SATB2 Haploinsufficiency in Patients with Cleft Palate

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Abstract: De novo translocation interrupting the transcription unit of SATB2 gene has been associated with cleft palate only (CPO). We tested for the presence of the copy number of SATB2 gene in a sample of 92 patients with CPO using a quantitative real-time PCR approach. In one patient (1%, 95% CI = 0.2% – 6%), a 19 Mb de novo deletion encompassing the SATB2 gene was detected. These results suggest that SATB2 gene deletions do not play an important role in the etiology of cleft palate.

Keywords: SATB2 • Haploinsufficiency • Deletion • Cleft palate • Real time PCR

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1. Introduction

Cleft palate is a common birth defect in humans, with a reported incidence of 1 in 1000 [1]. Statistical analysis of the chromosome deletion database pointed to the 2q32-33 region as one of the three regions within the genome for which haploinsufficiency results in cleft palate [2]. The de novo cleft palate associated translocation in the 2q32-q33 region has been shown to interrupt the transcription unit of the SATB2 gene [3]. SATB2 gene mutation analysis of 70 biologically unrelated patients with cleft palate did not reveal any coding region variants, but deletions could not be excluded [3]. Therefore, we decided to test the copy number of SATB2 gene in patients with cleft palate.

2. Material and Methods

We used a quantitative real-time PCR approach to search for the copy number of the SATB2 gene in 92 pediatric patients with cleft palate. Samples were obtained as a part of the EUROCRAN study. Written informed consent was obtained from patients’ parents prior to their participation in the study.

We developed single tube real-time quantitative PCR assay for rapid determination of gene dosage. This method involves a multiplex reaction using a FAM-labelled DNA minor groove binder (MGB) probe derived from the tested locus (SATB2) and a VIC-labelled MGB probe from the RNase P gene as internal reference. The copy number of the tested locus was determined by the comparative threshold cycle method (ΔΔCt) [4]. Each sample was run in triplicate. The number of cycles (Ct) at which the amplification plot representing the fluorescence emission of the reporter dye passed a fixed threshold, was determined for all PCR reactions. The threshold was set automatically within the logarithmic phase. The starting copy number of the unknown samples was determined relative to the known copy number of the calibrator sample using the formula:

\[ \Delta \Delta C_t = (\Delta C_t \text{ RNase } P \text{ (calibrator sample)}) - \Delta C_t \text{ SATB2 gene (calibrator sample)}) - (\Delta C_t \text{ RNase } P \text{ (unknown sample)}) - \Delta C_t \text{ SATB2 gene (unknown sample)}) \]

The relative gene copy number was calculated by $2^{(-\Delta \Delta C_t/a)}$, where $s$ represents the difference of the mean

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standard deviation (SD) of the Ct values of SATB2 gene and RNase P.

PCR was carried out using an ABI Prism 7000 sequence detection system and 96-well MicroAmp optical plates. The PCR was performed in total of 25 μl, containing 100 ng of genomic DNA, 12.5 μl of 2 X Taqman Universal PCR Master Mix, 1.25 μl PCR master mix for RNase P (TaqMan®RNase P Control Reagents Kit, part number 4316844) and 1.25 μl of PCR Reaction Mix 20X (Assays-by-Design™, part number 4332078) for SATB2. PCR conditions were 2 min at 50ºC, 10 min at 95ºC, 40 cycles consisting of 15 sec at 95ºC, and 1 min at 60ºC. The following oligonucleotides were used for the analysis:

SATB2 forward primer: acttagaccccattctttagcatttctt;
SATB2 reverse primer: acactgcagcagccattct;
SATB2 Taqman probe: aagctcccacaagaccca.

Any sample in which a deletion was found, and the samples of the parents of the patient with the deletion, were re-tested. Real-time PCR using SYBR® Green I Dye was performed according to the instructions (SYBR Green PCR Master Mix and RT-PCR Reagents Protocol (PN 4310251D). Three pairs of primers encompassing the region of introns 4/5 and 5/6 of SATB2 gene were used:

1. SATB2int4/5 F: gaaacgcagatatgggagcg,
   SATB2int4/5 R: agcatttggagctcatgaagc,
2. SATB2int5/6a F: caagacccaagaatggctgc,
   SATB2int5/6a R: cagggtccctgactgaagagg, and
3. SATB2int5/6b F: ggtagtggtggagcaaac,
   SATB2int5/6b R: ggggtctaagtgaaggaagg.

We determined the size of the deletion using the array-CGH method. Commercially available microarray Agilent Human Genome CGH Microarray 105A (Agilent Technologies, Santa Clara, CA, USA) with 21.7 kb overall median probe spacing was used. DNA restriction, labelling and hybridization were performed according to the manufacturer’s recommended protocols (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis, Protocol Version 5.0, June 2007, Agilent technologies, http://www.chem.agilent.com/Library/usermanuals/Public/G4410-90010_CGH Protocol_v5.pdf). Briefly, 1500 ng of genomic DNA was digested using restriction enzymes AluI and Rsal and fluorescently labelled using the Agilent DNA Labelling kit. After labelling, DNA was denatured and pre-annealed with Cot-1 DNA and Agilent blocking reagent and hybridized for 40 h at 65ºC in an Agilent hybridization oven. Standard wash procedures were followed. The array was scanned at 5 μm resolution using a Tecan scanner, and image analysis was performed using ArrayPro Analyzer (MediaCybernetics). Agilent CGH Analytics 3.4 software was used to identify the deletion boundaries. Commercially available Human Genomic Male DNA was used as a reference DNA (Promega GmbH, Germany).

### 3. Results

Only one SATB2 deletion was found in the population of 92 patients having cleft palate only (Table 1). This represented 1% (95% CI = 0.2% – 6%). Testing the mother and the father of the patient with the SATB2 deletion revealed a normal copy number of the SATB2 gene.

The deletion was confirmed using Real-time PCR with SYBR® Green I Dye.

Array-CGH confirmed an interstitial deletion, and showed that it encompasses not only the SATB2 gene, but includes much larger region of the long arm of chromosome 2, spanning 197.703.588 bp to 216.081.876 bp (UCSC Genome Browser; build hg18, March 2006) in the 2q33.1-q35 region.

The patient with the deletion was the first child of healthy parents, born at 37 weeks with a birth weight of 2650 g (25th centile) and birth length of 48 cm (25th centile). At the age of 4 months she was growth-retarded (weight and length <3rd centile) and hypotonic. She had low set, cup-shaped ears; a small mouth; a cleft palate; and an umbilical hernia.

### Table 1. SATB2 ratios detected by real-time PCR.

<table>
<thead>
<tr>
<th>Samples</th>
<th>SATB2 ratio</th>
<th>ΔΔCt lower range**</th>
<th>ΔΔCt upper range**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=91)</td>
<td>0.81-1.19</td>
<td>0.76</td>
<td>1.25</td>
</tr>
<tr>
<td>Deletion (n=1)</td>
<td>0.48</td>
<td>0.40</td>
<td>0.56</td>
</tr>
<tr>
<td>Father (n=1)</td>
<td>1.00</td>
<td>0.96</td>
<td>1.04</td>
</tr>
<tr>
<td>Mother (n=1)</td>
<td>0.96</td>
<td>0.9</td>
<td>1.02</td>
</tr>
</tbody>
</table>

*ΔΔCt ratio (2^ΔΔCt) defines the SATB2 gene copy number
**ΔΔCt upper range and ΔΔCt lower range (2^-ΔΔCt±s) includes the standard deviation (s). s represents the difference of the mean standard deviations SD of the Ct values of SATB2 and RNase P.