%, respectively, whereas those of U-*maspin* alone were 90.9% and 98.1%, respectively, and those for NT alone were 90.9% and 98.1%, respectively.

In addition, we observed comparable accuracies using the combined approach according to maternal age (Table 2). Among pregnant women under 35 years of age, the combined analysis of U-*maspin* levels and NT thickness identified 100% of fetuses with trisomy 18 (n=3), with an FPR of 2.3%. Among pregnant women 35 years of age or older, the combined analysis of U-*maspin* levels and NT thickness correctly identified both the control (n=11) and case groups (n=8) with a specificity of 100% and sensitivity of 100%.

Discussion

This study demonstrated the feasibility of combined testing using U-*maspin* levels as a fetal-specific epigenetic marker and NT thickness as an ultrasound marker in prenatal testing for
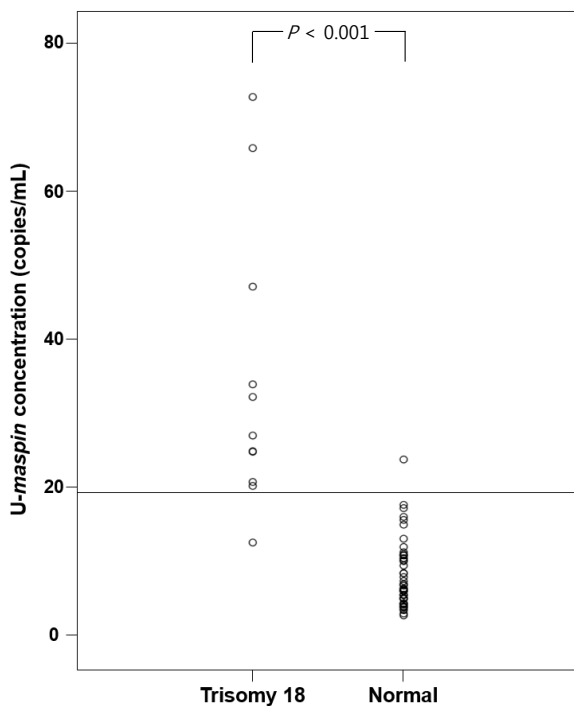

Fig. 1. Unmethylated-*maspin* (U-*maspin*) levels in the first trimester maternal plasma from cases and controls. The line represents the optimal cutoff value of U-*maspin* for the noninvasive detection of fetal trisomy 18.
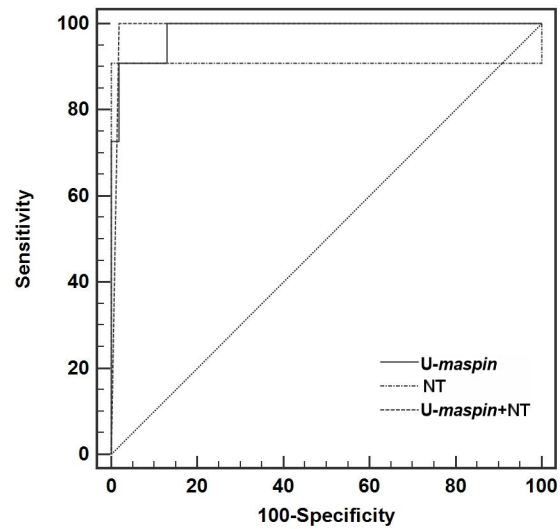

Fig. 2. Receiver operating characteristic curves of unmethylated-*maspin* concentration levels (U-*maspin*), nuchal translucency thickness (NT), and a combination of both (U-*maspin*+NT), which are represented by solid, dash-dotted, and dashed lines, respectively.

Table 2. Utility of measurements in the detection of trisomy 18

Measurement	Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	FPR (%)	FNR (%)	ACC (%)
NT (mm)	2.8	90.9	98.1	90.9	98.1	1.9	9.1	96.9
U-*maspin* (copies/mL)	19.19	90.9	98.1	90.9	98.1	1.9	9.1	96.9
NT+U-*maspin*		100	98.1	91.7	100	1.9	0.0	98.5
Maternal age <35 yr		100	97.7	75.0	100	2.3	0.0	97.8
Maternal age ≥35 yr		100	100	100	100	0.0	0.0	100

PPV, positive predictive value; NPV, negative predictive value; FPR, false positive rate; FNR, false negative rate; ACC, accuracy.

trisomy 18 during the first-trimester. We found that U-*maspin* levels and NT thickness were significantly elevated during the first trimester in pregnant women carrying fetuses with trisomy 18 when compared to those carrying normal fetuses. Furthermore, a combination of U-*maspin* levels and NT thickness correctly identified all pregnant women carrying fetuses with trisomy 18. Among pregnant women aged 35 years or older, this combined approach resulted in perfect classification of all pregnant women with normal and fetuses with trisomy 18, corresponding to 100% specificity and 100% sensitivity. Therefore, our results suggest that a combined approach using U-*maspin* levels and NT thickness may be a useful noninvasive screening method during the first trimester for fetal trisomy 18.

Increased NT thickness in the first trimester has been reported in trisomy 18 and other chromosomal abnormalities as well as many fetal malformations and genetic syndromes [18,19]. Sherod et al. [20] reported increased NT thicknesses in about 75% of fetuses with trisomy 18 in the first trimester. In a multicenter study, Snijders et al. [3] reported a detection rate of 74.8% with a 5% FPR for trisomy 18 by fetal NT thickness. Tul et al. [21] reported increased NT thickness in 39 out of 50 fetuses (78%) with trisomy 18 at 10 to 14 weeks of gestation. Recently, Sepulveda et al. [9] reported a 91% detection rate using NT measurements in first trimester pregnancies complicated by trisomy 18. In our study, the detection rate of 90.9% that was achieved by NT thickness alone was similar to the findings of Sepulveda et al. [9] and was higher than that in other reports [3,20,21]. The minor case of trisomy 18 revealed a normal NT thickness (1.1 mm), while U-*maspin* levels were within the abnormal range (20.46 copies/mL).

In a previous study, Chim et al. [22] demonstrated that *maspin* is unmethylated in the placenta but hypermethylated in maternal blood cells, and its epigenetic differences could serve as a universal fetal DNA marker. Tong et al. [15] described an epigenetic allelic ratio analysis, first by using bisulfite conversion followed by methylation-specific PCR and primer extension reactions designed to distinguish the A and C alleles of fetus-derived U-*maspin* based on size for the noninvasive prenatal detection of fetal trisomy 18. The test had a sensitivity of 100% with an FPR of 9.7% in placental tissues. However, the allelic ratio analysis was informative in only 38 of 187 placental tissue samples tested in the first or third trimester. Moreover, this study did not evaluate the clinical performance of the test in maternal plasma samples obtained in the first trimester. In our approach, U-*maspin* levels had a sensitivity of 90.9% in prenatal screening for fetal trisomy 18, with an FPR of 1.9% in maternal plasma

samples. Moreover, the test was informative in all maternal plasma samples obtained during the first trimester (less than 14 weeks of gestation). However, a single case of trisomy 18 was within the normal range (12.9 copies/mL) and showed an abnormal NT thickness (8.9 mm).

First trimester combined screening for trisomy 18 is based on a combination of maternal age, fetal NT thickness, and maternal serum biochemical markers such as human chorionic gonadotropin (hCG), and pregnancy-associated plasma protein A (PAPP-A), and has a predicted sensitivity of 70 to 90.9% with an FPR of less than 2% [23-25]. More recently, integrated testing using combinations of NT measurement and PAPP-A in the first trimester and various maternal serum biochemical markers, including alpha fetoprotein, unconjugated estriol, hCG, and inhibin-A in the second trimester have improved screening performance and achieved a detection rate of 92% with an FPR of 2.1% for trisomy 18 [2]. However, delaying screening until the second trimester unnecessarily increases maternal anxiety, as well as the likelihood that bonding with the fetus has already occurred. Moreover, the integrated testing may delay the early decision for invasive testing or therapeutic abortion. Therefore, there is an urgent need for the development of accurate screening strategies in the first trimester.

In the present study, we evaluated the utility and accuracy of noninvasive screening for fetal trisomy 18 using U-*maspin* levels and NT thickness during the first trimester of pregnancy. Our combined approach allowed detection of 11/11 (100%) fetuses with trisomy 18 with an FPR of 1.9%. This approach showed an improved detection rate compared with using NT thickness or U-*maspin* levels alone. Moreover, among women 35 years of age or older, the combined approach identified 100% of fetuses with trisomy 18, with an FPR of 0%. Thus, our results demonstrate that combining U-*maspin* levels and NT thickness not only is more sensitive than previous combined screening methods, but also allays parental anxiety by providing earlier results about chromosomal abnormalities in the first trimester. Furthermore, this combined approach is a relatively simple noninvasive screening method compared to previous screening tests that use a combination of four or more markers.

To our knowledge, this is the first study using a combination of a fetal-specific epigenetic marker and ultrasound marker analysis with U-*maspin* levels and NT thickness for noninvasive detection of trisomy 18 during the first trimester. Our findings suggest that this combination is useful in screening for fetal trisomy 18 in the first trimester of pregnancy. However, this study is limited by its small sample size; therefore, the 100% detection

rate in this study should be interpreted with caution. Further studies with a larger clinical cohort are required to validate the screening performance of this protocol.

Acknowledgements

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