Quantitative Real-Time PCR for Non-invasive Rapid and Reliable Diagnosis of Turner Syndrome

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Abstract. The aim of the study is a method of non-invasive for prenatal diagnosis, which is based on quantitative real-time PCR. Materials and Method: Genomic DNA were extracted from peripheral blood lymphocytes of Turner syndrome subjects (n=15), and normal controls (n=10) that were tested by quantitative real-time PCR. This technique was applied by a MGB TaqMan probe based real-time PCR assay for rapid diagnosis of monosomy X-linked status in Turner syndrome. In the present study, we have measured and determined the gene dosage of FVIII (target gene on X chromosome) relative to PMP22 (reference gene on 17p11.2). Results: The formula ratio = 2^{-ΔΔCt} applied for the calculation of the FVIII/PMP22 ratio. The
gene dosage ratio was quel 1.005±0.00342 and 0.4867±0.00797 for normal individuals and Turner syndrome, respectively.

**Conclusion:** This technique, including quantitative real-time PCR can be used as a rapid and standard and reliable method for rapid detection of prenatal diagnosis of monosomy X-linked

**Keywords:** Turner syndrome, quantitative real-time PCR, the gene dosage

**INTRODUCTION**

Turner syndrome is a genetic disorder resulting from the loss of the entire or a part of the X-chromosome, occurring in approximately 1:2500 female births[3,7]. In the most typical case, an individual with Turner syndrome has only one X chromosome (monosomy), less commonly a partial second X chromosome is present but is not structurally intact (e.g. ring chromosome or deleted chromosome). And in a minority of cases, some of the cells in the body have one X chromosome, but other cells have two or more (referred to as mosaicism)[5,9]. Possible symptoms in young infants include: swollen hand and feet, wide and webbed neck. The other symptoms may be seen in older females: anabsent or incomplete development at puberty, including spare public hair and small breast; braod, flat chest shaped like a shield, drooping eyelids; dry eyes; infertility; no periods (absent masturation); short height; vaginal dryness [3,5,7,9].

Turner syndrome can be diagnosed at any stage of life. It is better that is diagnosed before birth by prenatal testing[3,5,7,9], such as real-time PCR, cytogenetinc techniques. Although the cytogenetic method is a standard technique, but it is an invasive method that expose to danger of abortion. In order to prenatal diagnosis X monosomy, Turner syndrome, real-time PCR technique is a safe procedure[8].

**MATERIALS AND METHOD**

According to standard salting out protocol[4], genomic DNA were extracted from 2ml of peripheral blood lymphocytes of Turner syndrome cases (n=15) and normal control case (n=10). Using NanoDrop ND-1000 spectrophoto-meter (BioRad, USA) was measured the concentration and quality of DNA at 260 and 280 nm. DNA samples with the A 260/280 ratios of more than 1.7 were selected for further analysis. Samples aliquots were stored at -20°C and fresh working solutions (10ng/µL) were prepared immediately before each PCR run.
Quantitative real-time PCR: FVIII (human coagulation factor FVIII) gene located on X chromosome and PMP22 (peripheral myelin protein) gene located on 17p11.2-12 were selected as target and reference genes, respectively. Primers and probes were designed by Primer Express software Ver.3.0 (Applied Biosystem, Foster city, CA). Using BLAST tool (www.ncbi.nlm.nih.gov/blast), we underwent selected primers and probes to keep away from any significant homology with other known nucleotide sequences. PMP22 probe was conjugated at the 5’end with the reporter dye VIC. FVIII specific probe was synthesized with the reporter dye 6-carboxyfluorescein (FAM) covalently linked to the 5’ end (Applied Biosystem, UK) and TAMRA end 3’. PMP22 probe was minor groove binder DNA probes.

The characteristics of the primers and probes used in this study are summarized in table1.

PCR reaction master mix was purchased from Applied (BioSystems,UK). The master mix was optimized for TaqMan probe based reactions and consist of AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP Passive Reference, and optimized buffer components. PCR was taken out in optical grade 96-well plates (MicroAmp, Applied Biosystems, Singapore) at reaction volume of 20µl containing 10µl TaqMan universal master mix (Applied Biosystems, UK), 1µl of each primer, 0.7 µl of the specific probes and 5ng of genomic DNA. Reaction mixture was kept at 50°C for 2 min (for optimal AmpErase UNG activity), at 95°C for 10 min (for deactivation of AmpErase UNG and activation of AmpliTaq Gold), followed by 40 cycles consist of 95°C for 15 seconds and 60°C for 1 min (for probe/primer hybridization and extension). Each real-time PCR run contained three normal control sample in triplicate. To calculate the PCR efficiency of each gene, serial dilutions of the template DNA were prepared. Real-time was run for FVIII (target) and PMP22 (reference) gene in separate tubes, simultaneous using concentrations of 200, 100, 50 and 25 ng/µl DNA per reaction and run in triplicates for drawing standard curves then repeated for gene dosage analysis experiments, at least twice.

Table 1: Characteristics of the primers and MGB-TaqMan probes used in the Real-time PCR assays

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Reporter dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII F</td>
<td>GAACGTGCATGGGACTATATGCAAAG</td>
<td>None</td>
</tr>
<tr>
<td>FVIII R</td>
<td>CCTTCCTCCAAGCAGACTTACATC</td>
<td>None</td>
</tr>
<tr>
<td>FVIII P</td>
<td>TCTCGGTAGGCTGCTGTGGACG</td>
<td>FAM-TAMRA</td>
</tr>
<tr>
<td>PMP22F</td>
<td>GGAGGAGAGAGAGCCTGTAATGC</td>
<td>None</td>
</tr>
<tr>
<td>PMP22R</td>
<td>GTCCACATGCACACAGAAACG</td>
<td>None</td>
</tr>
<tr>
<td>PMP22P</td>
<td>TGCTAAGGTTGAGTTTCAT</td>
<td>VIC-MGB</td>
</tr>
</tbody>
</table>
**Data analysis:** Quantitative analysis was performed using the measurement of Ct values during the exponential phase of amplification. Analysis of data was done by the formula: $\text{Gene dosage ratio} = 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = [mCt_{F_{VIII} \text{ gene (test sample)}} - mCt_{PMP22 \text{ gene (test sample)}}] - [mCt_{F_{VIII} \text{ gene (test sample)}} - mCt_{PMP22 \text{ gene (test sample)}}][2]$. The Ct parameter was defined as the cycle number at which the amplification curve passed a fixed threshold line. In each assay, mCt was the mean Ct value of triplicate amplification.

Relative quantity of F_{VIII} gene was determined by comparative Ct method and ΔCt was calculated as the difference between the Ct values of the F_{VIII} gene and the Ct value of PMP22 gene of the same sample. Three normal samples were used as calibrator in each experiment and the gene dosage ratio were determined relative to the mean ΔCt value of the samples.

Data processing was performed by the ABI Prism 7300 Sequence Detection System and the SDS software Ver.1.2.3. (Applied Biosystems, UK). Statistical analysis and graph preparation were performed by Microsoft Excel 2007. The $\rho$ value of $<0.01$ was considered statistically significant for t-Test analysis.

**RESULTS**

Validation of Real-time PCR Assays: validation experiments were performed to determine the amplification efficiencies of F_{VIII} and PMP22 genes. Briefly, the log concentration of serially diluted template DNA was plotted against the corresponding Ct values acquire for each gene.

The slope of the best fit lines were within the acceptable range of -3.6<slope<-3.1 (http://www.gene-quantification.de), where slope of -3.3 corresponds to PCR efficiency of 100%. The slope for F_{VIII} and PMP22 genes were -3.149 and -3.190, respectively (Figure 1). The consistency of the PCR reactions through a wide range of template DNA concentrations (6-100ng) were assayed using plotting ΔCt values (Ct_{F_{VIII} \text{ gene}} - Ct_{PMP22 \text{ gene}}) against log amount of input DNA. The absolute slopes of the best fit lines were ≤0.1 for both genes, which indicated the validity of the ΔΔCt relative quantitation.
Discrimination Between Normal and Monosomy X-linked Samples: Diagnosis of monosomy X-linked was revealed by cytogenetic methods such as karyotype and G-banding. As expected from validation experiments, the amplification curves of FVIII and PMP22 genes crossed the threshold line at approximately the same point in normal samples (figure 2). This was showed to ΔCt values of 0-0.5 cycle with the mean value of 0.3±0.1 in date analysis. Using the formula: Gene dosage ratio=$2^{-\Delta\Delta C_{t}}$, the FVIII/PMP22 ratio was calculated for each sample. The mean ratio was obtained in both groups: 1.005±0.00342 for normal and 0.4867±0.00797 for monosomy X-linked cases.
DISCUSSION

In order to prenatal diagnosis is needed a reliable and rapid and non-invasive assay. Cytogenetic methods is a constancy and standard technique, but that is invasive method, in particular, in high risk pregnant women with gestational age, and needs two-weeks period for cell culture and subsequent analysis has proven to be associated with considerable parental anxiety and medical problems in those situations requiring therapeutic intervention [8]. Although molecular techniques were innovated such as QF-PCR and FISH, certain problems still are insolvable.

Aiming to develop a molecular method for diagnosis of Turner syndrome because early recognition allows appropriate therapy for short stature, puberty and also detection of associated malformation, minimizing sequels [6]. In this assay, it was validated a multiplex quantitative real-time PCR assay using MGB type TaqMan probes. Rocha et al.[6], recently, have applied real-time PCR, and, have used GAPDH gene as control (reference) gene, which have numerous pseudogenes (≈400 copies) on human genome. This might cause assay errors due considerable difference between target and reference gene copy number. Despite, we have used PMP22 gene as reference gene, which has no pseudogenes, to normalize FVIII gene dosage. Any deletional mutation or gene copy variation of this gene will result in obvious phenotypical outcome so that normal individual would certainly have intact copy of the gene [1].

We have implemented the \( \Delta \Delta C_{t} \) method instead of using standard curve method to determine target/reference gene dosage. Relative copy number of the target gene was calculated by comparative threshold cycle (\( \Delta \Delta C_{t} \)). Rocha et al. reported another real-time PCR assay in which the gained gene dosage ratio for Turner syndrome to normal subjects were entirely far from ideal values (0.5 vs theoretically expected ratio of 0.7). At least in part, this could happen, because of unequal PCR efficiencies. In our side, the mean gene dosage ratio of monosomy X-linked sample relative to normal sample was 0.4867±0.00797. In this present study, design and quality of the probes is important. Being generally dual-labeled with a fluorescent dye at 5’ end and a quencher dye at 3’ end. It might be kept the probe Tm (8-10°C) higher than primers Tm, the length of the probe should be longer than primers. This would result in highbackground fluorescence due to leakage of the fluorescent signal emitted by the reporter dye. Overcoming to this problem, it is recommended to use MGB TaqMan probes with a special non fluorescent quencher which binds to the minor groove of the template DNA.
REFERENCES


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