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Short Report

TBX22 mutations are a frequent cause of non-syndromic cleft palate in the Thai population

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Mutations in the *TBX22* gene underlie an X-linked malformation syndrome with cleft palate (CP) and ankyloglossia. Its mutations also result in non-syndromic CP in some populations. To investigate whether mutations in *TBX22* play a part in the formation of non-syndromic CP in the Thai population, we performed mutation analysis covering all the coding regions of the *TBX22* gene in 53 unrelated Thai patients with non-syndromic CP. We identified four potentially pathogenic mutations, 359G→A (R120Q), 452G→T (R151L), 1166C→A (P389Q), and 1252delG in four different patients. All mutations were not detected in at least 112 unaffected ethnic-matched control chromosomes and had never been previously reported. R120Q and R151L, found in two sporadic cases, were located in the DNA binding T-box domain. P389Q and 1252delG, found in two familial cases, were at the carboxy-terminal region, which has never been described. Our study indicates that *TBX22* mutations are responsible for a significant proportion of Thai non-syndromic CP cases confirming its importance as a frequent cause of non-syndromic CP across different populations.

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Orofacial clefts are common congenital birth defects seen in humans, with a reported prevalence of 1/700 live births worldwide (1–3). Orofacial clefts are generally considered a sporadic occurrence with multifactorial inheritance resulting from an interaction between genetic and environmental factors. This complex interaction between genes and the environment has hampered efforts to identify the underlying genetic predisposition to clefts. However, significant progress has been made with the identification of gene mutations in several forms of orofacial clefts by studying rare clefting syndromes and mouse mutants. Identification of these genes has provided an important source of candidate genes for more common non-syndromic orofacial clefts, as similar genes are also involved.

Orofacial clefts include cleft lip with or without cleft palate (CL/P) and non-syndromic cleft palate (CP). CL/P and CP are developmentally and genetically distinct. In Thai population, similar to many others, we found that several genes responsible for Mendelian clefting syndromes have roles in non-syndromic CL/P. These include the *IRF6*, *p63*, and *MSX1* (4–8). Conversely, the *SATB2* gene, which was first identified to be associated with non-syndromic CP (9), was recently found to underlie a syndromic cleft in a Thai patient (10).

Cleft palate with ankyloglossia (CPX; OMIM 303400) is inherited as a semidominant X-linked disorder. It is characterized by a cleft of the secondary palate and ankyloglossia. The *T-box transcription factor TBX22* was found to be mutated in families with CPX (11). It is expressed primarily in the palatal shelves and tongue during palatogenesis suggesting an important role in both palatal and tongue development (12, 13). In

addition to familial cases with suspected CPX, *TBX22* mutations have been identified in a significant proportion of non-syndromic CP cases (14). The spectrum of *TBX22* mutations found (nonsense, splice site, frameshift and missense) indicates that the phenotype is resulted from a complete loss of TBX22 function in men and haploinsufficiency in women (11, 12, 14). All these findings led us to hypothesize that some Thai patients with non-syndromic CP might harbor mutations in *TBX22*, a gene known to cause syndromic CP.

In this study, we performed mutation analysis of all the coding regions of the *TBX22* gene in 53 unrelated Thai patients with non-syndromic CP.

Material and methods

Patients

The study was approved by the local Ethics Committee, and written informed consent was obtained from all patients or their parents included in the study. Forty-seven sporadic cases of non-syndromic CP patients and six additional cases with a positive family history were enrolled. The control samples were Thai blood donors with no oral clefts, who denied history of oral clefts in other family members.

Mutation analysis

Genomic DNA was extracted from peripheral leukocytes according to standard protocols. Primers were designed within introns to allow genomic amplification and sequencing of exons 1–8 including exon–intron boundaries as shown in Table 1. We used 100 ng of genomic DNA, 1 × polymerase chain reaction (PCR) buffer (Fermentas, Burlington, ON, Canada), 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 0.15 μM of each primer, and 0.4 U Taq DNA polymerase (Fermentas) in a total volume of 20 μl. PCR products were treated with ExoSAP-IT (USP Corp., Cleveland, OH), according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. The sequence was analyzed using Sequencher (version 4.2; Gene Codes Corp., Ann Arbor, MI). For missense mutations, restriction enzyme digestions were used to confirm their presence in the patients and to screen more than 200 control chromosomes from unaffected ethnic-matched people. For the deletion mutation, 112 control chromosomes were screened by direct sequencing. In addition, parents or siblings were screened by restriction fragment length polymorphism for the same variant if DNA samples were available.

Protein sequence comparison

TBX22 orthologues were first identified through a Basic Local Alignment Search Tool using Homo sapiens TBX22, accession NP_058650, as the reference sequence. All known and complete TBX22 sequences were included from the vertebrate lineage. These files in FASTA format were then analyzed by CLUSTALX 1.81 program. The human TBX22 was aligned with mouse (accession NP_660259), chick (accession NP_989437), *Xenopus* (accession AAB49478), worm (accession BAD14918) Tbx22 as well as human TBX1 (accession NP_005983), human TBX15 (accession NP_689593) and human TBX18 (accession NP_001073977). The program classified amino acids by the variation in polarity, thereby assessing both amino acid class conservation and evolutionary conservation at any given site.

Results

The entire coding sequence of *TBX22* was analyzed by PCR-sequencing in all 53 unrelated CP Thai patients. Four different sequence variants in four different patients were identified (Table 2). There were 26 men and 27 women. The

Table 1. Oligonucleotides and PCR conditions for *TBX22* mutation analysis

Exon	Primer sequences for PCR 5′ to 3′		Annealing temperature (°C)
	Forward	Reverse	
1	TCCCTAACCCAGTTCAGGTT	ATGTGGCTGTCTGGCTGCGT	60
2	TGTCTGCTCCAAGATAGGCA	TACTGTATGTCATGGGAGTTG	60
3	CTGGAGTCAGCATTTGTCCA	GTCTGAAGGTCCAAATCCCT	60
4	CTGGAGTGAAGTCCTCAGGA	GCAGGGCTTGAACAGTTCCT	60
5/6	TGTGTGCACATGGTGGAGGT	AGTGAGACTCCATCTCAGGA	58
7	TGCTACTCACTATTAAGACC	ACATATCCCCTTGTGTAGTA	60
8	AAGGATGAAGCACAGATAGT	TGAAGCTCAAGGCCACTGTA	58

PCR, polymerase chain reaction.

Table 2. *TBX22* mutations identified in Thai patients with non-syndromic cleft palate

Mutation	Amino acid change	Number of the mutant allele identified in controls (chromosomes)	Characteristics of patients
359G→A	R120Q	0/286	Male, sporadic, cleft soft palate
452G→T	R151L	0/267	Male, sporadic, cleft soft palate
1166C→A	P389Q	0/254	Male, familial, incomplete cleft palate
1252delG	Frameshift	0/112	Female, familial, cleft soft palate

TBX22 mutations were identified in three men and one woman, thereby making the ratio of *TBX22* mutations found in male patients equal to 3 in 26 and in female patients equal to 1 in 27. This would be consistent with the fact that the incidence of CPs caused by an X-linked gene in men is higher than that of CPs in women. All mutations were located in the coding regions and were not present in ethnic-matched control individuals. To our knowledge, all these variants have never been previously reported. Two of the identified mutations were found in patients with a positive family history for clefts and the other two in patients with no known family history.

A single-base transition 359G→A was detected in exon 3 in a male patient with sporadic cleft of soft palate. This was expected to result in an arginine to glutamine substitution at position 120 (R120Q) (Fig. 1, left panel). This mutation is located at a highly conserved residue within the T-box gene family and throughout evolution (Fig. 2). Another male patient with sporadic cleft of soft palate was found to carry a non-synonymous missense mutation, a G→T transversion at base pair 452 in exon 3 (452G→T) expected to result in an arginine to leucine substitution (R151L) (Fig. 1, middle panel). The mutation occurs within the T-box domain at the evolutionarily conserved residues found in other T-box genes and species (Fig. 2). The third mutation was found in a male patient with CP and a positive family history for clefts. This was a single-base transversion 1166C→A, which expected to result in the substitution of a proline by a glutamine at position 389 (P389Q) (Fig. 1, right panel). His

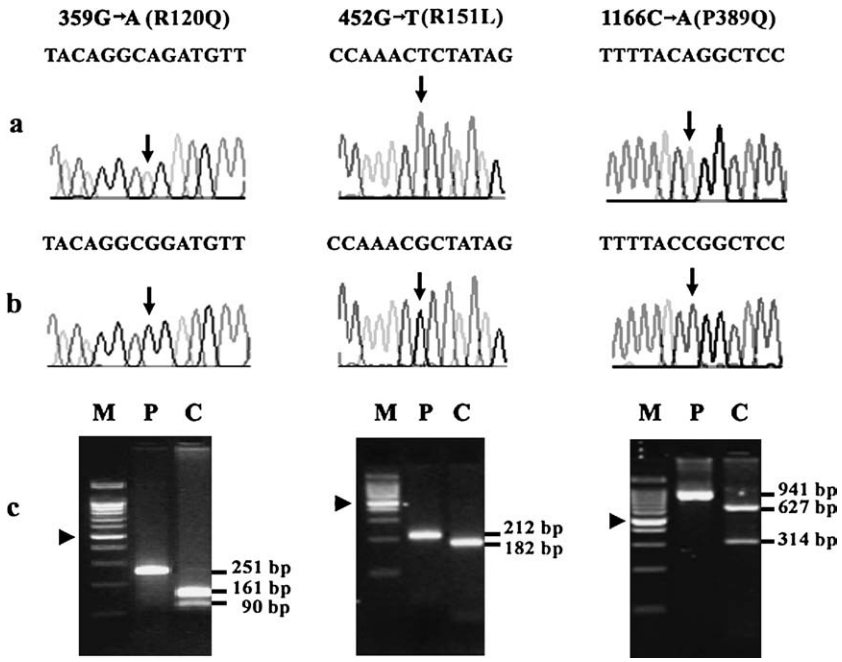

Fig. 1. Mutation analysis: the left, middle and right panels relate to 359G→A (R120Q), 452G→T (R151L), and 1166C→A (P389Q) mutations, respectively. (**a**) Electropherograms of patients showing the mutations (arrows). (**b**) Electropherograms of controls showing normal genotypes (arrows). (**c**) Restriction enzyme digestion analysis, M: 100-bp marker. The 500-bp band is indicated by an arrow head. P: patient. C: unaffected control. In the left panel, *Fok*I digested the wild-type allele of a control into 161 and 90-bp products. The 359G→A mutation in the patient eliminates the *Fok*I site, leaving the intact 251-bp product. In the middle panel, *BstU*I digested the wild-type allele of a control into 182 and 30-bp products (the 30-bp band was invisualized). The 452G→T mutation in the patient eliminates the *BstU*I site, leaving the intact 212-bp product. In the right panel, *Msp*I digested the wild-type allele of a control into 627 and 314-bp products. The 1166C→A mutation eliminates the *Msp*I site, thereby leaving the intact 941-bp product.

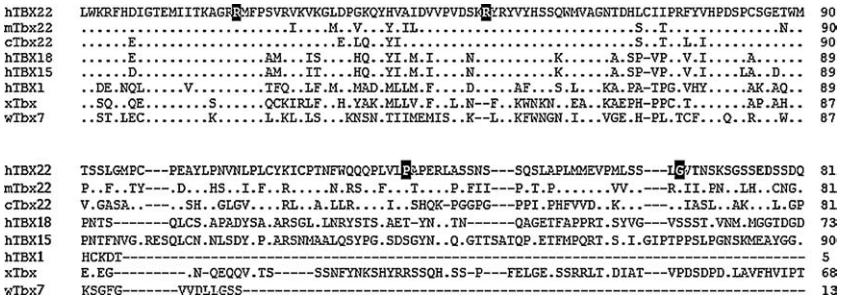

Fig. 2. Sequence alignment of the T-box domain (upper) and the carboxy-terminal region (lower) from different T-box family members and different species. The site of the amino acid variant found in this study is indicated in shaded boxes in the human TBX22 sequence. Sites that are conserved are indicated by dots (.). h, human; m, mouse; c, chicken; x, *Xenopus laevis*; w, worm (*Caenorhabditis elegans*).

mother was found to be clinically unaffected, while a maternal aunt was reported to have CP. Samples from the mother and aunt were unavailable. Finally, a single-base deletion at nucleotide position 1252 (1252delG) in exon 8 (Fig. 3, upper panel) was detected in a female patient with cleft of soft palate. The loss of a guanine residue causes a frameshift that introduces a premature stop codon at position 485, which is likely to result in either an absent or truncated protein. The proband's father had bifid uvula and her paternal uncle was reported to have CP only. Mutation analysis of additional family members revealed that her father carried the mutation (Fig. 3, middle panel), while her unaffected mother and brother had only the normal alleles (Fig. 3, lower panel).

Discussion

Human non-syndromic CP is etiologically complex with both genetic and environmental factors playing a role. Identification of genes for rare developmental syndromes has provided an important source of candidate genes for more common birth defects as similar genes are also involved. *TBX22* is a major genetic determinant for familial CP, particularly with the presence of ankyloglossia. In addition, it has been recently demonstrated that *TBX22* makes a significant contribution to the prevalence of CP at least in the Brazilian and the North American cohorts (14). In this study, we investigated the presence of *TBX22* mutations in 53 unrelated Thai patients with non-syndromic CP by direct sequencing of the entire coding exons. To date, 15 different *TBX22* mutations have been reported in patients with CP and/or ankyloglossia (http://www.hgmd.cf.ac.uk). These include small deletions/insertions, nonsense, splice site, frameshift and missense alterations. Our studies have identified four different novel mutations. Three missense and one deletion mutations were not detected in at least 112 unaffected control chromosomes (Table 2). Two were located in the DNA binding T-box domain, while the others were located at the carboxy-terminal region, which has never been described.

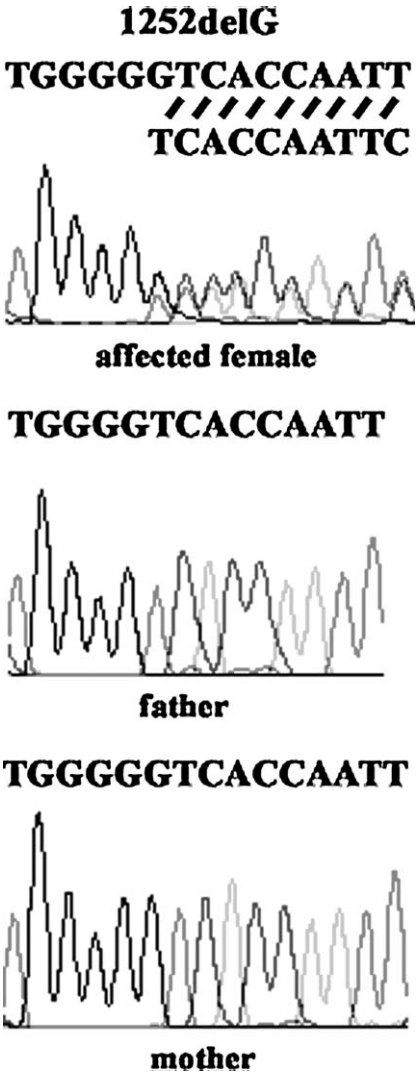

Fig. 3. Mutation analysis of the family with *TBX22* 1252delG mutation. The sense sequence electropherograms from genomic DNA amplified from exon 8 are of the cleft palate female proband showing delG/wild-type (upper panel), affected father showing delG (middle panel) and unaffected mother showing only wild-type alleles (lower panel).

A single-base 359G→A transition in exon 3 resulting in arginine to glutamine substitution at position 120 (R120Q) was detected in our study. This nucleotide substitution was next to the one that has been previously described in Tunisian CPX family (the 358C→T transition resulting in an amino acid substitution R120W) (15). This finding confirmed a significant role of this critical residue. From the crystal structure of the Brachyury T domain (16), this position lies within a β barrel and is predicted to be involved in DNA binding. It is likely that R120Q interferes with the capability of the protein to bind DNA contributing to the loss of transcriptional activation. We also identified another novel missense mutation, R151L, located within the c strand of T-box domain (16). It forms polar interactions with the target DNA sequence. Our findings emphasize the importance of conserved residues within the DNA-binding domain. Interfering with DNA binding is likely to be one of the major mechanisms underlying the CP with or without ankyloglossia as evidenced by a number of missense mutations found in the DNA-binding domain of TBX22.

The third and fourth mutations identified in our study, the *TBX22* 1166C→A (P389Q) and 1252delG mutations are in the C terminus outside the T-box domain. There have been no mutations in *TBX22* previously described in this region. The P389Q possibly affects TBX22 function through the mechanisms involving protein–protein interaction or nuclear localization as evidenced in functional analysis of other *TBX* genes (17–20). Recent studies revealed that *TBX5* missense mutations including the ones located outside the T-box domain caused Holt–Oram syndrome by impairing nuclear localization leading to reduced transcriptional activation (20). Additional pathogenic mechanisms of missense mutations found in *TBX3, TBX5* and *TBX19* include interfering with the ability to form stable interactions as dimers or with other binding partners (17–20).

The 1252delG is expected to result in subsequent changes of 67 amino acids and truncation at amino acid 485. The truncated protein retains its T-box domain. No apparent functional motif has been detected in the C-terminus region. Functional studies when available will elucidate the significance of these changes. It is possible that certain functional domain may exist. Future studies implicating other regions as critical residues involved in the protein–protein interaction and nuclear localization will provide important insights into the function of TBX22 and other T-box proteins as well as their roles in regulating transcription. Another possible pathogenic effect of the frameshift 1252delG is that the mutation causes unstable RNA resulting in loss of TBX22 function.

Previous studies revealed no genotype–phenotype correlation (14). In addition, there was an intrafamilial and interfamilial phenotypic variability (11, 14). Men carrying the mutations show variation ranging from a complete cleft of the secondary palate, submucous cleft, bifid uvula to high-arched palate. A previous study reported ankyloglossia in 79% of male patients and 45% of female patients with *TBX22* mutation. CP, however, may be the sole presenting feature in 6% of female patients (14). The heterozygous women show greater phenotypic variability. It has been proposed that the variable expression of CPX is likely to be influenced by modifiers or environmental factors. Non-random X inactivation could be another factor modifying the phenotype in women. In Marcano's paper (14), a range of severity was seen within the families with several members harboring the mutations. A possible example of a non-expressing obligate affected men in an X-linked isolated non-syndromic CP family was also described although another group pointed out that non-expression is not proved as the obligate men was not examined for bifid uvula or submucous CP (21, 22).

The family with 1252delG found in our study is particularly interesting as this mutation is novel, and the phenotype is more severe in affected women compared with her affected father. Further exploration might help elucidate the perplexing finding. Nevertheless, our study further confirmed the variable expression of CPX.

TBX22 mutations are responsible for a significant proportion of Thai CP cases confirming its importance as a frequent cause of CP. This study further supports that *TBX22* is the gene responsible for non-syndromic CP across different populations.

Supplementary material

Fig. S1. A patient with isolated cleft palate.
Fig. S2. An affected relative with isolated cleft palate.
Fig. S3. A patient with ankyloglossia (anterior view).
Fig. S4. A patient with ankyloglossia (lateral view).

Supplementary materials are available as part of the online article at http://www.blackwell-synergy.com

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