X chromosome dosage by quantitative fluorescent PCR and rapid prenatal diagnosis of sex chromosome aneuploidies

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Introduction
Quantitative fluorescent PCR (QF-PCR) is an assay designed to perform rapid prenatal diagnoses of common chromosome aneuploidies (Mansfield, 1993; Pertl et al., 1994). This method is based on the PCR amplification of selected chromosome-specific short tandem repeats (STR) (Adinolfi et al., 1997, 2000). During PCR amplification, a fluorochrome is incorporated into the products, which are then visualized and quantified using an automated DNA sequencer (Adinolfi et al., 1997). Several investigations have documented the accuracy of QF-PCR tests for the rapid prenatal diagnoses of numerical disorders affecting chromosomes 21, 13 and 18 (Perzl et al., 1996, 1999; Adinolfi et al., 1997, 2000; Verma et al., 1998; Mann et al., 2001).

Due to the previous unavailability of highly polymorphic STR markers, it is only in recent times that it has been possible to detect X and Y chromosome abnormalities by QF-PCR (Cirigliano et al., 1999, 2001a; Schmidt et al., 2000).

For the detection of Turner’s syndrome (45,X), several X-linked markers must be included in a multiplex QF-PCR assay in order to produce a pattern documenting the presence of a single X chromosome. However, in some rare cases, due to the gene frequency of the selected markers, the test may fail to distinguish between a sample retrieved from a 45,X and a normal female fetus homozygous for all X markers (Cirigliano et al., 2001a,b).

We have developed a new method for the rapid detection of X chromosome copy number based on QF-PCR amplification of the X-linked HPRT (hypoxanthine phosphoribosyl transferase) marker, with the autosomal D21S1411 used as internal control for its quantification. This assay, which does not require additional markers, allows one to distinguish between the QF-PCR patterns generated from a normal female and a 45,X fetus independent of the heterozygosity, as well as to perform rapid prenatal diagnosis of rare normal fetuses homozygous for chromosome 21 STR.

Materials and methods
In a preliminary study, 22 amniotic samples from normal female fetuses were used to evaluate the hypothesis that a close correlation could be detected between the amount of fluorescent activity generated by an X-linked marker, such as X22 or HPRT and those of an autosomal STR. Several markers were employed in different QF-PCR conditions, until the fluorescent products of the HPRT sequence and those of D21S1411 were found to be comparable and to allow quantification of X chromosome copy number. Once these preliminary tests had documented the reproducibility of the comparative method, 128 selected amniotic fluids, collected at ~16 weeks gestation, were investigated; 64 samples were retrieved from mothers with normal females and 64 samples from mothers with normal males. The QF-PCR diagnoses were all confirmed by conventional cytogenetic analysis.

Two more amniotic fluid samples and 11 blood samples from adults with Turner’s syndrome, that had been frozen and stored for a few years, were also included in the study (Cirigliano et al., 1999). While this work was in progress, two amniotic fluid samples were received for routine prenatal tests; the first showed a QF-PCR pattern suggesting prenatal diagnosis of 45,X and

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the second one was found to be homozygous for five chromosome 21 STR. Both samples were then analysed with the new approach.

Whole genomic DNA, extracted from bloods and uncultured amniotic fluids using InstaGene Matrix (Bio-Rad Laboratories, CA, USA), underwent multiplex QF-PCR amplification using STR markers for chromosomes 21, 13, 18, X and Y as previously described (Cirigliano et al., 2001b). The sex chromosome markers included the pentanucleotide repeat X22, mapped in the PAR2 region of the X and Y chromosomes, as well as HPRT, DXS6803 and DXS6909 (Cirigliano et al., 1999, 2001a,b). Sex was assessed for all samples using the amelogenin non-polymorphic marker (AMXY; here we will indicate the fluorescent products specific for chromosomes X and Y respectively as AMX and AMY). PCR reactions were performed in a final volume of 25 µl containing 5–40 pmol of each primer (Roche, TIB Molbiol, Berlin, Germany), 200 µmol/l dNTP, 2 mmol/l MgCl₂, in 1×buffer and one unit of Taq polymerase (Promega, Madison, WI, USA). After denaturation at 95°C, hot start PCR was performed for 22 or 27 (bloods or amniotic fluids) repeating cycles at 95°C for 30 s, 57°C for 35 s and 72°C for 35 s on a GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA). Capillary electrophoresis and analysis of the fluorescent PCR products and size standards were performed with an ABI Prism 310 automated DNA sequencer and Gene-Scan 3.1 (Applied Biosystems) as previously described (Cirigliano et al., 1999, 2001a). Additionally the D21S1411 was used as autosomal internal control by calculating the ratios between the fluorescent intensity of the correspondent PCR products with the X-specific peaks generated by the HPRT marker. These sequences generate alleles of very similar size, but they can be readily identified using primers labelled with different fluorochromes (Sherlock et al., 1998).

Results

In a preliminary study of 22 amniotic samples from female fetuses, the fluorescent activities of X chromosome markers (e.g. X22 or HPRT) were compared with those of each autosomal STR (e.g. D21S1411, D18S386 or D13S634) that had been included in the same QF-PCR multiplex assay. The aim of this analysis was to assess whether a close correlation between the amount of fluorescent activities produced by an X chromosome marker and an autosomal STR could be detected. In this case, the peaks of fluorescent activities of an X chromosome marker in a sample from a normal heterozygous female would be identical to the activities of the two products of an autosomal STR (e.g. ratio X chromosome markers/autosomal STR = 1:1 to 1:1). In the first batch of 22 amniotic samples from female fetuses, a good correlation could be noticed only when the HPRT and chromosome 21-specific D21S1411 was used with the PCR amplification limited to 27 cycles. All other comparisons between sex and autosomic markers showed unrelated and non-reproducible fluorescent ratios.

According to the heterozygosity or homozygosity of each HPRT/D21S1411 marker, ratios equal to (i) 1:1 to 1:1, (ii) 1:1, (iii) 2 to 1:1, or (iv) 1:1 to 2 were detected, documenting that the tested samples had been collected from female subjects: (i) heterozygous for both markers (1:1 to 1:1), (ii) samples homozygous for both sequences (1:1), (iii) homozygous for the first and heterozygous for the second (2 to 1:1), or (iv) vice versa (1:1 to 2) (Table I and Figure 1).

When the fluorescent activities of the two markers, HPRT and D21S1411, were compared in the group of 64 amniotic fluid samples retrieved from normal female fetuses, the expected ratios of two

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPRT/D21S1411 ratios</th>
<th>AMY Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal female</td>
<td>1:1 to 1:1</td>
<td>1:1 to 2</td>
</tr>
<tr>
<td>Normal male</td>
<td>1 to 1</td>
<td>2 to 1:1</td>
</tr>
<tr>
<td>Turner’s syndrome</td>
<td>1 to 1</td>
<td>AMY positive</td>
</tr>
</tbody>
</table>

Table I. Expected ratios between HPRT and D21S1411 STR markers in normal fetuses and Turner’s syndrome

![Figure 1. Electrophoretograms showing QF-PCR products of HPRT and D21S1411 (arrows) and small tandem repeats (STR) on normal male and female fetuses. These markers are part of a multiplex QF-PCR including X22, D18S386 and D13S634 STR. The ratios between peaks of the two sequences are reported in each lane. First lane: Male sample heterozygous for the autosomal STR. The single X peak has the same fluorescent intensity of the two 21 alleles. Second lane: Male sample homozygous for the autosomal STR. The 21 peak (from two chromosomes) has double the intensity of the X peak (from one chromosome). Third lane: Female double homozygous. The two X chromosomes generate the same amount of PCR product as the two chromosomes 21. Fourth lane: Female sample homozygous for the X-linked and heterozygous for the autosomal marker. The fluorescent intensity of the X product is in a double dose compared with the autosomal alleles. Fifth lane: Opposite situation to the previous one. Sixth lane: Female sample heterozygous for both STR. The fluorescent intensity is the same for the four alleles.](image-url)
Figure 2. Electrophoretograms of HPRT and D21S1411 (arrows) detecting normal females and Turner’s syndrome.

Table II. Results of testing 128 normal controls and 13 Turner’s syndrome with HPRT and D21S1411, and range variations between the QF-PCR products of the two sequences

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPRT/D21S1411 zygosity</th>
<th>HPRT/D21S1411 range</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>46,XX</td>
<td>Double heterozygous</td>
<td>1:1–1.2 to 1:1</td>
<td>46</td>
</tr>
<tr>
<td>46,XX</td>
<td>HPRT homozygous</td>
<td>1.7–2 to 1:1</td>
<td>9</td>
</tr>
<tr>
<td>46,XX</td>
<td>D21S1411 homozygous</td>
<td>1:1 to 2–2.4</td>
<td>5</td>
</tr>
<tr>
<td>46,XY</td>
<td>Double homozygous</td>
<td>0.8–1.2 to 1</td>
<td>4</td>
</tr>
<tr>
<td>46,XY</td>
<td>D21S1411 homozygous</td>
<td>1 to 1.8–2.3</td>
<td>4</td>
</tr>
<tr>
<td>46,XY</td>
<td>D21S1411 heterozygous</td>
<td>0.7–1.3 to 1:1</td>
<td>60</td>
</tr>
<tr>
<td>45,X</td>
<td>D21S1411 heterozygous</td>
<td>0.7–1.2 to 1:1</td>
<td>13</td>
</tr>
</tbody>
</table>

While this study was in progress, an amniotic fluid received for routine prenatal diagnosis was found to produce single peaks for all X-linked markers (X22, HPRT, DXS6803 and DXS6809) in the absence of any Y chromosome-specific product. While this pattern could have been interpreted as evidence that the fetus had Turner’s syndrome, a comparison of the ratios between HPRT and D21S1411 products (1.8 to 1:1) revealed that it was from a rare case of normal female homozygous for all X-specific markers employed. This diagnosis was later confirmed by conventional cytogenetic analysis.

The multiplex QF-PCR assay of another amniotic fluid sample showed single fluorescent peaks for five chromosome 21 STR. In this case the ratio between the HPRT and D21S1411 markers revealed a normal diploid chromosome 21 complement. The rare possibility of uniparental disomy for this chromosome was also excluded by testing both parents with the same markers and demonstrating the presence of a common allele for all sequences compatible with the homozygosity observed in the fetus.

Discussion

Rapid prenatal diagnoses by QF-PCR of Turner’s syndrome (45,X) can be performed using a set of STR, including the X22, on the PAR 2 region of the X and Y chromosomes and at least three more amelogenin product (Table I, Figures 2 and 3). Two amniotic and 11 blood samples from subjects with pure Turner’s syndrome (45,X) showed only one dose of HPRT marker and two of chromosome 21-specific D21S1411 in absence of the Y chromosome specific product (Tables I and II, Figures 2 and 3).

Figure 3. Electrophoretogram showing eight small tandem repeat (STR) markers and AMXY detecting a Turner’s syndrome fetus. All autosome markers are informative for normal chromosome copy number. The X22 and HPRT show only one allele in absence of the Y-derived product of the amelogenin. Highlighted is the HPRT peak in single dose if quantified using the D21S1411 alleles (arrows).
X-linked markers such as HPRT, DXS6803 and DXS6809. The QF-PCR pattern of samples from 45,X fetuses will show only single X-derived peaks of fluorescent activity in absence of the Y-specific AMXY product (Cirigliano et al., 1999, 2001a). However, according to the heterozygosity of the X/Y STR used, it is expected that a very small proportion of normal females (~0.2%) could be homozygous for all the sex chromosome markers employed (Cirigliano et al., 2001b). Consequently, although very low, there is the risk that a normal female fetus may be indistinguishable from a fetus affected by Turner’s syndrome. The only way to further reduce the possibility of misinterpretation of the QF-PCR pattern is to use an autosomal sequence as an internal control for X chromosome quantification.

In preliminary studies, when the ratios of fluorescent activities between several X-linked and autosomal polymorphic sequences were compared, only one chromosome 21-specific STR (D21S1411) was found to provide an accurate measure of the X chromosomes present in a sample. These findings were confirmed when a further group of 128 amniotic fluids were tested. All 64 samples from female fetuses produced HPRT/D21S1411 ratios documenting the presence of double doses of the X-chromosome marker. Samples from male fetuses showed instead ratios close to 1 to 1:1 or 1 to 2, as expected. All samples from fetuses or adults with pure Turner’s syndrome (45,X) produced HPRT/D21S1411 ratios 1 to 1:1 or 1 to 2, thus documenting the presence of a single X chromosome in the absence of a Y-specific marker.

Only one STR on chromosome 21 (D21S1411) co-amplified with the X-linked HPRT was found to provide an accurate measure of the X chromosomes present in a sample. It was expected that very few autosomal sequences could amplify with the same PCR efficiency of an X-linked marker because, in contrast to quantification between two alleles of an STR (amplified with the same PCR primers), the quantification between unrelated sequences, generated from different primer pairs, can be hampered by the dissimilarity of PCR curves or by artefacts such as pronounced preferential amplification of the shorter sequence for amplicons of greatly differing lengths.

The main advantage of using this approach is demonstrated by the analysis of two rapid prenatal tests performed in the course of this study. In one case, the QF-PCR was characterized by the presence of only single peaks for X22, DXS6803, DXS6809 and HPRT as well as the absence of the Y-specific AMXY product. Consequently, the fetus was suspected to have a 45,X chromosome complement. However, the analysis of the ratios between HPRT and D21S1411 showed a normal double dose of the X chromosome from a rare normal female homozygous for all the X-linked STR employed.

The HPRT product can also be used as an internal control to quantify the D21S1411, for the assessment of chromosome 21 copy number. This was shown in a rare case of amniotic fluid retrieved from a fetus homozygous for five chromosome 21 STR.

One of the advantages of this new method for the X chromosome dosage is that no markers are required in addition to that already included in the same multiplex QF-PCR assay developed to detect common numerical variations involving chromosomes 21, 18, 13, X and Y (Cirigliano et al., 2001b).

Prenatal detection of 47,XXY and 47,XYY fetuses can readily be performed using the pseudautosomal X22 and the modified AMXY markers that are also included in the present multiplex assay.

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References

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