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(54) Non-invasive detection of fetal genetic traits

(57) Blood plasma of a pregnant woman contains both fetal and maternal circulatory extracellular DNA, the latter forming the major part (generally > 90%) thereof. The majority of the circulatory extracellular fetal DNA has now been found to have a small size of approximately 500 base pairs or less whereas the majority of the circulatory extracellular maternal DNA has a size greater than approximately 500 base pairs. Separation of circulatory extracellular DNA fragments which are smaller than approximately 500 base pairs provides a possibility to enrich for fetal DNA sequences from the vast bulk of circulatory extracellular maternal DNA.

There is thus provided a sample of the blood plasma or serum (which preferably is substantially cell-free) of a pregnant woman in which extracellular DNA comprising 500 base pairs or less is enriched; the manufacture of such sample by subjecting a sample of the blood plasma or serum of a pregnant woman to a size separation so as to enrich extracellular DNA comprising 500 base pairs or less; and the use of such sample for the non-invasive detection of fetal genetic traits.

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Description

[0001] The presence of circulatory extracellular DNA in the peripheral blood is a well established phenomenon. In this context, it has been shown that in the case of a pregnant woman extracellular fetal DNA is present in the maternal circulation and can be detected in maternal plasma or serum. Studies have shown that this circulatory fetal genetic material can be used for the very reliable determination, e.g. by PCR (polymerase chain reaction) technology, of fetal genetic loci which are completely absent from the maternal genome. Examples of such fetal genetic loci are the fetal RhD gene in pregnancies at risk for HDN (haemolytic disease of the fetus and newborn) or fetal Y chromosome-specific sequences in pregnancies at risk for an X chromosome-linked disorder e.g. haemophilia or fragile X syndrome.

[0002] The determination of other, more complex fetal genetic loci (e.g. chromosomal aberrations such as aneuploidies or chromosomal aberrations associated with Down's syndrome, or hereditary Mendelian genetic disorders and, respectively, genetic markers associated therewith, such as single gene disorders, e.g. cystic fibrosis or the haemoglobinopathies) is, however, more problematic. The reason for this difficulty is that the major proportion (generally >90%) of the extracellular DNA in the maternal circulation is derived from the mother. This vast bulk of maternal circulatory extracellular DNA renders it difficult, if not impossible, to determine fetal genetic alternations such as those involved in chromosomal aberrations (e.g. aneuploidies) or hereditary Mendelian genetic disorders (e.g. cystic fibrosis or the hemoglobinopathies) from the small amount of circulatory extracellular fetal DNA.

[0003] An examination of circulatory extracellular fetal DNA and circulatory extracellular maternal DNA in maternal plasma has now shown that, surprisingly, the majority of the circulatory extracellular *fetal* DNA has a relatively small size of approximately 500 base pairs or less, whereas the majority of circulatory extracellular *maternal* DNA in maternal plasma has a size greater than approximately 500 base pairs. Indeed, in certain instances the circulatory DNA material which is smaller than approximately 300 base pairs appears to be almost entirely *fetal*. Circulatory extracellular fetal DNA in the maternal circulation has thus been found to be smaller in size (approximately 500 base pairs or less) than circulatory extracellular maternal DNA (greater than approximately 500 base pairs).

[0004] This surprising finding forms the basis of the present invention according to which separation of circulatory extracellular DNA fragments which are smaller than approximately 500 base pairs or less provides a possibility to enrich for fetal DNA sequences from the vast bulk of circulatory extracellular maternal DNA.

[0005] This selective enrichment, which is based on size discrimination of circulatory DNA fragments of approximately 500 base pairs or less, leads to a fraction which is largely constituted by fetal extracellular DNA. This permits the analysis of fetal genetic traits including those involved in chromosomal aberrations (e.g. aneuploidies or chromosomal aberrations associated with Down's syndrome) or hereditary Mendelian genetic disorders and, respectively, genetic markers associated therewith (e.g. single gene disorders such as cystic fibrosis or the haemoglobinopathies), the determination of which had, as mentioned above, so far proved difficult, if not impossible. Size separation of extracellular fetal DNA in the maternal circulation thus facilitates the non-invasive detection of fetal genetic traits, including paternally inherited polymorphisms which permit paternity testing.

[0006] The present invention thus provides

- a sample of the blood plasma or serum (which preferably is substantially cell-free) of a pregnant woman in which extracellular DNA comprising 500 base pairs or less is enriched;
- the manufacture of such sample by subjecting a sample of the blood plasma or serum of a pregnant woman to a size separation so as to enrich extracellular DNA comprising 500 base pairs or less; and
- the use of such sample for the non-invasive detection of fetal genetic traits.

Circulatory extracellular fetal DNA can be enriched from the bulk of the circulatory extracellular maternal DNA in maternal plasma or serum samples by size separation. Said serum or plasma sample is preferably substantially cell-free, and this can be achieved by known methods such as, for example, centrifugation or sterile filtration.

The size separation of the extracellular DNA in said serum or plasma sample can be brought about by a variety of methods, including but not limited to

- chromatography or electrophoresis such as chromatography on agarose or polyacrylamide gels, ion-pair reversed-phase high performance liquid chromatography (IP RP HPLC, see Hecker KH, Green SM, Kobayashi K, J. Biochem. Biophys. Methods 2000 Nov. 20; 46(1-2): 83-93), capillary electrophoresis in a self-coating, low-viscosity polymer matrix (see Du M, Flanagan JH Jr, Lin B, Ma Y, Electrophoresis 2003 Sep; 24(18): 3147-53), selective extraction in microfabricated electrophoresis devices (see Lin R, Burke DT, Burn MA, J. Chromatogr. A. 2003 Aug 29; 1010(2): 255-68), microchip electrophoresis on reduced viscosity polymer matrices (see Xu F, Jabasini M, Liu S, Baba Y, Analyst. 2003 Jun; 128(6): 589-92), adsorptive membrane chromatography (see Teeters MA, Conrardy SE, Thomas BL, Root TW, Lightfoot EN, J. Chromatogr. A. 2003 Mar 7; 989(1):165-73) and the like;
- density gradient centrifugation (see Raptis L, Menard HA, J. Clin. Invest. 1980 Dec; 66(6): 1391-9); and
- methods utilising nanotechnological means such as microfabricated entropic trap arrays (see Han J, Craighead HG, Analytical Chemistry, Vol. 74, No. 2, January 15, 2002) and the like.

[0007] The sample thus obtained not only permits the subsequent determination of fetal genetic traits which had already been easily detectable in a conventional manner such as the fetal RhD gene in pregnancies at risk for HDN (haemolytic disease of the fetus and the newborn), or fetal Y chromosome-specific sequences in pregnancies at risk for an X chromosome-linked disorder such as haemophilia, fragile X syndrome or the like, but also the determination of other, more complex fetal genetic loci, including but not limited to

- chromosomal aberrations (e.g., aneuploidies or Down's syndrome) or hereditary Mendelian genetic disorders and, respectively, genetic markers associated therewith (e.g. single gene disorders such as cystic fibrosis or the haemoglobinopathies); and
- fetal genetic traits which may be decisive when paternity is to be determined.

[0008] Such determination of fetal genetic traits can be effected by methods such as, for example, PCR (polymerase chain reaction) technology, ligase chain reaction, probe hybridisation techniques, nucleic acid arrays (so-called "DNA chips") and the like.

[0009] The following Examples are further illustrating the invention but are not to be construed as limitating its scope in any way.

Example 1: Detection of male fetal DNA in maternal plasma by real-time quantitative Polymerase Chain Reaction (PCR) after size fractionation of DNA by agarose gel electrophoresis

Materials and Methods

Subjects and Sample processing

[0010] Seven women pregnant in the third trimester with a male fetus were recruited for this study. 16 - 18 ml blood samples were collected into EDTA tubes. 6 - 9 ml of plasma were obtained after centrifugation at 1600 g for 10 minutes and a second centrifugation of the supernatant at 16000 g for 10 minutes.

DNA isolation

[0011] DNA from 5 - 7 ml plasma was extracted using the QIAgen Maxi kit, according to the manufacturers' protocol. DNA was eluted in a volume of 1.5 ml.

DNA precipitation

[0012]

1. To the plasma DNA were added: 1/10 volume NaAc (3M, pH 5.2), 2 volumes absolute ethanol, $MgCl_2$ to a final concentration of 0.01 M and Glycogen to a final concentration of 50 μg / ml. The solution was thoroughly mixed by vortexing.
2. The solution was stored overnight at -70° C.
3. The DNA was recovered by centrifugation at 20000 g for 30 minutes at 4° C.
4. The supernatant was carefully removed and the pellet washed with 500 μl 70% ethanol.
5. The pellet was air dried and dissolved in 35 μl distilled water.

DNA separation

[0013]

1. A 1 % agarose Gel (Invitrogen, Cat No: 15510-027)was prepared for DNA electrophoresis.
2. 28 μl DNA solution were loaded on the gel.
3. The gel was electrophoresed at 80 Volt for 1 hour.
4. The Gel was cut into pieces corresponding to specific DNA sizes according to the DNA size markers (New England Biolabs, 100 bp ladder and Lamda Hind III digest). The DNA sizes contained by the specific gel fragments were: 90 - 300 bases, 300 - 500 bases, 500 - 1000 bases, 1.0 - 1.5 kilobases ("kb"), 1.5 - 23 kb and > 23 kb.
5. The DNA was purified from the agarose gel pieces using the QIAEX II Gel Extraction kit (Qiagen, Cat No. 20021) and eluted in 35 μl Tris-HCl (pH 8.0, 10mM).

Real-time PCR

[0014] Sequences from the Y chromosome (SRY) and from chromosome 12 (GAPDH gene) were amplified with the Applied Biosystems (ABI) 7000 Sequence Detection System by real-time quantitative PCR to quantify amounts of fetal and total DNA in the size-separated fractions. The TaqMan system for SRY consisted of the amplification primers SRY_Fwd: TCC TCA AAA GAA ACC GTG CAT and SRY_Rev: AGA TTA ATG GTT GCT AAG GAC TGG AT and a FAM labeled TaqMan MGB (Minor Groove Binder) probe SRY_MGB: TCC CCA CAA CCT CTT. The TaqMan System for the GAPDH gene consisted of the following primers and VIC-labeled probe: GAPDH_Fwd: CCC CAC ACA CAT GCA CTT ACC, GAPDH_Rev: CCT AGT CCC AGG GCT TTG ATT and GAPDH_MGB: TAG GAA GGA CAG GCA AC.

[0015] TaqMan amplification reactions were set up in a total reaction volume of 25 µl, containing 6 µl of the sample DNA solution, 300 nM of each primer (HPLC purified, Mycrosynth, Switzerland) and 200 nM of each probe (ABI) at 1 x concentration of the Universal PCR reaction mix (ABI). Each sample was analyzed in duplicate for each of the two amplification systems. A standard curve containing known amounts of genomic DNA was run in parallel with each analysis.

[0016] Thermal cycling was performed according to the following protocol: An initial incubation at 50 °C for 2 minutes to permit Amp Erase activity, 10 minutes at 95 °C for activation of AmpliTaq Gold, and 40 cycles of 1 minute at 60 °C and 15 seconds at 95 °C.

[0017] Amplification data collected by the 7000 Sequence Detection System was quantified using the slope of the standard curve as calculated by the sequence detection software and the results of a standard DNA solution used in the dilution curve with similar DNA copy numbers as the sample reactions as a reference sample for copy number calculations.

Results

[0018] Table 1 shows that in the five pregnancies examined, DNA fragments originating from the fetus were almost completely of sizes smaller than 500 base pairs with around 70 % being of fetal origin for sizes smaller than 300 base pairs.

[0019] These results demonstrate that free DNA of fetal origin circulating in the maternal circulation can be specifically enriched by size separation of the total free DNA in the maternal blood. Depending on the downstream application the DNA size chosen for the enrichment of fetal DNA will be smaller than 300 or smaller than 500 bases.

Table 1

Size of DNA	% of fetal DNA in each fragment	% of maternal DNA in each fragment
<0.3 kb	73.2 (22.22-87.06)	26.8 (12.94-77.78)
0.3-0.5 kb	18.95 (6.43-31.42)	81.05 (68.58-93.57)
0.5-1 kb	2.81 (0.00-7.75)	97.19 (92.25-100)
1.0-1.5 kB	0.00 (0.00-12.50)	100 (87.5-100)
1.5-23 kb	0.00 (0.00-8.40)	100 (100-100)

[0020] The abbreviation "kb" appearing in the first column of this table stands for 1000 base pairs, and the figures given in its second and the third column are the median values of the percentages and, in brackets, the ranges.

Example 2: Detection of fetal DNA after agarose gel electrophoresis by Polymerase Chain Reaction (PCR) of microsatellite markers, also called "Short Tandem Repeats" (STRs)

Materials and Methods

Subjects and samples

[0021] 18 ml blood samples from pregnant women were collected into EDTA tubes and plasma separated by centrifugation as described in example 1.The maternal buffy coat (i.e. the white colored top layer of the cell pellet obtained after the first centrifugation of 1600g for 10min.) was washed twice with PBS.

DNA isolation

[0022] DNA from the plasma was extracted using a modification of the High Pure DNA template kit from Roche, the whole sample was passed through the filter usually used for 200 µl using a vacuum. The DNA was eluted in a volume of 50 µl elution buffer.

[0023] Maternal DNA was isolated from the buffy coat, using the High Pure DNA template kit, and eluted into 100 µl.

DNA separation

[0024] The DNA was size-separated by electrophoresis on an agarose gel and purified as described in Example 1.

PCR specific for Short Tandem Repeats

[0025] From the fraction of sizes smaller than 500 bases, sequences from tetranucleotide repeat markers on Chromosome 21 were amplified in a PCR reaction as described in Li et al. Clinical Chemistry 49, No. 4, 2003. Because of the low concentration of plasma DNA, the fetal DNA in maternal plasma was examined by using a semi-nested PCR protocol.

[0026] The maternal and paternal pairs were genotyped using total genomic DNA to monitor microsatellite markers on chromosome 21.

[0027] The STR markers used were:

D211 S11;
D21S1270;

[0028] The resulting DNA fragments were then size separated by capillary electrophoresis on a sequencer, and the peak areas representing each allele for a specific marker were measured by the software.

Results

[0029]

Table 2

Detection of fetal alleles specific for the microsatellite marker (Short Tandem Repeat) D21S11 on chromosome 21.		
	Maternal alleles detected (D21S11)	Fetal alleles detected (D21S11)
Maternal genomic DNA	232 bp 234 bp	N/A
Total extracellulear DNA (unseparated)	232 bp 234 bp	No fetal alleles detectable
Size-separated extracellular DNA (<300 bp)	232 bp 234 bp	228 bp 232 bp
Size-separated extracellular DNA (300-500 bp)	232 bp 234 bp	228 bp 232 bp

[0030] Only in the size-separated fractions (<300 bp and 300-500 bp) could the fetal alleles for D21 S 11 be detected, namely the paternally inherited 228 bp allele and the maternally inherited 232 bp allele, i.e. one allele from each parent.

Discussion

[0031] Analysis of the STR fragments can allow for the detection of paternal alleles that are distinct in length from the maternal repeat sequences, and by calculating the ratios between the peak areas it can be possible to identify patterns that are not consistent with a normal fetal karyotype. The identification of fetal STR allele in the maternal circulation can allow the detection of certain chromosomal aberrations non-invasively e.g. trisomy 21. Also paternity testing can be accomplished prenatally in a non-invasive manner.

Claims

1. A sample of the blood plasma or serum of a pregnant woman in which extracellular DNA comprising 500 base pairs or less is enriched.

2. A sample according to claim 1 which is substantially cell-free.

3. A process for the manufacture of a sample according to claim 1 or 2 which comprises subjecting a sample of the blood plasma or serum of a pregnant woman to a size separation so as to enrich extracellular DNA comprising 500 base pairs or less.

4. A process according to claim 3 wherein the size separation is carried out by chromatography or electrophoresis, by density gradient centrifugation or by methods utilising nanotechnological means.

5. A process according to claim 4 wherein the chromatography and, respectively, electrophoresis is chromatography on agarose or polyacrylamide gels, ion-pair reversed-phase high performance liquid chromatography (IP RP HPLC), capillary electrophoresis in a self-coating, low-viscosity polymer matrix, selective extraction in microfabricated electrophoresis devices, microchip electrophoresis on reduced viscosity polymer matrices or adsorptive membrane chromatography.

6. A process according to claim 4 wherein the method utilizing nanotechnological means is making use of microfabricated entropic trap arrays.

7. The use of a sample according to claim 1 or 2 for the non-invasive detection of fetal genetic traits.

8. The use according to claim 7 wherein the fetal genetic trait to be detected is the fetal RhD gene in a pregnancy at risk for HDN (haemolytic disease of the fetus and the newborn) or a fetal Y chromosome-specific sequence in a pregnancy at risk for an X chromosome-linked disorder.

9. The use according to claim 7 wherein the fetal genetic trait to be detected is a chromosomal aberration, a hereditary Mendelian genetic disorder and, respectively, a genetic marker associated therewith, or a fetal genetic trait which may be decisive when paternity is to be determined.

10. The use according to claim 8 wherein the X chromosome-linked disorder is haemophilia or fragile X syndrome.

11. The use according to claim 9 wherein the chromosomal aberration is an aneuploidy.

12. The use according to claim 9 wherein the chromosomal aberration is associated with Down's syndrome.

13. The use according to claim 8 wherein the hereditary Mendelian genetic disorder is a single gene disorder.

14. The use according to claim 13 wherein the single gene disorder is cystic fibrosis or a haemoglobinopathy.

15. The use according to any one of claims 7 to 14 wherein the detection of the fetal genetic traits is carried out by PCR (polymerase chain reaction) technology, ligase chain reaction or probe hybridisation techniques or by means of nucleic acid arrays.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 03 40 5742

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	SIVA SASHI C ET AL: "Evaluation of the clinical usefulness of isolation of fetal DNA from the maternal circulation." THE AUSTRALIAN & NEW ZEALAND JOURNAL OF OBSTETRICS & GYNAECOLOGY. AUSTRALIA FEB 2003, vol. 43, no. 1, February 2003 (2003-02), pages 10-15, XP009024979 ISSN: 0004-8666 * page 11 - page 13; figures 1,2 * ---	1-7	C12Q1/68
X	LO Y M D ET AL: "PRESENCE OF FETAL DNA IN MATERNAL PLASMA AND SERUM" LANCET, XX, XX, vol. 350, 16 August 1997 (1997-08-16), pages 485-487, XP001037118 ISSN: 0140-6736 * page 486 * ---	1-7	
X	SMID MADDALENA ET AL: "Evaluation of different approaches for fetal DNA analysis from maternal plasma and nucleated blood cells" CLINICAL CHEMISTRY, vol. 45, no. 9, September 1999 (1999-09), pages 1570-1572, XP002268967 ISSN: 0009-9147 * page 1570 - page 1571 * --- -/--	1-7	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	20 February 2004	Costa Roldán, N

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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E : earlier patent document, but published on, or after the filing date
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EP 03 40 5742

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X	PERTL BARBARA ET AL: "Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats" HUMAN GENETICS, vol. 106, no. 1, January 2000 (2000-01), pages 45-49, XP002268968 ISSN: 0340-6717 * the whole document * ---	1-7	
A	LO Y M DENNIS: "Fetal DNA in maternal plasma: Biology and diagnostic applications" CLINICAL CHEMISTRY, AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY. WINSTON, US, vol. 46, no. 12, December 2000 (2000-12), pages 1903-1906, XP002247511 ISSN: 0009-9147 * the whole document * ---	1-15	
A	PERTL BARBARA ET AL: "Fetal DNA in maternal plasma: Emerging clinical applications" OBSTETRICS AND GYNECOLOGY, vol. 98, no. 3, September 2001 (2001-09), pages 483-490, XP001179125 ISSN: 0029-7844 * abstract * * page 486 - page 487 * --- -/--	1-15	TECHNICAL FIELDS SEARCHED (Int.Cl.7)

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A	HOUFFLIN-DEBARGE VERONIQUE ET AL: "High sensitivity of fetal DNA in plasma compared to serum and nucleated cells using unnested PCR in maternal blood" FETAL DIAGNOSIS AND THERAPY, vol. 15, no. 2, March 2000 (2000-03), pages 102-107, XP009025236 ISSN: 1015-3837 * the whole document * ---	1-15	
A	HAHN S ET AL: "MULTIPLEX AND REAL-TIME QUANTITATIVE PCR ON FETAL DNA IN MATERNAL PLASMA A COMPARISON WITH FETAL CELLS ISOLATED FROM MATERNAL BLOOD" ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, NEW YORK ACADEMY OF SCIENCES, NEW YORK, NY, US, vol. 906, 2000, pages 148-152, XP008007707 ISSN: 0077-8923 * the whole document * -----	1-15	
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EPO FORM 1503 03.82 (P04C01)

专利名称(译)	胎儿遗传性状的非侵入性检测		
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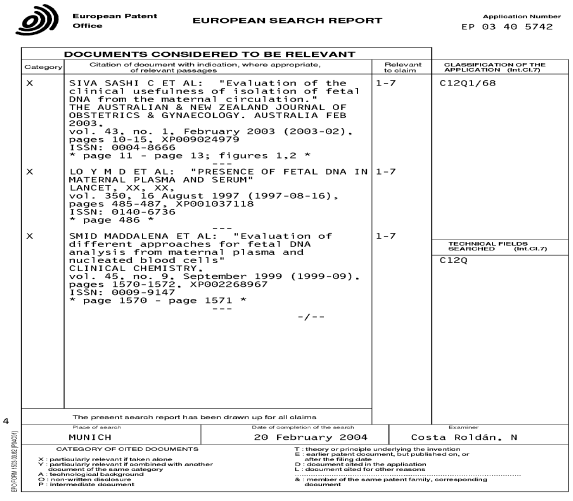
其他公开文献	EP1524321B1 EP1524321B2		
外部链接	Espacenet		

摘要(译)

孕妇的血浆含有胎儿和母体循环细胞外DNA，后者形成其主要部分（通常> 90％）。现在已发现大多数循环细胞外胎儿DNA具有约500碱基对或更小的小尺寸，而大多数循环细胞外母体DNA具有大于约500个碱基对的大小。小于约500个碱基对的循环细胞外DNA片段的分离提供了从大量的循环细胞外母体DNA中富集胎儿DNA序列的可能性。因此，提供了孕妇的血浆或血清（优选基本上无细胞）的样品，其中富含包含500个碱基对或更少的细胞外DNA;通过对孕妇的血浆或血清样品进行大小分离以便富集包含500个碱基对或更少的细胞外DNA来制造这种样品;以及这种样品用于胎儿遗传性状的非侵入性检测的用途。



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