Early fetal gender determination using convention nested PCR assay of maternal serum: New technologies and clinical applications in Nasiriya province.

Hiba abdul-adheem

College of medicine\ cancer research unit \ University of Thiqar, Iraq

Abstract

This work was carried out in the cancer research unit laboratories, college of medicine, Thi-Qar University, Iraq. This study demonstrates that a reliable, non-invasive sex determination can be achieved by PCR analysis of maternal serum during the first trimester of pregnancy. In contrary to the risks of invasive methods that affect both mother and fetus, applying cell-free fetal DNA (cffDNA) is proven highly effective with lower risk. One of the applications of prenatal diagnosis is fetal gender determination, which is important in fetuses at risk of sex-linked genetic diseases. In such cases by obtaining the basic information of the gender, necessary time management can be taken in therapeutic to significantly reduce the necessity of applying the invasive methods. The aim of this study was to use simple multiplex PCR protocol of fetal gender at early stage of gestation applying a multiplex PCR amplification of the *ATL*1 locus in the FMR1 gene located on the long arm of X-chromosome5 and the Y-chromosome-specific sequence.

A total of 52 pregnant women participated in this study, The *ATL1*-specific sequences were detected in all 52 serum specimens, while the Y-chromosome-specific fragment were generated in 23 of serum samples. The Y-specific fragment was not detected in any of the 29 serum samples of serum.

التحديد المبكر لجنس الجنين بواسطة فحص الـ PCR التقليدي المتداخل: تقنيات جديدة وتطبيقات سريرية في محافظة الناصرية

م. هبة عبد العظيم عبد الامير

وحدة بحوث السرطان /كلية الطب / جامعة ذي قار

المستخلص

تم تنفيذ هذا العمل في مختبرات وحدة بحوث السرطان، كلية الطب، جامعة ذي قار. تبين هذه الدراسة امكانية تحقيق طريقة غير جراحية يمكن الاعتماد عليها لتحديد الجنس عن طريق تحليل مصل دم الام بواسطة (PCR) خلال الثلث الاول من الحمل. في مقابل مخاطر الطرق الجراحية التي تؤثر على الام والجنين، فأن تطبيق طريقة الدنا الخالي من الخلايا تثبت فاعلية عالية بمخاطر قليلة. واحدة من تطبيقات التشخيص قبل الولادة هو تحديد جنس الجنين وهو أمر مهم في للأجنة التي في خطر الاصابة بالأمراض الوراثية المرتبطة بالجنس. في مثل هذه الحالات من خلال الحصول على المعلومات الأساسية للجنس، وإدارة الوقت اللازمة يمكن أن تؤخذ في العلاج لحد بشكل كبير من ضرورة تطبيق الطرق العلاجية. وكان الهدف من هذه الدراسة هو استخدام بروتوكول متعدد PCR بسيط لجنس الجنين في مرحلة مبكرة من الحمل بواسطة تطبيق تضخيم متعدد PCR من موضع *ATL1* في الجين *FMR1* تقع على الذراع الطويلة من X- الكروموسوم 5 وتسلسل محدد للكروموسوم Y.

وقد شارك في هذه الدراسة ما مجموعه 52 امرأة حامل، وتم الكشف عن التسلسل المحدد ل *ATL1* في جميع عينات مصل الدم ال 52، في حين أن القطع الخاصة بالكروموسوم Y ولدت في 23 عينة من عينات المصل. لم يتم الكشف عن جزء Y محددة في أي من عينات المصل 29 من المصل.

Introduction

Molecular analysis of plasma DNA during human pregnancy has led to the discovery that maternal plasma contains both fetal and maternal DNA[1], so prenatal diagnosis of genetic diseases by means of noninvasive approaches has been getting increasingly closer to becoming a reality in clinical practice[2]. Prenatal diagnosis was introduced in the early 1970s with the primary aim of diagnosing aneuploidies such as trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome) and trisomy 13 (Patau syndrome), as well as aneuploidies related to X and Y-chromosomes (for example, Klinefelter syndrome, trisomy X and Turner syndrome) [3-5]. In addition, the use of DNA markers such as single nucleotide polymorphisms (SNPs) has allowed the detection of fetal genetic abnormalities. One such example is the detection of paternally inherited SNPs located in the β-globin locus for Non-invasive prenatal diagnosis NIPD of β-thalassemia[6,7].

Free fetal DNA has been used successfully for the determination of fetal sex and fetal Rhesus D status in maternal plasma[8,9]. Many studies

have demonstrated that the amounts of cell-free fetal DNA circulating in maternal plasma are relatively high in early pregnancy and gradually rises with a sharp increase before delivery[10]. The aim of this study was to use simple multiplex PCR protocol of fetal gender at early stage of gestation applying a multiplex PCR amplification of the *ATL*1 locus in the FMR1 gene located on the long arm of X-chromosome5 and the Y-chromosome-specific sequence[11].

Methods

Among pregnant women, who attended Bent-Alhuda hospital for maternity and child in Thi-Qar province/Iraq, 52 pregnant women were enrolled following specific criteria of selection. Gestational age was between 11 and 12 weeks of gestation, calculated from last menstruation and confirmed by ultrasound. All the women were healthy and had a singleton and physiological pregnancy.

Blood sample collection and DNA preparation

Peripheral blood (5 mL) was drawn and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The blood samples were centrifuged at 1600 g for 10 min, then the supernatant was collected in a 1.5 mL tube and it was centrifuged again at 16 000 g for 10 min to pellet any remaining cellular debris. The serum samples obtained were divided in aliquots of 500 μL and stored at −20 ◦C until their use. serum DNA was extracted from 200 μL serum samples using QIAmp Blood Mini Kit (QIAGEN Inc., Hilden, Germany) as described previously[11,13]

Primers and PCR analysis

The semi nested PCR protocol was used for amplification of the *ATL*1 gene, whereas the nested PCR was used to generate the Y-specific sequence[14,]. The first multiplex PCR mixture. The first multiplex PCR mixture (25 μL) contains 5 μL serum DNA, 1.25 μL of primer X1 & X3 and Y1.5 &1.6, 10 μL GoTaq® Hot Start Green Master Mix and 5 μL of Nuclease free water. A total of 40 PCR cycles (94°C one minute, 55°C one minute, and 72°C one minute) was carried out on the thermal cycler (PCR) (eppendorf, Germany). In a second PCR, 2 μL of the first PCR

product was re-amplified in a total volume of 25 µL with nested primers X2 & X3 and Y1.7 & Y1.8 to generate fragments of 261 bp X and 198 bp Y specific, respectively, using the same PCR cycling conditions. Ten microlitres of amplicons was separated on 1.5% agarose gel electrophoresis in Tris-acetate-EDTA (TBE) buffer with electrophoresis system Biotech Fischer (Germany), for 30 min and visualized under UV light after ethidium bromide (0.5 µg/mL) staining. No template control was also performed throughout the PCR process using water. The PCR amplification mixture showed in Table 1

Table 1 Primer sequences used in PCR

Primer Name	Sequence (5' → 3')
X1	CCCTGATGAAGAACTTGTATCTC
X2	TCGCCTTTCTCAAATTCCAAG
X3	GAAATTACACACATAGGTGGCACT
Y1.5	CTAGACCGCAGAGGCGCCCAT
Y1.6	TAGTACCCACGCCTGCTCCGG
Y1.7	CATCCAGAGCGTCCCTGGCTT
Y1.8	CTTTCCACAGCCACATTTGTC

Results

DNA was extracted from maternal serum; The PCR product was electrophoresed on 0.8 % agarose (75V and 120 min.) and directly visualized with ethidium bromide under UV light, (figure 1).

Table 2 The overall results of fetal sex prediction from maternal serum DNA analysis

Fetal sex prediction in maternal serum	No. of samples
Male	23
Female	29

A total of 52 pregnant women participated in this study. Maternal serum samples, collected between five to 12 weeks of gestation were analysed by a multiplex nested PCR approach described above. Figure 2 is a representative gel electrophoresis of this multiplex PCR system. As

shown in the figure, while the 261 bp ATL1-specific fragments were observed in all cases, the 198 bp Y-specific fragments were identified only on serum samples of male pregnancies. Therefore, the 261 bp ATL1-specific fragment could be assigned as an internal control of PCR amplification. The system was applied to the 52 maternal serum samples and the result was summarised in Table 2. The *ATL1*-specific sequences were detected in all 52 serum specimens, while the Y-chromosome-specific fragment were generated in 23 of serum samples. The Y-specific fragment was not detected in any of the 29 serum samples of serum.

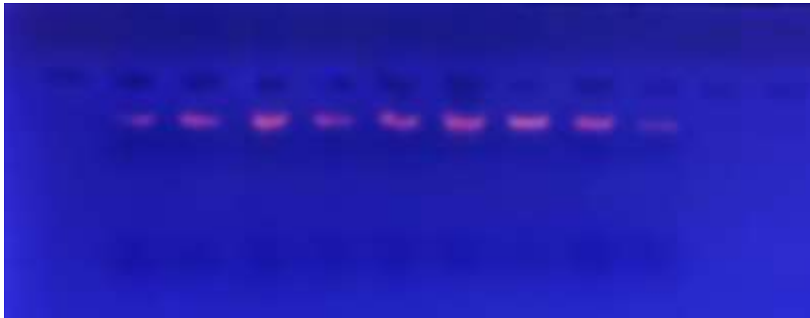

Figure 1 extracted serum DNA Analyzed by 0.8 % agaroseAgarose Gel Electrophoresis

L 1 2 3 4

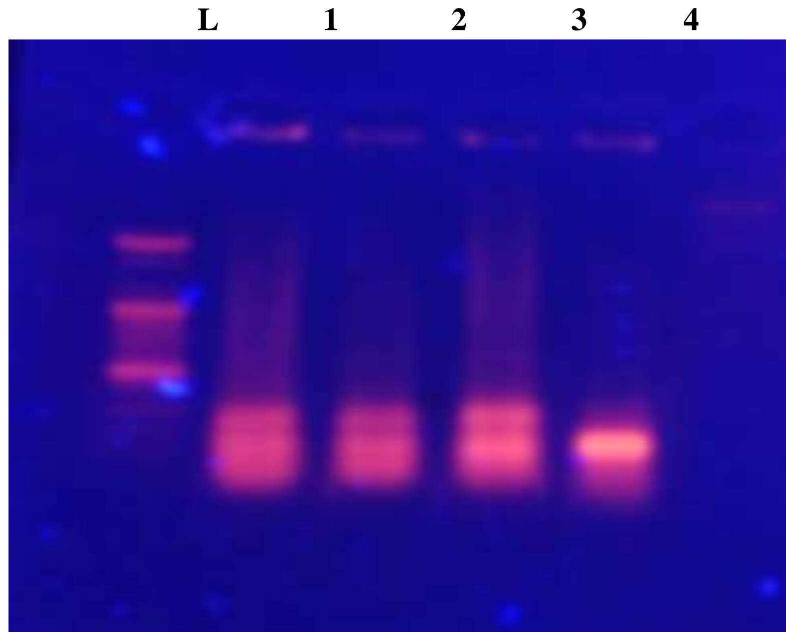

Figure 2 Arepresentative 1.5% agarose gel electrophoresis of fetal sex prediction of ten maternal plasma specimens using the multiplex nested polymerase chain reaction assay for the ATL1 sequence on X-chromosome (261 bp) and the Y-specific sequence (198 bp). Predictions of male pregnancies were made for 4 and female pregnancies for 1, 2, 3, respectively. L represents DNA ladder 2000 bp.

Discussion

This study used to diagnosis the gender of embryo from pregnant female circulation this done by extraction the DNA from maternal serum. Sex determination is one of the important methods, which help to determine the sex of the developing embryo. So Determination of fetal sex during early pregnancy has important implication for prenatal diagnosis of sex-linked abnormalities. The presence of Y-chromosome-specific DNA fragments in maternal serum samples are assumed to originate from male fetuses. Increased concentrations of the male-specific DNA in maternal serum have been reported in certain pregnancy-associated complications such as fetal trisomy 21, preterm labour and pre-exclampsia[15]. Although, identification of male-specific DNA in maternal plasma is relatively simple, results differ significantly with several analytical factors and methods used[14]. Our method is based on the presence of fetal DNA in maternal serum and plasma, a finding that was reported for the first time by Lo et al[16]. The high sensitivity for detecting fetal DNA with conventional PCR is attributable to a high concentration of fetal DNA in maternal plasma and serum. DNA samples were extracted from maternal serum but not plasma because many studies reported a lower efficiency of DNA extraction from plasma than from serum[14]. Regarding the second possibility, i.e., that low concentrations of fetal DNA in maternal plasma may lead to incorrect diagnosis of fetal gender. In order that, our sample were collected at an early gestational age (11and12 weeks), so the fetal DNA has lower concentration in maternal plasma than serum; therefore, the results may be incorrect if we used maternal plasma.

Our results differ from those of Lo et al[16], and Houfflin-Debarge et al[17]. Lo et al[15] found no significant difference in accuracy for diagnosing fetal gender between their maternal plasma-based method and their maternal serum-based method. Houfflin-Debarge et al[17] found that the

accuracy of their method for fetal gender diagnosis was significantly higher when they used maternal plasma rather than maternal serum.

The detection of viral DNA in human plasma or serum is similar to that of fetal DNA in maternal plasma or serum in regard to “foreign DNA” in host plasma or serum. Hamprecht et al.[19] indicated that viral DNA in serum could be detected with greater sensitivity than could viral DNA in plasma. Patel et al.[20] showed that the sensitivity for detecting viral DNA was similar for serum and plasma. However, Boom et al.[21] found that viral DNA could be detected in serum with less sensitivity than viral DNA in plasma. Because these three reports differed in their methods of DNA extraction, the lack of consistent superiority of plasma or serum in terms of sensitivity for detecting viral or fetal DNA suggests that the extraction efficiencies for plasma and serum might differ according to the DNA extraction method used. Because this DNA extraction method is more efficient for serum than for plasma, we believe that maternal serum is more suitable than plasma for fetal gender diagnosis.

We have used a simpler method using multiplex nested PCR assay for X- and Y-chromosome-specific sequences, applying conventional PCR protocol. We found that this simple and simultaneous amplification of the ATL1 gene of the X-chromosome and the Y-specific sequence could provide a satisfactory result for prenatal fetal gender determination. It is also noteworthy that the use of multiplex nested PCR assay of the ATL1 gene and the Y-specific sequence described here could greatly improve the sensitivity and specificity of prenatal fetal sex determination, with only 300 μL of maternal plasma. In addition, the inclusion of the X-specific ATL1 gene amplification as an internal control of the nested PCR system greatly improved the reliability of the fetal sex prediction as without this ATL1-specific fragment, a reliable prediction would not be obtained.

For further studies, we recommend to confirm PCR results by follow up the the sonographical results of fetal gender for women enrolled in the study.

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