SATB2 Haploinsufficiency in Patients with Cleft Palate

Research Article

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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*

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 loci was determined by the comparative threshold cycle method (ΔΔCt) [4]. Each sample was run in triplicate. The number of cycles (C_t) 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:

ΔΔCt=[ΔCt *RNase P* (calibrator sample)-ΔCt *SATB2* gene (calibrator sample)]-[ΔCt *RNase P* (unknown sample)-ΔCt *SATB2* gene (unknown sample)]

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

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

Samples	SATB2 ratio		
	ΔΔCt*	ΔΔCt lower range**	ΔΔCt upper range**
Normal (n=91)	0,81-1,19	0,76	1,25
Deletion (n=1)	0,48	0,40	0,56
Father (n=1)	1,00	0,96	1,04
Mother (n=1)	0,96	0,9	1,02

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

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[SM], 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. *SATB2*int4/5 F: gaaacgcagatatgggagcg, *SATB2*int4/5 R: agcatttggagctcatgaagc,

2. *SATB2*int5/6a F: caagacccaagaatggctgc, *SATB2*int5/6a R: cagggtccctgactgaagagg, and

3. *SATB2*int5/6b F: ggatgagtgggatgacaaac, *SATB2*int5/6b 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 RsaI 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 65°C 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.

4. Discussion

The *SATB2* gene has been previously reported as the cleft palate candidate gene [3]. There is strong evidence supporting an important role of this gene in palate development: (1) cleft palate is a very common finding in patients with a 2q32-q33 deletion [5]; (2) *de novo* translocation interrupting the transcription unit of *SATB2* gene has been reported to be associated with cleft palate only [3]; (3) whole mount *in situ* hybridization to mouse embryos shows site- and stage-specific expression of *SATB2* in the developing palate [3]; and (4) mice jaw development is *Satb2*-dosage sensitive [6].

The susceptibility of the 2q32-q33 region for the chromosomal rearrangements (interstitial deletions, translocations) is probably associated with genomic features leading to recombination errors. Although segmental duplications, which serve as a substrate for non-allelic homologous recombination, were not detected in the 2q33 region (UCSC, March 2006, Segmental Dups Track Setting), other mechanisms, such as non-homologous end joining might constitute a stimulatory effect for deletion formation [7].

Three genes *TBX22*, *IRF6* and *SUMO1* have been identified as playing a role in the etiology of isolated cleft palate, with TBX22 mutations found in up to 4% of unselected cleft palate cases [8-10].

The decision to categorize cleft palate as non-syndromic or syndromic might be very difficult, particularly if it is made at a very early age. In our patient with an *SATB2* deletion, clef palate was firstly categorized as non-syndromic, but later in the child's development, dysmorphic features and growth and developmental retardation were noticed, which were in concordance with the detected deletion.

The deletion encompasses 80 RefSeq genes (UCSC, March 2006, Genes and gene prediction tracks, status reviewed and validated) and two of them, *SATB2* and *SUMO1*, have already been reported as the cleft palate candidate genes [3,11]. Two patients with *SUMO1* haploinsufficiency have been described in the literature: a girl with cleft lip and palate who carried a balanced translocation disrupting *SUMO1* gene [11], and a child with CPO with 124,5 kb deletion including genes *SUMO1* and *NAP5/NOP58* [10]. Moreover, animal studies have confirmed that *Sumo1* gene haploinsufficiency results in cleft lip and palate [11]. Importantly, many proteins involved in the palatogenesis are sumolyated. One of them is also SATB2; the SUMO modification of this gene appears to modulate its activity as a transcriptional factor [12].

We concluded that *SATB2* gene deletions do not play an important role in the etiology of cleft palate; however, given that *SATB2* and *SUMO1* genes are the only known candidate genes for cleft palate in the deleted region, that might support their contribution to clefting.

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