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Prenatal Fetal Sex Determination from Maternal Peripheral Blood Using Polymerase Chain Reaction

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We have investigated the use of a nested polymerase chain reaction(PCR) assay with Y-specific sequence from the DYS 14 locus on the short arm of Y-chromosome for prenatal sex determination in the peripheral blood of 22 pregnant women who participated in the antenatal genetic diagnosis program. The sensitivity and specificity of the nested PCR using DYS 14 locus primers($Y_{1.5}$,$Y_{1.6}$, and $Y_{1.7}$,$Y_{1.8}$) were 76.4% and 55.5%, respectively. In terms of gestational age,positive predictive values of 66.6%, 66.6%, and 80% were obtained for the first, second, and third trimester respectively. The corresponding negative predictive values were 50%, 50%, and 100% respectively. Male specific band was positive in three of the six cases of female bearing women and male specific band was negative in three of the seven cases of male bearing women during 9-16 gestational weeks showing low sensitivity. But all cases except one show the male specific band during the male fetus and all female fetuses did not show the male specific 198 base pair band during 18~40 gestational weeks. This study suggests that prenatal sex determination by PCR employing maternal peripheral blood was usually possible in late pregnancy but less reliable in early pregnancy. It seems that if we used a method separating fetal cells from maternal blood and then run PCR on these cells with DYS 14 locus primers we could make a fairly accurate fetal sex determination.

Key Words: Nested polymerase chain reaction, DYS 14 locus, fetal sex determination, prenatal diagnosis

It is well known that there are small amount of interchangeable blood exist between the mother and fetus and so minute trace of various fetus derived cells circulating in the maternal blood can be detected. If we can detect the fetal cells in the maternal blood during the early pregnancy stage, it can be easily substituted for invasive diagnostic methods, such as chorionic villus sampling (CVS) or amniocentesis, and this method can be useful for prenatal diagnosis and management of many X-linked genetic disorders, such as classic Hemophilia A and Duchenne muscular dystrophy.

Several researchers demonstrated that fetal cells in the maternal blood (Walknowska *et al.* 1969) and even very small amount of fetal cells could be detect by recent development of polymerase chain reaction (Saiki *et al.* 1988).

The fetal sex determination from maternal blood by using dual amplification of Y-chromosome specific DNA sequence was first demonstrated by Dr. Lo in 1989. Since then

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various similar reports using Y-chromosome specific DNA sequence, such as DYZ 1, DYS 14, and ZFY, with sensitivity ranges from 60% to 90% (Lo *et al.* 1989; Wachtel *et al.* 1991; Kao *et al.* 1992; Suzumori *et al.* 1992).

Due to high sensitivity of PCR, unexpected false positive and false negative rates were currently unsolved problems. These unexpected results were probably due to the sensitivity of the DNA primer used, laboratory settings and working conditions and, different fetal cell concentration as gestational weeks gets older. In order to overcome these problems, highly specifically sensitive primer development and the improvement of PCR method is urgently required.

In this study, we have investigated the clinical application of PCR in prenatal sex chromosome analysis and fetal sex determination of 22 pregnant women who underwent prenatal genetic study by nested PCR of DYS 14 locus on the short arm of Y-chromosome from the maternal peripheral blood.

MATERIALS AND METHODS

Twenty-two pregnant women who received antenatal care at the department of OB. & GYN., Yonsei University College of Medicine were selected for this study from period of Jan. through Dec. 1994. According to the gestational age, 5 cases were between 9~10 weeks of gestation, 8 cases were of 15~16 weeks, 2 cases were of 18~20 weeks, 3 cases were of 27~28 weeks and 4 cases were between 31~40 weeks. A sample of 1 ml of whole blood was collected from each subjects.

Isolation of chromosomal DNA

DNA was extracted from maternal blood by the standard method (Maniatis *et al.* 1982). 3 ml of blood buffer(55 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) was added to 1 ml of blood and mixed well and put on ice for 15 minutes and centrifuged at 3,500 rpm for 15 minutes. The supernatant was removed and 3 ml of blood buffer was added to the pellet, mixed well, spun and again washed with blood buffer.

1 ml of SE buffer(75 mM NaCl, 25 mM EDTA) was added to the pellet and mixed and then 25 μl of 20% SDS, 10 μl of proteinase K (10 mg/ml) was added and mixed and incubated at 25℃ for the overnight.

Equal amount of phenol-chloroform was added and mixed on the nutator for 3~4 hours and centrifuged at 15,000 rpm(10,000xg) for 15 minutes and transferred the upper aqueous layer and this procedures were repeated two times. After phenol-chloroform extraction, the upper aqueous layer was collected and 2 volume of ethanol was added.

DNA was precipitated by centrifuge and added 200 μl of TE(10 mM Tris-HCl pH 8.0, 1 mM EDTA) for dissolving DNA and used it for PCR.

Polymerase chain reaction (PCR)

First PCR: The reaction mixture consisted of DNA 1.5 μl(=1 μg), 20pmol first primer pair ($Y_{1.5}$, $Y_{1.6}$) 1.5 μl, 5 mM dNTP 1.2 μl, 10x PCR buffer 3 μl, Taq polymerase 1.5 units, and finally water was added to 30 μl reaction volume.

First PCR consisted of the first cycle at 94℃ for 5 minutes, at 55℃ or 57℃ for 1.5 minutes, at 72℃ for 2 minutes, 28 or 38 cycles at 94℃ for 1 minute, at 55℃ or 57℃ for 1.5 minutes, 72℃ for 1.5 minutes, last cycle at 94℃ for 1 minute, at 55℃ or 57℃ at 1.5 minutes, and at 72 μ for 10 minutes. An aliquot of 1~5 μl of the first PCR product was diluted 40 times and transferred into the second PCR.

Second PCR: The reaction mixture consisted of 3 μl of 40 times diluted first PCR product, 20pmol second nest set primers($Y_{1.7}$, $Y_{1.8}$) 1.5 μl each, 5 mM dNTP 1.2 μl, 10x PCR buffer 3 μl, Taq polymerase 1.5 units and water to 30 μl reaction volume.

Second PCR consisted of first cycle at 94℃ for 5 minutes, at 57℃ for 1.5 minutes, at 72℃ for 1.5 minutes, then 19 or 22 cycles at 94℃ for 1 minute, at 57℃ for 1.5 minutes, at 72℃ for 1.5 minutes, last cycle at 94℃ for 1 minute, at 57℃ for 1.5 minutes, and at 72℃ for 10 minutes. The total reaction was 20 or 30 cycles.

The base sequences of primers used in first

and second PCR were as following and we ordered and synthesized them from Korea Bioengineering LTD.

Y-specific sequence primers in DYS 14 locus
$Y_{1.5}$: 5′-CTAGACCGCAGAGGCGCCAT-3′
$Y_{1.6}$: 5′-TAGTACCCACGCCTGCTCCGG-3′
$Y_{1.7}$: 5′-CATCCAGAGCGTCCCTGGCTT-3′
$Y_{1.8}$: 5′-CTTTCCACAGCCACATTTGTT-3′

Confirmation of PCR results

An aliquot of 5 μl of the second PCR product was analysed by gel electrophoresis on 1.2 % agarose-TBE.

Fetal sex determination was performed by karyotyping CVS or amniocentesis for genetic indication of 9~28 weeks gestation and after 31 weeks gestation, the sex of newborns were compared with PCR results.

Prevention of contamination

To avoid the risk of contamination of male DNA, all procedures were performed by women doctors or researchers and all reagents were irradiated with ultraviolet light and frequent changing of disposable gloves.

RESULTS

PCR with $Y_{1.5}$, $Y_{1.6}$ and $Y_{1.7}$, $Y_{1.8}$ primers

The nested PCR was performed in 22 pregnant women between 9 and 40 weeks gestation with Y-specific sequence $Y_{1.5}$, $Y_{1.6}$, and $Y_{1.7}$, $Y_{1.8}$ primers located on the DYS 14 locus of the short arm of the Y-chromosome. After the first PCR with $Y_{1.5}$, $Y_{1.6}$ primers, several nonspecific bands were appeared.

In the second PCR with $Y_{1.7}$, $Y_{1.8}$ primers under stringent condition, male specific 198 base pair DNA band was detected(Fig. 1).

Ten of the thirteen cases of male bearing women showed 198 base pair band with the sensitivity of 76.9%, however, four of nine cases of female bearing women showed PCR positive band with the specificity of 55.5%. The positive and negative predictive values for Y-chromosome detection were 71.4% and 62.5%(Table 1). The predictive values accord-

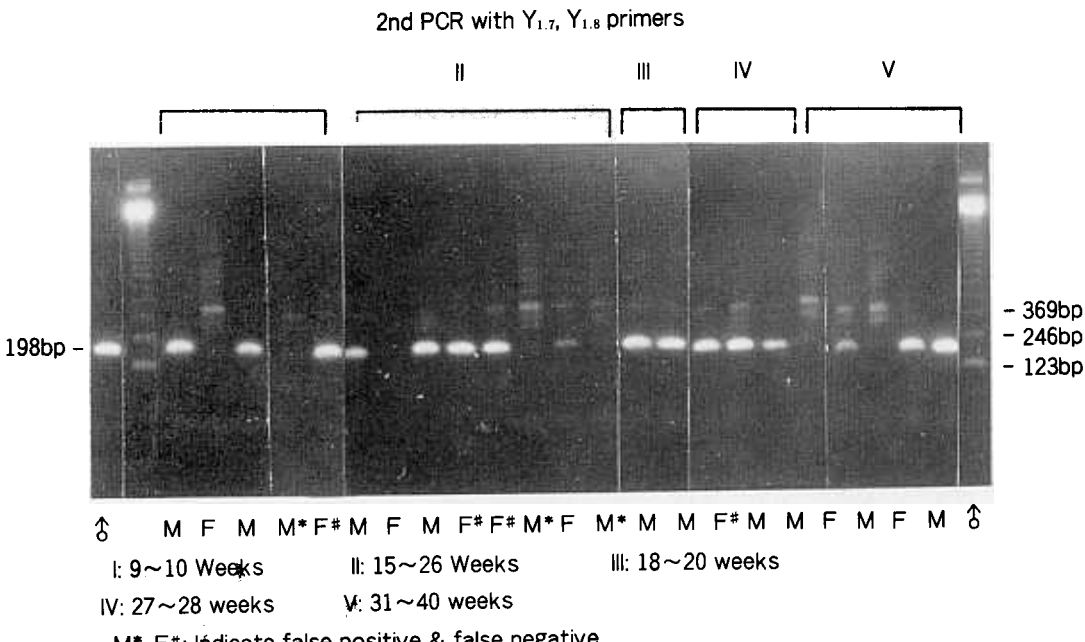

Fig. 1. Detection of male specific DNA fragment by nested PCR with Y-specific primers $Y_{1.5}Y_{1.6}$ and $Y_{1.7}$, $Y_{1.8}$

Table 1. Detection of Y-chromosome sequence, DYS 14 target from maternal peripheral blood at various stage of pregnancy

No. of cases	Gest. ages (weeks)	Positive bands by PCR	
		Mothers with male fetus	Mothers with female fetus
5	9~10	2/3	1/2
8	15~16	2/4	2/4
2	18~20	2/2	0/0
3	27~28	2/2	1/1
4	31~40	2/2	0/2

*Sensitivity: 76.9%, Specificity: 55.5%
Positive predictive value: 71.4%
Negative predictive value: 62.5%

ing to gestational age were relatively low. The male specific band was positive in three of six cases of female bearing women and the male specific band was negative in three of seven cases of male bearing women in 9~16 gestational weeks(Fig. 1-I,II).

But all cases except one showed male specific band in male fetuses and all female fetuses did not show 198 base pair band in 18~19 gestation weeks(Fig. 1-III), 27~28 gestational weeks(Fig. 1-IV), and 31~40 gestational weeks(Fig. 1-V).

In according to the gestational age, positive predictive values of 66.6%, 66.6%, and 80% were obtained for the first, second, and third trimesters,respectively. The corresponding negative predictive values were 50%, 50%, and 100 % respectively. The positive and negative predictive values were lower in the first and second than the third trimester.

DISCUSSION

Since Dr. Walknowska found fetal cells in the maternal blood of male bearing women in 1969 (Walknowska *et al.* 1969),many efforts were made in order to detect fetal cells in maternal blood (Schroder *et al.* 1972; Parks and Herzenberg, 1982; Covene *et al.* 1988).

Prenatal diagnosis of many diseases with maternal peripheral blood had been limited primarily due to very few fetal cells in the maternal peripheral blood until the recent development of the polymerase chain reaction (Saiki *et al.* 1988). PCR opens up new possibilities for detection of very small amount of fetal cells from maternal peripheral blood which allows new approach for prenatal diagnosis.

The possible source of false negative and false positive results were unresolved problems. False negative result from removal of fetal cells by maternal immune system that regarded the fetal cells as foreign substances (Bianchi *et al.* 1992) or the variable amount of fetal cells existence in maternal peripheral blood as gestational weeks gets older.

And false positive results from the cross reaction between Y-chromosome and autosome (Nakagome *et al.* 1990) and the amplification of male DNA contamination (Lo *et al.* 1989; Lo *et al.* 1990) or fetal cells remaining from previous male pregnancy (Hook,1990), and in case of vanishing male of female twin pregnancy (Mueller *et al.* 1990).

DNA studies by PCR are successful at the concentration of fetal to maternal cell at more than 1 in 70,000 (Adinolfi *et al.* 1989), and various different results were reported by different researchers suggested that difficulty of clinical application of PCR (Parks and Herzenberg, 1982; Nakahori *et al.* 1986; Yeoh *et al.* 1989; Bianchi *et al.* 1990; Holzgreve *et al.* 1990; Nakagome *et al.* 1991). On the other hand, based on the PCR amplification method with DNA sequence of Y-chromosome specific ZFY gene, permitted detection was as few as a single male cell among 1,000,000 female cells (Kao *et al.* 1992).

Three different Y-targets have been used for prenatal sex determination from unsorted maternal peripheral blood; DYZ 1 (Lo *et al.* 1989; Arinami *et al.* 1991; Merel *et al.* 1991), DYS 14 (Lo *et al.* 1989; Wachtel *et al.* 1991) and ZFY (Kao *et al.* 1992).

In 1989, Lo *et al.* have first reported the success of prenatal sex determination by PCR with DYZ 1 sequence (Lo *et al.* 1989), and in 1992, Suzumori et al. have reported the sensitivity value of PCR with DYZ 1 sequence of

66.7%, with the specificity of 81.8%, positive predictive value and negative predictive value were 75% each respectively. And they concluded that more sophisticated primer and technical development of PCR is needed in order to improve numerical results (Suzumori *et al.* 1992).

In 1992, Kao *et al.* have reported positive result of 20 cases in 23 women bore male baby by PCR with Y-chromosome specific ZFY (Zinc-finger-Y) and there was no false positive case (Kao *et al.* 1992). We also carried out nested PCR with Y-chromosome specific ZFY gene primer. But after 30 cycles of second PCR with ZFY primers, all cases demonstrated 307 base pair band in all women bearing regardless of sex of fetus. This obtained results were not compatible with the previous results of Kao *et al* (This data was not showen in this paper).

In 1990, Dr. Lo designed new primers $Y_{1.5}$ and $Y_{1.6}$ for external pair and internal pair of primers $Y_{1.7}$ and $Y_{1.8}$ which were male specific DNA sequence primers located on the DYS 14 locus of the short arm of Y-chromosome and with use of this new primers, no false positive results were reported (Arnemann *et al.* 1987; Lo *et al.* 1990). And in 1993, he also amplified the Y-chromosome specific DNA sequence gene DYS 14, with the new primers in various stages of pregnancy and they have reported positive predictive value of 86%, 67%, and 87% in the first, second, and third trimesters respectively. The corresponding negative predictive values were 83%, 64%, and 67% (Lo *et al.* 1993).

It is important to note that the first trimester, which is the most relevant to prenatal diagnosis is no better than the later trimester. In this study with the same primers, the sensitivity of PCR in the gestation of 9-16 weeks were lower than that of after 18 weeks gestation. And the positive predictive values were 66.6%, 66.6%, and 80% for the first, second, and third trimester each respectively. And the corresponding negative predictive values were 50%, 50%, and 100%.

Various groups have reported PCR accuracies of 65% in the first and second trimester (Wachtel *et al.* 1991), 68% and 86% in second trimester (Arinami *et al.* 1991; Merel *et al.* 1991) and 92% in the first trimester (Kao *et al.* 1992). It is interesting to note that there were different results even in the same gestational weeks (Kao *et al.* 1992).

In this study demonstrated relatively high sensitive detection of fetal cell from the maternal blood in the late pregnancy, but the sensitivity became lower in the early pregnancy. This is probably due to presence of low concentration of fetal cells in maternal peripheral blood in early pregnancy than later pregnancy. Therefore, need of more sensitive primer could overcome this problems and possibly increased accuracy of PCR. From this it seems that if we by using a method separating fetal cells from maternal blood and then by running PCR on these cells with $Y_{1.5}$, $Y_{1.6}$ and $Y_{1.7}$, $Y_{1.8}$ primers could make a fairly accurate fetal sex determination.

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