Non-syndromic cleft palate: analysis of TBX22 exon 5 gene mutation

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Abstract

Introduction: This study aimed to investigate the mutation of T-box transcription factor TBX22 exon 5 in children with non-syndromic cleft palate. Four mutations in TBX22 exon 5 in X-linked cleft palate with ankyloglossia (CPX) patients had been identified in the previous studies. The study used the syndromic cleft palate susceptibility gene as a candidate gene for more common non-syndromic cleft palate.

Material and methods: A family-based study with parents and their children composing parent-child trios was performed in this research. Twenty children with non-syndromic cleft palate and 38 healthy parents were enrolled. TBX22 exon 5 was examined by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing. The peaks of the sequence diagrams were analyzed using chromas221 and the results of sequencing were proofread using dnastar6.13. The index of the transmission disequilibrium test (TDT) was calculated through McNemar testing.

Results: We have not found the presence of any mutation of TBX22 exon 5 reported in syndromic cleft palate patients in references. The index of TDT was 0.56 and showed no statistically significant difference ($p < 0.05$). No TBX22 exon 5 mutation was found in the 20 children.

Conclusions: Mutation of TBX22 exon 5 is not associated with non-syndromic cleft palate in the population of Jiangzhe areas in China.

Key words: non-syndromic cleft palate, TBX22, transmission disequilibrium test.

Introduction

Cleft lip and/or cleft palate is a common birth defect with a reported prevalence of 1 in 700 births worldwide [1]. The aetiology is complex and is thought to involve both major and minor genetic influences with variable interactions from environmental factors. The embryology of cleft lip and of cleft palate are largely distinct. Where one or more additional features are involved, clefts are referred to as syndromic; according to any associated anomalies, cleft palate is divided into syndromic and non-syndromic cleft palate. Significant progress has been made with the identification of gene mutations by studying syndromic cleft palate lately [2]. Some important findings have recently come from studies involving syndromic forms of the disorder. These include several genes which have now been shown to contribute a major effect to the aetiology. These genes can also be used to demonstrate a significant overlap between syndromic and non-syndromic clefts. It is clear that mutations in these genes may con-

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tribute to a proportion of the non-syndromic cleft cases [3].

X-linked cleft palate with ankyloglossia (CPX) (MIM 303400) is syndromic and inherited as a Mendelian, semi-dominant X-linked disorder affecting male patients and approximately one third of female carriers having been described in several large families from different ethnic origins. Recently the underlying genetic defect in CPX was identified, where unique mutations were found in the T-box-containing transcription factor TBX22. TBX22 mutations underline a chromosome X-linked syndrome with cleft palate and ankyloglossia. The cleft phenotype predominantly affects males, who show variation ranging from a complete cleft of the secondary palate, submucous cleft, or bifid uvula to high arched palate. Although ankyloglossia is a common phenotype associated with TBX22 mutations, its occurrence is not a prerequisite for cleft palate and the two features are independent effects of the loss of TBX22 function. Mutations within families could result in either cleft palate only, ankyloglossia only, or both. The T-box transcription factor TBX22 is essential for normal craniofacial development, as demonstrated by the finding of nonsense, frameshift, splice-site, or missense mutations in patients with CPX. TBX22 mutations are reported in well-defined CPX families and in 4-8% of non-syndromic cleft palate patients. Four mutations in TBX22 exon 5 in CPX patients were identified including three missenses and one small deletion. It has been recently demonstrated that TBX22 makes a significant contribution to prevalence of cleft palate at least in the Brazilian, North American and Thai cohorts. [4-7] The objectives of the study were to investigate whether TBX22 exon 5 mutation might be responsible for the formation of non-syndromic cleft palate in Chinese.

Material and methods

Patients

This was a family-based study with parents and their children composing parent-child trios. Informed consent was obtained from the parents before the study and it was approved by the Ethical Committee of Zhejiang University. We randomly selected 20 cases from 50 patients with non-syndromic cleft palate who were treated in our hospital, excluding single-parent families. A total of 20 children with non-syndromic cleft palate, with the exception of one who had repaired cleft lip, and 38 parents participated in this study. There was a twin in the team. The patients were aged from 12 to 36 months (average: 18.2 ±6.2 months): 9 were boys and 11 girls. The cleft palate patients were divided into a bifid uvula or soft cleft palate group (8 persons) and a soft and hard cleft palate group (12 persons). Their mothers were aged from 22 to 36 years (average: 28.1 ±4.3 years) and fathers aged from 25 to 40 years (average: 30.1 ±4.6 years); they all denied a positive family history except one.

For each patient, a detailed phenotype was obtained. Cleft palate patients with ankyloglossia syndrome, Pierre-Robin syndrome, Stickler syndrome, chromosomal anomalies and kabuki make-up syndrome were excluded from the study.

Amplification and sequencing

Fifty-eight blood samples (1 ml) were obtained. DNA was extracted from blood samples according to standard procedures. Primers were designed for genomic amplification and sequencing of exon 5 as described previously [5]. The primers were as follows: forward: 5'-ACATGGTGGAGGTGGTCAGGA-3'; reverse: 5'-ACATATCCCCTTGTGTAGTA-3'. The PCR reaction system (total 30 μl) contained 2.0 μl of genomic DNA, 3.0 μl of each primer, 1.0 μl of each dNTP, 3.0 μl of Mg^{2+}, 2 U of Taq DNA polymerase, 3.0 μl of 10× buffer.

Each PCR product (10 μl) was subjected to conventional non-denaturing polyacrylamide gel electrophoresis and silver staining before observation and recording the results. The PCR products were purified with Millipore membrane and 3 μl of purified product was sequenced using a MegaBACE 1000 sequencing instrument.

Sequence comparison and analysis

The data of 58 cases of PCR product were measured, and the peaks of the sequence diagrams were analysed using chromas221 (version 2.21 technelysium). The results of sequencing were proofread using dnastar6.13. Statistical tests were based on two-sided probabilities with a significant $\alpha < 0.05$. Baseline differences between parent-child trios were using the transmission disequilibrium test (TDT). Finally, the index of the transmission disequilibrium test (TDT) was calculated through McNemar testing. The TDT is a useful method to detect linkage between a marker locus and a disease susceptible locus. It is not prone to producing false positive results due to population stratification [8].

Results

In the family-based study of 58 samples, we have detected the sequences of the child team ($n = 20$) and the parents team ($n = 38$). Especially, we have checked the sequences of the family with a twin. Sequencing results were aligned with the standard sequence of TBX22 exon 5 in GenBank. We have found no mutations in TBX22 exon 5 in our research, though four mutations were identified in TBX22 exon 5 in CPX patients in previous studies. The first one's codon change was
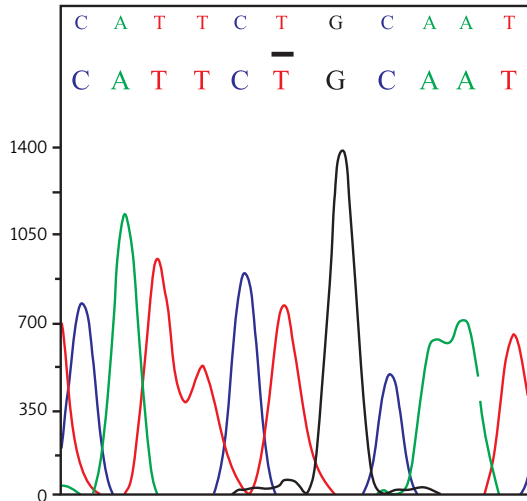
Figure 1. Non-syndrome cleft palate children and the mutation of T-box transcription factor TBX22 exon 5: there is no missense mutation (T→C)
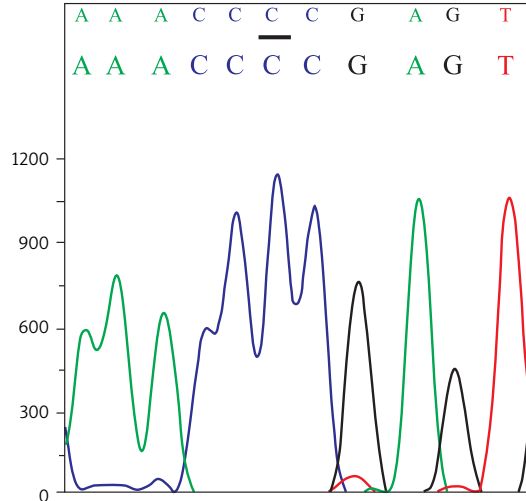

Figure 2. Non-syndrome cleft palate children and the mutation of T-box transcription factor TBX22 exon 5: there is no small deletion (delC)
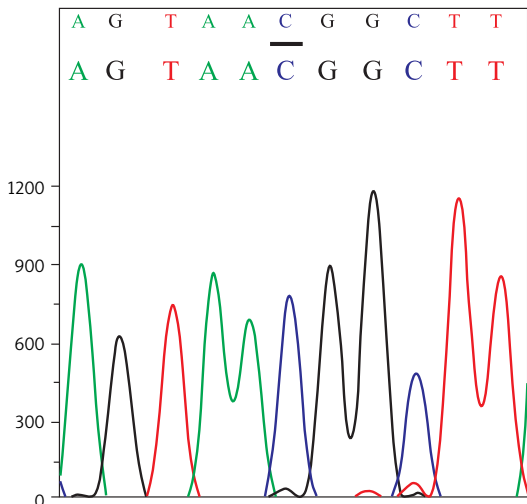

Figure 3. Non-syndrome cleft palate children and the mutation of T-box transcription factor TBX22 exon 5: there is no missense mutation (C→T)
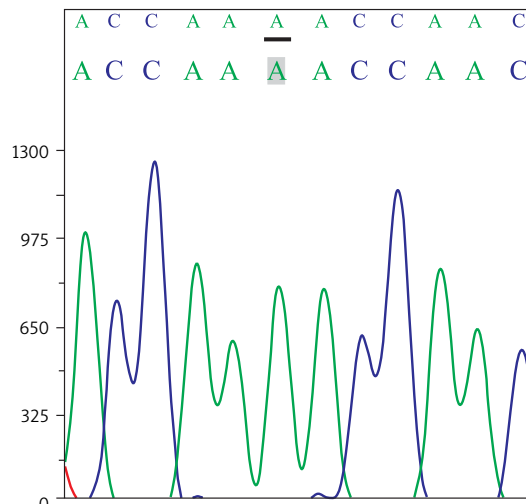

Figure 4. Non-syndrome cleft palate children and the mutation of T-box transcription factor TBX22 exon 5: there is no missense mutation (A→T)

641T→C(CTG→CCG), amino acid change was Leu→Pro(L214P) and its accession number was CM022464 [4]; the second one's codon change was 671delC (AGTAC^AAACCcCGAGTGCACG) and its accession number was CD013205 [5]; the third one's codon change was 785C→T(ACG→ATG), amino acid change was Thr→Met (T260M) and its accession number was CM013049 [5]; the last one's codon change was 790A→T (aAAC→TAC), amino acid change was Asn→Tyr (A264T) and its accession number was CM040122 [6]. In the corresponding base site of TBX22 exon 5, we found 646T, 671C, 785C and 796A, which can be seen in the pictures below (Figures 1-4). There were no missense mutations or small deletions identified. No clinical sig-nificance was found in calculating the index of the transmission disequilibrium test of 20 parent-child trios ($p > 0.05$) and the index of TDT was 0.56.

Discussion

In our study we used the syndromic cleft palate susceptibility gene as a candidate gene for more common non-syndromic cleft palate. Efforts to iden-tify the genetic factors have been most successful for monogenic, syndromic clefts, such as in Van der Woude syndrome (involving IRF6); Margarita Island syndrome (PVRL1); Kallmann syndrome (FGFR1); ectrodactyly, ectodermal dysplasia, and cleft lip and/or palate syndrome (p63); cleft lip and/or

palate with hypodontia (MSX1); and X-linked cleft palate (TBX22) [3, 9]. The value of translating a mutation causing a rarely observed syndromic condition onto more common non-syndromic forms of the disorder has been elegantly demonstrated in the study by Sözen *et al.* [10] for the role of PVRL1 in cleft lip with or without cleft palate. Phenotypic penetrance within families is often variable, and has not been explained by the type or position of mutations within the genes [11].

Considerable success has been achieved in identifying aetiological genes for syndromic forms of orofacial clefting with approximately 15-20% cloned to date. TBX22 is the important susceptibility gene currently found in X-linked cleft palate – ankyloglossia syndrome (CPX) and it is a major genetic determinant for familial cleft palate, particularly when combined with ankyloglossia. Linkage and/or haplotype analysis has been used to demonstrate that the cleft palate and ankyloglossia phenotype was X-linked and mapped to the approximate interval of CPX in Xq21 [4, 5, 12-14]. As a transcription factor, TBX22 is expressed specifically in tissues related to palatogenesis and it is confirmed as a major genetic influence in normal palate development. TBX22 expression was reported to disappear just prior to palatal shelf fusion and was thought instead to be required for mesenchymal proliferation and shelf elevation. Identification of the downstream target genes remains a priority [3, 6, 15].

TBX22 clearly makes a significant contribution to the prevalence of cleft palate and it will be important to study its biological role in the process of craniofacial development. To date, 13 different TBX22 mutations have been reported in CPX patients (http://www.hgmd.cf.ac.uk), including missense, nonsense, splicing, regulatory, small deletions and small insertions. There were four mutations located in TBX22 exon 5: 641T→C(L214P) [4], 671delC(221) [5], 785C→T(T260M) [5], 790A→T(A264T) [6]. In our research the sequence alignment did not reveal any credible mutation. We have not examined all the exons of TBX22, so mutations may also be harboured in the other exons. Suphapeetiporn *et al.* [7] in their study performed mutation analysis that covered all the coding regions of the TBX22 gene in 53 unrelated Thai patients with non-syndromic cleft palate, and identified four potentially pathogenic mutations: 359G→A (R120Q), 452G→T (R151L), 1166C→A (P389Q) and 1252delG in four different patients. There were also no mutations in exon 5 in the Thai population with non-syndromic cleft palate.

A better understanding of the cellular biology will help us to afford appropriate advice, therapy and ultimately prevention. A missense variant (359G→A; R120Q) was detected in the Thai population with ankyloglossia alone, the same mutation being previously reported in Thai patients with non-syndromic cleft palate. This nucleotide substitution was next to the one that has been described in a Tunisian CPX family (358C→T; R120W). From the crystal structure of the Brachyury T domain, this position lies within the $\alpha\beta$ barrel and is predicted to be involved in DNA binding. The amino acid of another missense variant in a CPX family (790A→T; A264T) is located in the T box domain at the start of the α helix 3, which is the position to recognise the target DNA in the minor groove and bridges the DNA backbone. Interfering with DNA binding is likely to be one of the major mechanisms underlying cleft palate with or without ankyloglossia as evidenced by a number of missense mutations found in the DNA-binding domain of TBX22 [6, 7, 14-16].

Based on the family-based study which refers to the patients and their healthy parents, the parent-child trio becomes the research object. Transmission of a particular allele from heterozygous parents to an offspring is expected to be approximately 50%, when there is no association of the allele and the disease. If the allele is associated with an increased risk of the disease, excess transmission to the offspring is expected. This approach accounts for the non-independence of parental transmissions to affected offspring [17, 18]. The suspected correlation was subjected to validation using the transmission disequilibrium test. The allele is not transmitted to affected children. As a control with a pair of alleles for a 1 : 1 matched case-control study, TDT eliminates the case-control population stratification problem, largely avoiding the phenomenon of the occurrence of false positives and improving the accuracy of gene targeting [19-22].

In conclusion, we found no mutations in TBX22 exon 5 in our patients with non-syndromic cleft palate, but the sample size is limited, and mainly concentrated in the selection of patients within Jiangzhe areas in China. As coverage is not wide enough, these restrictions may have some influences and induce negative results.

Acknowledgments

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