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Rapid detection of common autosomal aneuploidies by quantitative fluorescent PCR on uncultured amniocytes

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Prenatal diagnosis of chromosomal abnormalities by cytogenetic analysis is time consuming, expensive, and requires highly qualified technicians. Rapid diagnosis of aneuploidies followed by reassurance for women with normal results can be performed by molecular analysis of uncultured foetal cells in less than 24 h. Today, all molecular techniques developed for a fast diagnosis of aneuploidies rely on the semi-quantification of fluorescent PCR products from short tandem repeat (STR) polymorphic markers. Our objective was to test a chromosome quantification method based on the analysis of fluorescent PCR products derived from non-polymorphic target genes. An easy to set up co-amplification of portions of DSCR1 (Down Syndrome Critical Region 1), DCC (Deleted in Colorectal Carcinoma), and RB1 (Retino-blastoma 1) allowed the molecular detection of aneuploidies for chromosomes 21, 18 and 13 respectively. Quantitative analysis was performed in a blind prospective study of 400 amniotic fluids. Four samples (1%) could not be analysed by PCR probably because of a low concentration of foetal DNA. Follow up karyotype analysis was done on all samples and molecular results were in agreement with the cytogenetic data with no false – positive or false – negative results. Our gene based fluorescent PCR approach is an alternative molecular method for a rapid and reliable detection of aneuploidies which can be helpful for the clinical management of high-risk pregnancies.
European Journal of Human Genetics (2002) **10**, 462 – 466. doi:10.1038/sj.ejhg.520833

Introduction

Trisomies are the largest group of chromosome abnormalities, and trisomies for chromosomes 13 (Patau's syndrome), 18 (Edward's syndrome), or 21 (Down's syndrome) are a major concern in prenatal diagnosis because they are the only three autosomal trisomies found with any significant frequency among live births.

For more than 25 years, the prenatal diagnosis of chromosomal abnormalities has been based on full karyotyping of foetal cells from amniotic fluid. Foetal karyotyping remains the gold standard for prenatal diagnosis of chromosomal abnormalities, but the significant delay in providing the diagnosis (about 10 – 20 days)[1] leads to an inevitable anxiety period for the parents.

More recently, several methods based on fluorescence *in situ* hybridisation (FISH) or semi-quantitative fluorescent PCR (QF – PCR) of short tandem repeats have been developed for a rapid detection of aneuploidies in high-risk pregnancies, allowing a prenatal diagnosis of chromosomal abnormalities within 24 – 48 h. Introduced in the late 1990s, FISH analysis of uncultured interphase foetal cells with commercially available multicolour specific probes is presently successfully routinely applied.[2,3] Although fast

and reliable, the prenatal detection of aneuploidies by FISH remains expensive and its use is mainly restricted to pregnancies with anomalies detected by ultrasonography. The use of QF–PCR of STR for the fast detection of aneuploidies has been applied to trisomy 21.[4] Since 1993, this molecular approach has been extensively used by several groups on a research basis[5–10] and the diagnosis of aneuploidies by QF–PCR of STRs has now been validated as a reliable method applicable in many laboratories.[9] The use of STRs for the molecular diagnosis of aneuploidies on uncultured amniocytes requires the PCR amplification of several polymorphic markers for each chromosome tested. Since a minimum of two informative markers is preferable for a confident diagnosis of trisomy,[9] this leads to complex multiplex PCR assays. Although the comparative quantification of non-polymorphic loci has been already extensively used to investigate gene copy number, this approach has only been applied once to the detection of sex chromosome aneuploidies.[11]

In this study, we present a comparative quantitative assay for the prenatal detection of aneuploidies of chromosomes 13, 18, and 21 based on the analysis of fluorescent PCR products derived from three genes, DSCR1, DCC and RB1 located on chromosome 21, 18 and 13 respectively, this is the first time that the comparative quantification of non-polymorphic loci is used for autosomal aneuploidy testing. For each gene, a relative comparative quantification is performed by using the two other genes as internal controls, thus allowing us to calculate two ratios per chromosome. This method has been successfully applied in a blind prospective study of 400 amniotic fluids.

Materials and methods
Samples
Four hundred pregnant women underwent amniocentesis for prenatal chromosome analysis between gestational weeks 14 and 17. These women were referred to the prenatal diagnosis unit of the university hospitals of Nancy, Montpellier and Clermont-Ferrand. The referral criteria were maternal age (38 years which is the French cut-off age for free-of-charge karyotyping), positive biochemical screening for DOWN syndrome, abnormal foetal ultrasonic scan, positive family history of chromosomal rearrangement, or parental anxiety. Ethical approval for the study was obtained from each centre under the assumption that PCR results were not reported to the referring clinicians.

DNA extraction and fluorescent PCR
Genomic DNA extraction was performed on the cell pellet obtained from 1–2 ml of amniotic fluid after centrifugation at 2400 g for 10 min with the QIAamp DNA blood mini kit according to the manufacturer's recommendations (Qiagen, Germany), elution was performed in 50 μl of elution buffer. We did not estimate DNA concentrations as we expected to obtain samples of extremely low and varying DNA concen-

tration. A single tube multiplex PCR was performed with PCR primer pairs for the DSCR1 gene (21q22.1–q22.2), for the DCC gene (18q21.1) and for the RB1 gene (13q14.3). We chose the DSCR1 gene for chromosome 21 because a minimal critical region for Down syndrome has been proposed in 21q22.[12] DCC[13] and RB1[14] were selected for gene copy number quantification because the amplified regions do not show any significant homology with sequences in the human genome. For each primer pair, the forward oligonucleotide was labelled at the 5′ end with 6-carboxy-fluorescein (FAM) dye, the primer sequences and PCR product lengths are listed in Table 1. The PCR reaction was performed in a total volume of 25 μl containing 2 μl DNA (the volume of the DNA solution should not exceed one tenth of the final PCR reaction volume), 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.5 μM of each primer, and 2 U Taq Polymerase (Taq-Gold, Applied Biosystems). The reaction mixture was subjected to PCR amplification in a Applied Biosystems 9700 thermal cycler, PCR conditions were 5 min denaturation at 95°C followed by 24 cycles with 1 min denaturation at 94°C, 45 s annealing at 58°C and 1 min 30 s elongation at 70°C. A final extension step of 40 min at 60°C ended the PCR cycle to avoid peak splitting caused by the incomplete adding of adenine by Taq polymerase to the end of the PCR products.

Comparative quantitative analysis
One μl of fluorescent PCR product was mixed with 12 μl formamide and 0.4 μl of Genscan Rox 400HD size standard (Applied Biosystems, USA), denatured 3 min at 95°C and chilled on ice until capillary electrophoresis on an ABI 310 genetic analyser with POP 4 (Applied Biosystems, USA). The DSCR1, DCC, and RB1 derived PCR products were analysed with Genescan and Genotyper softwares (Applied Biosystems, USA), and the chromosome copy number was estimated by calculating the peak area ratios: DSCR1:DCC and DRSC1:RB1 for chromosome 21, DCC:DRSC1 and DCC:RB1 for chromosome 18, and RB1:DRSC1 and RB1:DCC for chromosome 13. All amniotic fluids were analysed in duplicate and the mean values are given.

Results
The 400 amniotic fluid samples were analysed by comparative quantitative fluorescent PCR before completion of cytogenetic analysis. Trisomy 21 was diagnosed by standard cytogenetic methods in seven cases, trisomy 18 in two cases, and trisomy 13 in one case. Our quantitative method successfully identified all aneuploidies involving chromosomes 13, 18 and 21. Moreover, all karyotypically normal samples were correctly diagnosed as disomic for the three chromosomes tested in our assay. Four amniotic fluids (1%) could not be analysed, probably because of very low DNA concentration.

Figure 1 depicts characteristic normal and trisomic patterns. The peak area ratios did not overlap (Table 2)

Table 1 Oligonucleotides used in this study

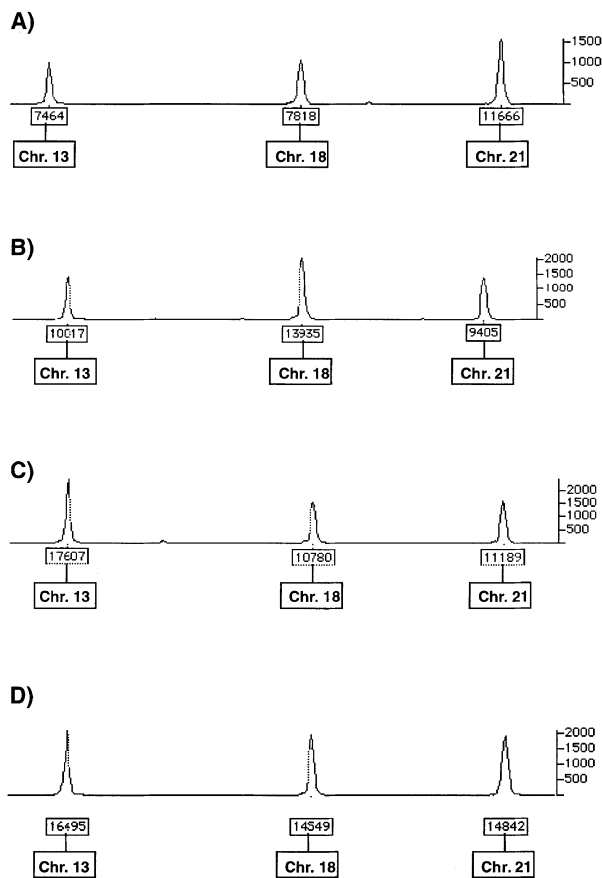| Chromosome	Locus	Primer	PCR product length
13	RB1	F 5′- cca caa ccg tct gga aga c-3′ R 5′-ggt tat gga gtg taa gga gc-3′	124 bp
18	DCC	F 5′-aat gcg ggg agg aaa tgt c-3′ R 5′-gtt ttg tat cag cag aga cc-3′	150 bp
21	DSCR1	F 5′-cgt gtc tgt ctt gga tgg t -3′ R 5′-aca ggg gaa caa gat tac ga-3′	169 bp

Figure 1 Typical electrophoretograms for normal and trisomic amniotic fluids. (**A**) Trisomy 21, (**B**) trisomy 18, (**C**) trisomy 13, (**D**) euploid sample. The x axis shows the lengths of the PCR products that are 124 bp for chromosome 13, 150 bp for chromosome 18 and 169 bp for chromosome 21. Peak areas are depicted under each peak (Genotyper), the y axis shows fluorescent intensities in arbitrary units.

and allowed an unequivocal determination of the chromosome copy number in our series of amniotic fluids.

PCR based strategies rely on the fact that the amount of PCR products is proportional to the quantity of initial target sequence. Our quantification method is based on comparable and stable PCR efficiencies for the three target genes. Thus, amplification efficiencies were tested every cycle between 20 to 30 rounds of amplification on DNA from healthy volunteers. Results showed, as expected,[15] lower variations of PCR product ratios during the proximal part of the exponential amplification phase (data not shown). Moreover, peak splitting was observed after 26 cycles on the DCC and RB1 amplificatied PCR peaks and could not be corrected with a final extension step (see Material and methods). Therefore, as the three ratios were close to one at 24 cycles, all amniotic fluids were analysed with a 24 cycles PCR program.

We discarded all amniotic fluids suspected of blood cell contamination as determined by macroscopic examination for red blood cells. In our series, seven blood stained samples (1.6%) were not analysed because of probable maternal cell contamination, although Schmidt *et al*[7] found less than 2% of red stained amniotic fluids to be really contaminated with maternal DNA.

In diploid cells, each gene can be considered as a two copy reference test whereas in trisomic cells, the genes located on the extra chromosome are present in three copies. For example, the theoretical ratios for a trisomy 21 sample would be 3:2 (1.5) for DSCR1:DCC and DSCR1:RB1 and 2:2 (1) for DCC:RB1. As shown on Figure 1D, peak areas for the three target genes were very similar in normal diploid samples from our study population: the mean ratios for 21:13, 21:18 and 18:13 were found at 0.91 (SD 0.087, range 0.81–1.13), 1.00 (SD 0.071, range 0.86–1.12) and 1.01 (SD 0.074, range 0.76–1.09) respectively (Table 2). With trisomic samples, the PCR area for the target gene was increased, giving abnormal 1.5 ratios when compared to the two other genes which were considered as internal controls. This is illustrated in Figure 1A–C for trisomic amniotic fluids.

Discussion

The quantitative molecular detection of aneuploidies for chromosomes 13, 18 and 21 presented in this study is fast and reliable. It allows prenatal identification of the most frequent trisomies within 24 h after sampling. When compared to the labour-intensive and time-consuming FISH, this method is simple and less expensive. Before this study, several groups had already proved the usefulness and the accuracy of QF–PCR for the rapid detection of aneuploidies.[5–9] They all used the same STRs based strategy which

Table 2 Relative semi-quantification of aneuploidies

| | Relative peak area | | | | | | | | |
| | 21:18 | | | 21:13 | | | 18:13 | | |
Samples (n=400)	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Euploid (*n*=390)	1.00	0.071	0.86–1.12	0.91	0.087	0.81–1.13	1.01	0.074	0.76–1.09
Trisomy 13 (*n*=2)	1.01	0.05	0.98–1.05	0.62	0.02	0.62–0.63	0.61	0.01	0.60–0.62
Trisomy 18 (*n*=1)	0.67	–	–	0.94	–	–	1.39	–	–
Trisomy 21 (*n*=7)	1.67	0.07	1.58–1.70	1.79	0.12	1.62–1.94	1.07	0.04	1.14–1.03

Three relative peak ratios (21/18, 21/13 and 18/13) were calculated for each amniotic fluid sample, for each chromosome, a relative semi-quantification was performed by using the two other loci and internal controls, this allowed us to calculate two ratios per chromosome. This table shows data for disomic and trisomic samples.

detects trisomic foetuses either with a three alleles of equal intensity pattern or with a two allele pattern characterized by a 2 : 1 ratio. Up to now, the STRs based strategy has been the method of choice for a molecular detection of aneuploidies. Although attractive, this method has several small disadvantages. The QF–PCR with fluorescent STRs approach needs at least two highly informative microsatellites per chromosome because homozygosity at one locus could impair the diagnosis of aneuploidy.[6] This results in a complex one tube PCR with up to 12 markers amplified at once[9] or to several (3 to 4) more simple multiplex PCR reactions.[8,7] Levett *et al*[8] in a large prospective study of 5000 amniotic fluids were not able to conclude for one chromosome in 2% of the samples because seven out of eight markers showed a homozygous peak. Detection of diallelic trisomies can be tricky, particularly with dinucleotide repeat markers which generate stutter alleles (−2 bp, −4 bp). Moreover the ratio of peak areas can vary from one allele to another[4] and there is a preferential amplification of small PCR fragments. We chose to use non polymorphic sequences instead of STRs for the comparative quantification of aneuploidies. With our approach, the PCR products resulting from target genes do not show stutter bands.

A technical limitation of our gene based semi-quantitative PCR comes from its inability to identify maternal DNA contamination. This is a disadvantage when compared to the QF–PCR with fluorescent STRs. Although detected, samples with evidence of a maternal genotype cannot be interpreted according to several studies.[8,9] However, for Schmidt and collaborators, identification of maternal contamination does not exclude a correct interpretation.[7] We believe that macroscopic examination of amniotic fluid allows the detection of the vast majority of bloody or brownish samples. Also, traces of maternal cells should not preclude the correct diagnosis of aneuploidies by PCR based approaches. During the study period, we decided to exclude all blood stained samples suspected of maternal contamination (1.6%), and for the remaining samples without major contamination we had no discrepancy between the cytogenetic and the molecular results. It is noteworthy that even apparently clear amniotic fluids could be contaminated with maternal DNA.[7]

Non-diagnosis of trisomies could result from sequence variant residing within a priming site leading to incomplete/non amplification of one allele, this rare event has already been reported in multiplex PCR approach for the detection of deletions within the DMD gene.[16,17] This problem inherent to the PCR technique could be circumvented by the amplification of two loci per chromosome.

Another weakness of this comparative quantitative analysis of non polymorphic DNA markers is that polyploidy (particularly triploidy) and mosaicism cannot be detected. In addition, testing for sex chromosome abnormalities by means of comparative quantitative analysis is technically feasible,[11] X- and Y-chromosomal genes could be introduced in our PCR protocol in this purpose.

Lastly, our approach as other molecular methods based on QF–PCR with fluorescent STRs, cannot detect small segment imbalances.

Our assay was designed for a rapid and cost-effective detection of common trisomies of chromosome 13, 18 and 21 for high-risk pregnancies with anomalies detected by ultrasonography, and not as a large-scale prenatal diagnosis of the common aneuploidies. This comparative quantification assay is an attractive alternative to the commercially available multi-FISH kits.

We are convinced that none of the different molecular approaches will replace chromosome analysis and karyotyping on cultured amniocytes. Non-invasive prenatal diagnosis based on amplification of foetal DNA isolated from maternal blood, also promising, has proved very difficult to develop.[18,19] However, PCR-based diagnostic methods on uncultured amniotic cells are reliable and accurate adjuncts for a rapid detection of trisomies 13, 18 and 21 which are a major concern in prenatal diagnosis particularly when pregnancy is relatively advanced. In the future, fluorescent PCR diagnosis could become a method of choice for a rapid and non-invasive detection of selected aneuploidies. Although not appropriate for the detection of maternal cell contamination and mosaicism, our molecular approach based on comparative quantification of gene derived PCR products is a robust and easy to set up technique for the molecular prenatal diagnosis of aneuploidies in less than 24 h.

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