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PAX6 polymorphisms in 20 Chinese children with supernumerary teeth in the maxillary incisor area

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Aim. To search for variants of *PAX6* gene in patients with nonsyndromic supernumerary teeth in the maxillary incisor area (NSST) and compare genotypes and allele frequencies of the detected polymorphisms between the patients and controls.
Design. Twenty children with NSST and 31 controls were included. Genomic DNA was extracted from buccal epithelial cells of each individual. Sequencing analysis of all exons and exon/intron boundaries of *PAX6* gene were performed in patients. Genotypes and allele frequencies of the single nucleotide polymorphisms detected in patients were compared between the two groups using chi-square tests.
Results. Of the 20 patients examined, six showed heterozygous for rs667773 and rs3026393 simultaneously. Among them, four possessed two supernumerary teeth and the other two possessed one. Another six patients showed heterozygous for rs3026393, five of which possessed only one supernumerary tooth and the other one possessed two. Of another six patients with homozygous rs3026393, three possessed one supernumerary tooth and the other three possessed two. The distributions of genotypes and alleles frequencies of single nucleotide polymorphisms rs667773 and rs3026393 showed no significant difference between the two groups.
Conclusions. The present study did not find evidence of PAX6 polymorphisms being associated with supernumerary teeth in the population studied.

Introduction

Supernumerary teeth are those that are additional to the normal complement of human dentition[1]. These teeth can be an isolated finding or may be part of developmental disorders. The prevalence of supernumerary teeth ranges from 0.1 to 3.6% in different ethnic populations, and most of these teeth located in the maxillary incisor area[1,2]. Several theories were put forward in the literature to explain the occurrence of supernumerary teeth. The most widely accepted theory is the combination of genetic and environmental factors in human odontogenesis[3,4]. To date, several genes associated with supernumerary teeth have been identified: *NHS* gene involved in Nance-Horan syndrome[5] (MIM 302350), *RUNX2* in cleidocranial dysostosis[6,7](MIM 119600), *TRPS*I in trichorhinophalangeal syndrome[8,9] (MIM 190351), and other genes involved in corresponding syndromes. The aetiology of supernumerary teeth, however, remains unknown, especially in the aspect of genetic factors of nonsyndromic supernumerary teeth.

The mouse model provides insight to explore the complex genetics of tooth development in human dentition. Recent studies have reported that supernumerary teeth were observed in paired box gene 6 (*Pax6−/−*) mouse/rat fetuses in their upper incisor region[10–12]. Differentiation of ameloblasts and odontoblasts and formation of predentin could be seen in the supernumerary teeth as well as in the normal incisors[11,12]. Evidence from these animal studies suggests that Pax6 may play an important role in the development of maxillary incisors and might associate with the occurrence of supernumerary teeth in the maxillary incisor area.

PAX6 gene (Genbank accession number: NG_008679) spans a 33-kb region in 11p13 and contains 14 exons, including an alternatively spliced exon5a that encodes 14 amino

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acids, and encodes a protein of 422 amino acids. PAX6/Pax6 is a transcription factor containing the paired and homeobox DNA-binding domains and is essential for the development of tissues including the eyes, central nervous system, and endocrine glands of vertebrates and invertebrates[13]. Heterozygous deletions or loss-of-function mutations in *PAX6* can result in ocular anomalies[14,15]. PAX6 is expressed in the pancreas, and mutations in *PAX6* may result in abnormal glucose metabolism and defective processing of proinsulin[16]. Heterozygous mutations in *PAX6* may also result in auditory processing deficits related to corpus callosum anomalies[17]. Only two articles in the literature described dental anomalies in individuals with mutations in PAX6, which were the high-arched palate and dental crowding[18,19].

Up to the present, relationships of PAX6 and supernumerary teeth have not been studied in human subjects. In this study, sequencing analysis of all exons and exon/intron boundaries of *PAX6* gene were performed in 20 Chinese children with nonsyndromic supernumerary teeth in the maxillary incisor area (NSST), with the aim of searching for variants of *PAX6*. The single nucleotide polymorphisms (SNPs) detected in patients were also evaluated in 31 control individuals to compare genotypes and allele frequencies between the two groups.

Material and methods

Study participants

Twenty unrelated Chinese children (18 boys and two girls; aged from 5 to 13 years old) referred to the Department of Paediatric dentistry at Peking University School and Hospital of Stomatology were included in this study. The diagnosis of NSST was made on the basis of oral examination and the periapical radiograph and/or panoramic radiograph. All the participants have no complaints of abnormality in their head, eyes, ears, nose, throat, thyroid, trunk, and extremities. Individuals with cleft lip and palate, retention of primary teeth and delayed eruption of permanent tooth, congenital absence of teeth and disturbances in form of teeth were excluded.

Thirty-one healthy individuals (14 males and 17 females; aged from 11 to 39 years old) without supernumerary teeth recruited from the Department of orthodontics at Peking University School and Hospital of Stomatology were used as control. The exclusion criteria were as described earlier.

This study was approved by the Ethics Committee of Peking University Health Science Center (approval number: IRB00001052-10056) and carried out according to the guidelines of the World Medical Association Declaration of Helsinki (version, 2002 http://www.wma.net/e/policy/b3.htm). Informed consent was obtained from all individuals and their parents.

DNA sequence analysis of the PAX6 gene

To obtain buccal epithelial cells, buccal swabs were placed in the patients' mouth and rotated against the inside of the cheek for approximately 20 rounds (Jinzhang, Tianjin, China). Then, the swabs were dried at room temperature overnight, placed in the swab holder, and stored at −20°C. Genomic DNA was extracted from buccal swabs of each individual with the use of a TIANamp Swab DNA mini kit (Tiangen, Beijing, China) according to the manufacturer's instructions.

All the exons and exon/intron boundaries of *PAX6* were amplified by polymerase chain reaction (PCR). PCR was performed with an initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 53.7–62.9°C (optimized for each primer pair) for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min, with GoTaq® DNA Polymerase (Promega, Madison, WI, USA). PCR products were purified and then sequenced in an ABI 3730 XL automatic sequencer. The primers used for amplification were described previously (Table 1).

SNPs analysis in control group

To compare genotypes and allele frequencies of SNPs between patients and controls, two SNPs detected in patients were evaluated in 31 control individuals. Collection of buccal epithelial cells, extraction of genomic DNA, and

Table 1. Primers used for polymerase chain reaction amplification and sequencing of *PAX6* gene (Genbank accession number: NG_008679).

Primer name	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)
Exon1a-F	AAAACTAGTCGGCGCAGAGCT	432	56
Exon1a-R	TCCCTCAGTAACTCGCTTCCAT		
Exon 1-F	CAAACGGACCAATTGCACCA′	449	58
Exon 1-R	GGTTGGTGTGTGAGAGCAATTCTC		
Exon 2-F	TCACCAATCAGCATAGGTGTGC	466	58
Exon 2-R	TAGAAAGTTTGGGCTCCTGCG		
Exon 3-F	TGACTGAGCCCTAGATGCATGTG	364	56
Exon 3-R	TCCCCAATCTGTTTCCCCTACAT		
Exon 4-F	GAACGGAGATTCTCCTGTCCTA	363	53.7
Exon 4-R	CAGTATCGAGAAGAGCCAAGC		
Exon 5-F	AGGATGCATTGTGGTTGTCTCCTC	405	56
Exon 5-R	TGGGGGGGTCCATAATTAGCA		
Exon 5a, 6-F	GTTTTGATGCATCTTCAGGCAG	707	56
Exon 5a, 6-R	AGTCAGGGCATTCCTCTCTGTT		
Exon 7-F	GGTGTATCTGCAAATCCACCCA	470	58
Exon 7-R	CAATGTGGTCGATGTGTCCCA		
Exon 8-F	AAGGCTGACAGTTACCTTGGGAA	398	56
Exon 8-R	TCTTCTATGCAAAGGGCCCTG		
Exon 9-F	TTGGTTGGAGGTAATGGGAGTG	334	59
Exon 9-R	TGGCAGCAGAGCATTTAGCAG		
Exon 10, 11-F	CTGCTAAATGCTCTGCTGCCA	614	59
Exon 10, 11-R	CGACTTGACTGGTCAAGCCAAT		
Exon 12-F	AGCTCGAGGCCCAATCTTAGAT	436	56
Exon 12-R	AGGGACAAGGAAAGCAAGGAGT		
Exon 13-F1	CTTTTCCTTTGGATTGGGGTG	654	60.9
Exon 13-R1	CACAGATCAAACATCCATCCAGTC		
Exon 13-F2	CCTATAAATTTGTATTCCATGTC		
Exon 13-R2	CTTGGCCAGTATTGAGACATATC		

Primers used in this study were described previously. Exon 13-F2 and Exon 13-R2 were used only for sequencing[20].

performance of PCR were done as described earlier. Genotyping of rs667773 was carried out by restriction digestion of the PCR products with BccI (NEB, Beverly, MA, USA) and analysed by 2% agarose gel electrophoresis. For rs3026393, the PCR products were purified and sequenced.

Statistical methods

All data were processed by SPSS software (16.0; SPSS Inc., Chicago, IL, USA). Genotypes and allele frequencies in patients and controls were compared with chi-square tests. *P*-values <0.05 were considered as statistically significant.

Results

Clinical details

Oral examination and a periapical radiograph and/or a panoramic radiograph were taken to confirm the diagnosis of NSST for the 20 study individuals. Of the 20 patients examined, twelve possessed a single supernumerary tooth in the upper incisor region; eight children possessed two supernumerary teeth. All the supernumerary teeth were dysmorphic compared with normal incisors. No other tooth abnormalities were observed except supernumerary teeth in maxillary incisor area.

Table 2. Genotypes of two single nucleotide polymorphisms in 20 patients with nonsyndromic supernumerary teeth in the maxillary incisor area.

rs667773 (IVS9-12C>T)	rs3026393 (IVS12 + 43T>G)	Number of supernumerary teeth	Number of patients
C/T	T/G	1	2
		2	4
C/C	T/G	1	5
		2	1
C/C	G/G	1	3
		2	3
T/T	T/T	1	1
C/C	T/T	1	1

DNA sequence analysis of the PAX6 gene

Genomic sequences of *PAX6* from 20 participants with supernumerary teeth were analysed by direct sequencing. Whereas sequencing of PAX6 coding DNA did not reveal any putative aminoacid change, two already known SNPs were found in noncoding regions (Table 2). Of the 20 individuals, six showed heterozygous for IVS9-12C>T (SNP-rs667773) and IVS12 + 43T>G (SNP-rs3026393) simultaneously. In these six patients, four possessed two supernumerary teeth and the other two possessed one. Another six patients showed heterozygous for rs3026393 only, five of which possessed only one supernumerary tooth and the other one possessed two. Of another six patients with homozygous rs3026393, three possessed one supernumerary tooth and the other three possessed two (Fig. 1).

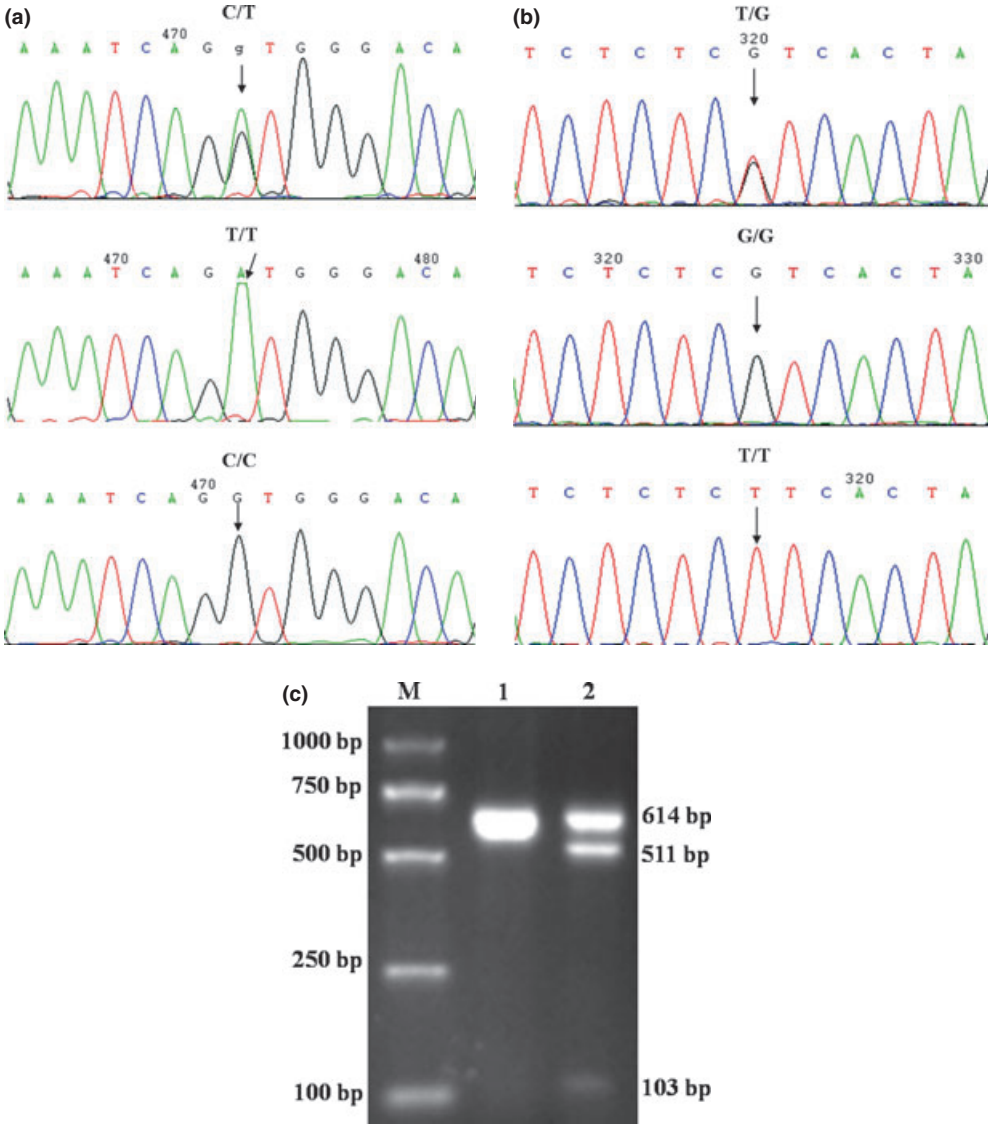

Fig. 1. Analysis of *PAX6* polymorphisms. (a, b) DNA sequencing chromatogram showed the genotypes of rs667773 and rs3026393 in Intron 10 and Intron 13, respectively. (c) Polymerase chain reaction–restriction fragment length polymorphism analysis of the rs667773 in controls. The Exon 10/11 polymerase chain reaction products (614 bp) were digested with BccI. Homozygous C alleles were represented by a DNA band with size at 614 bp. Homozygous T alleles were represented by two DNA bands with sizes at 511 and 103 bp. Whereas heterozygous genotype displayed a combination of both alleles (614, 511 and 103 bp). Lane 1: homozygous CC; lanes 2: heterozygous CT.

Analysis of two SNPs in two groups

To explore preliminarily the relationship between rs667773 and rs3026393 and susceptibility to supernumerary teeth, genotypes and alleles frequencies of two SNPs in patients and controls were compared. Of the 31 control individuals, four showed heterozygous for rs667773. Another four individuals showed heterozygous for rs667773 and rs3026393 simultaneously. Eight individuals showed heterozygous and ten showed homozygous for rs3026393. Using the chi-square test, distributions of genotypes and alleles frequencies of SNPs rs667773 and rs3026393 showed no significant difference between the two groups (Table 3).

Discussion

Twenty Chinese children with NSST were included to screen variants in the exons and potential regulatory regions of the *PAX6* gene. The distributions of genotypes and alleles frequencies of SNPs rs667773 and rs3026393 were compared between patients and controls.

Supernumerary teeth appear with a higher frequency in males than in females with literature reporting rates of between 2 : 1 and 6 : 1 depending on the respective population[21–24]. *PAX6* gene is located in an autosome (11p13), so sex ratio of samples would not have effect on PAX6 sequencing analysis in our study.

Table 3. Comparison of genotypes and alleles frequencies of rs667773 and rs3026393 between patients and controls.

	Patients (n = 20), %	Controls (n = 31), %	P
rs667773			
Genotypes			
C/C	13 (65.0)	23 (74.2)	0.482
C/T, T/T	7 (35.0)	8 (25.8)	
Alleles			
C allele	32 (80.0)	54 (87.1)	0.336
T allele	8 (20.0)	8 (12.9)	
rs3026393			
Genotypes			
T/T	2 (10)	8 (25.8)	0.243
T/G	12 (60)	12 (38.7)	
G/G	6 (30)	11 (35.5	
Alleles			
T allele	16 (40.0)	28 (45.2)	0.607
G allele	24 (60.0)	34 (54.8)	

The chi-square test was used to determine whether significant differences (*P*-value) were observed when patient group was compared with control subjects.

Sequencing of all the exons and exon/intron boundaries of *PAX6* did not show nucleotide alterations in the protein coding sequence in the present study. Our results showed for the first time that the SNPs rs667773 and rs3026393, especially rs3026393, frequently presented in patients with NSST, however. In a study of 164 nuclear families with 170 highly myopic offspring and their parents, the SNP rs3026393 showed significant association with high myopia in Han Chinese nuclear families[25]. But exact function of rs667773 and rs3026393 is unknown.

To assess preliminarily whether rs667773 and rs3026393 were associated with susceptibility to supernumerary teeth, we evaluated these two SNPs in 31 control individuals. Genotypes and alleles frequencies of SNPs rs667773 and rs3026393 showed no significant difference between the two groups. Clinically, we found that patients (four of six) who showed heterozygous for rs667773 and rs3026393 simultaneously tended to possess two supernumerary teeth, however. And patients (five of six) who only showed heterozygous for rs3026393 tended to possess one supernumerary tooth.

A number of transcription factors, signalling molecules, growth factor receptors, and extracellular matrix molecules are expressed in the first branchial arch and involved in the regulation of tooth development[26]. Fibroblast growth factor antagonists Spry2 or Spry4, ectodysplasin (Eda) or its receptor, a bone morphogenic protein, and/or Wnt inhibitor and Runx2 were all found to be associated with supernumerary teeth[27–30]. Functions of these other genes could not be excluded in our patients.

In conclusion, rs667773 and rs3026393 were found for the first time in the patients with supernumerary teeth in the maxillary incisor area. Our results did not reveal any putative aminoacid change, however. The present study did not find evidence of *PAX6* polymorphisms being associated with supernumerary teeth in the population studied.

What this paper adds
- Sequence analysis of *PAX6* gene showed for the first time rs667773 and rs3026393 in the patients with NSST.

Why this paper is important to paediatric dentists
- This study provides information for paediatric dentists who are devoted into searching for genetic factors of supernumerary teeth.

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