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Cleft Lip with Cleft Palate, Ankyloglossia, and Hypodontia are Associated with *TBX22* Mutations

ABSTRACT

X-linked cleft palate and ankyloglossia (CPX) are caused by mutations in the *TBX22* transcription factor. To investigate whether patients with ankyloglossia alone or in the presence of other craniofacial features including hypodontia or CLP might be caused by *TBX22* mutations, we analyzed 45 Thai patients with isolated ankyloglossia, 2 unusual CPA families, and 282 non-syndromic Thai and UK patients with CLP. Five putative missense mutations were identified, including 3 located in the T-box binding domain (R120Q, R126W, and R151L) that affects DNA binding and/or transcriptional repression. The 2 novel C-terminal mutations, P389Q and S400Y, did not affect *TBX22* activity. Mutations R120Q and P389Q were identified in patients with ankyloglossia only, while R126W and R151L were present in families that included CLP. Several individuals in these families were also found to have micro/hypodontia. This study has expanded the phenotypic spectrum of *TBX22*-related mutations to include dental anomalies and cleft lip.

KEY WORDS: ankyloglossia, cleft lip and palate, dental anomaly, hypodontia, microdontia, *TBX22*.

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INTRODUCTION

X-linked cleft palate and ankyloglossia (CPX: OMIM 303400) are caused by mutation in the T-box transcription factor *TBX22* (Braybrook *et al.*, 2001). Nonsense, frame-shift, splice site, and missense changes have all been described, with the latter usually occurring in the highly conserved 180-amino-acid T-box domain. These lead to a loss of function, mediated through impaired DNA binding, but may also affect the ability of the protein to undergo post-translational modification with SUMO1 (Andreou *et al.*, 2007). An increased risk of cleft palate (CP) with ankyloglossia has also been associated with sequence variation resulting in reduced *TBX22* promoter activity (Pauws *et al.*, 2009b).

The palate phenotype can vary, involving either a complete cleft of the secondary palate or a submucous cleft, with or without a bifid or absent uvula. These different forms have been described within a single family (Moore *et al.*, 1987; Bjornsson *et al.*, 1989; Stanier *et al.*, 1993), suggesting that environmental factors or other genetic modifiers may influence the outcome. However, we recently observed overt clefts and submucous clefts in littermates of inbred mice lacking *TBX22*, with no obvious explanation for the difference (Pauws *et al.*, 2009a). Ankyloglossia is also variable, being present in classic CPX but not always in relatives carrying the same mutation. These individuals might otherwise be described as non-syndromic. Consequently, *TBX22* mutations are reported in well-defined CPX families and in 4-8% of non-syndromic CP patients (Marçano *et al.*, 2004; Suphapeetiporn *et al.*, 2007).

The expression of *TBX22* is first seen at CS16, approximately 37 days post-ovulation (Braybrook *et al.*, 2002). At early stages, it is expressed in the developing somites and the first pharyngeal arch (Haenig *et al.*, 2002; Bush *et al.*, 2004). Expression is later observed in the medial portion of the posterior palatal shelves and in the base of the tongue around the site of attachment to the lower jaw. Craniofacial expression is also detected in the ventral portion of the nasal septum, the tooth buds, and extra-ocular tissue (Braybrook *et al.*, 2002). *TBX22* expression was reported to disappear just prior to palatal shelf fusion (Braybrook *et al.*, 2002; Bush *et al.*, 2004) and was thought instead to be required for mesenchymal proliferation and shelf elevation. However, in most *Tbx22*^{null} mouse embryos, shelf elevation and fusion progress normally, while patterning of the post-fusion mesenchymal bridge and subsequent palatal ossification is abnormal (Pauws *et al.*, 2009a).

The genetic basis of isolated ankyloglossia is rarely studied, but it is not unusual within CPX families, especially female carriers (45%) and occasionally males (4%) (Marçano *et al.*, 2004). Mutations have yet to be reported where there is no family history of CPX, but this could be of great significance for future genetic counseling. There is also mounting evidence for a role for *TBX22* in the occurrence of cleft lip and palate (CLP). Apart from a lone female with CLP and ankyloglossia in a large CPX family (Braybrook *et al.*, 2001), a genome-wide CLP sib pair analysis reported a suggestive multipoint LOD score (2.89) for the locus surrounding *TBX22* (Prescott *et al.*, 2000). Meanwhile, recent evidence from the chick shows that ectopic expression of *hTBX22* in the frontonasal mass results in cleft lip (Higashihori *et al.*, 2010).

The objectives of the study were to investigate whether ankyloglossia alone or in the presence of other craniofacial features, including hypodontia or CLP, might be caused by *TBX22* mutations. Therefore, we selected patients with isolated ankyloglossia or CLP in whom to sequence *TBX22*. We identified 2 novel missense mutations and 3 others that had previously been reported in classic CPX. Several patients were also noted to have dental anomalies. Computational and biochemical analysis was used to provide functional evidence for a causal role of these variants.

MATERIALS & METHODS

Patients and Samples

The study was granted ethical approval by the committees of the Faculty of Dentistry, Chiang Mai and Mahidol University, and by the Institute of Child Health/Great Ormond Street Hospital. Written consent was obtained from the participants. Blood samples from patients with isolated ankyloglossia (n = 45) or sporadic, isolated CLP (n = 90) were collected from the Thai population. Ankyloglossia and hypodontia were evaluated by one of the authors, who is a pediatric dentist (M.P.). Ankyloglossia was reported when the lingual frenum was very short with significant restriction of tongue movement, or the frenum was attached from the tip of the tongue to the anterior alveolar ridge. Hypodontia was indicated when the tooth or teeth were missing clinically and radiographically. A further 192 single or multiple affected UK families with non-syndromic CLP were enrolled in the study (Prescott *et al.*, 2000). In addition, two unusual familial cases, one of cleft palate and ankyloglossia (CPA) from Thailand and one complex CPA/cleft lip and palate with ankyloglossia (CLPA) family from the UK, were included. Control samples without hypodontia or ankyloglossia consisted of 100 Thai and 192 UK individuals (Apostolidou *et al.*, 2007). The coding regions and flanking intronic sequences of *TBX22* were amplified from genomic DNA and sequenced as previously described (Braybrook *et al.*, 2001).

Mutation Analysis

Putative missense mutations were analyzed *in silico*, and compared by 3 Web-based programs: PANTHER (http://www.pantherdb.org/), PolyPhen (http://genetics.bwh.harvard.edu/pph/), and SIFT (Ng and Henikoff, 2003) (http://sift.jcvi.org/). Protein sequence alignments were constructed by CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/).

Plasmid expression constructs containing full-length *TBX22* cDNA (pcDNA3.1.*TBX22*.V5/His) or missense mutations (E187K, R120W, N264Y) were as previously described (Andreou *et al.*, 2007). New mutations were introduced with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Transcriptional repression was investigated with a luciferase reporter construct (pGL3, Promega, Madison, WI, USA) containing -683 bp to +115 bp of the *MyoD* promoter (amplified with *MyoD*_F, 5′-ATTCTCGAGACCCGGAGTTTGAGCAGAAT-3′ and *MyoD*_R, 5′-ATTCTCGAGACAAAGGTTCTGTGGGTTGG-3′). DNA binding-independent effects were investigated by means of a GAL4/LexA inducible repression assay with wild-type and mutant constructs. Reporter assays were performed as previously described (Andreou *et al.*, 2007) by co-transfection into 293T cells with FuGene6 (Roche, Mannheim, Germany). Results were statistically tested by ANOVA with significance set at $p < 0.01$.

Chromatin-immunoprecipitation was used to detect interaction between *TBX22* and the *MyoD* promoter. 293T cells were transfected with full-length wild-type or mutant (N264Y, R120Q, R126W, R151L, P389Q, S400Y) pcDNA3.1.*TBX22*.V5/His constructs and incubated for 48 hrs. Cross-linking with 0.8% formaldehyde was stopped by the addition of glycine to a final concentration of 0.125 M. The DNA was sonicated (Bioruptor, Diagenode, Liège, Belgium) and protein-DNA complexes immunoprecipitated with anti-V5 antibody (Invitrogen) and protein G agarose (Pierce, Rockford, IL, USA). Protein-DNA complexes were unlinked and the DNA recovered with the QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA). PCR amplification then used the primers: *MyoD*_ChIPF, 5′-ATTCTCGAGTCCGAGTTTGGAGAGATTGG-3′; and *MyoD*_ChIPR, 5′- TGAGGAGTGAGACCGTGAAA-3′.

RESULTS

TBX22 coding exons were sequenced in 45 patients with isolated ankyloglossia. Two potentially pathogenic changes were identified. A heterozygous missense variant in exon 8 (1166C>A; P389Q) was identified in a 9-year-old Thai girl (Fig. 1A). Inheritance could not be established, since the mutation was not present in her clinically unaffected mother, and DNA was not available from her unaffected father, who is deceased. The second missense variant in exon 3 (359G>A; R120Q) was present in a 13-year-old Thai boy (Fig. 1B). This variant was present in his mother, who has microdontia of the maxillary permanent left lateral incisor. Interestingly, both variants have previously been reported in Thai patients with non-syndromic cleft palate (Suphapeetiporn *et al.*, 2007). A different missense change at the same location, R120W, was reported in a Tunisian family with classic CPX (Chaabouni *et al.*, 2005). The arginine residue of the R120Q variant is highly conserved in all species and T-box family members, with the only exception being Drosophila OMB, where a glutamine residue is found (Muller and Herrmann, 1997). In contrast, the C-terminal P389Q variant, which is conserved among human, mouse, and chicken, shares little homology with other species or other T-box proteins (Fig. 2L).

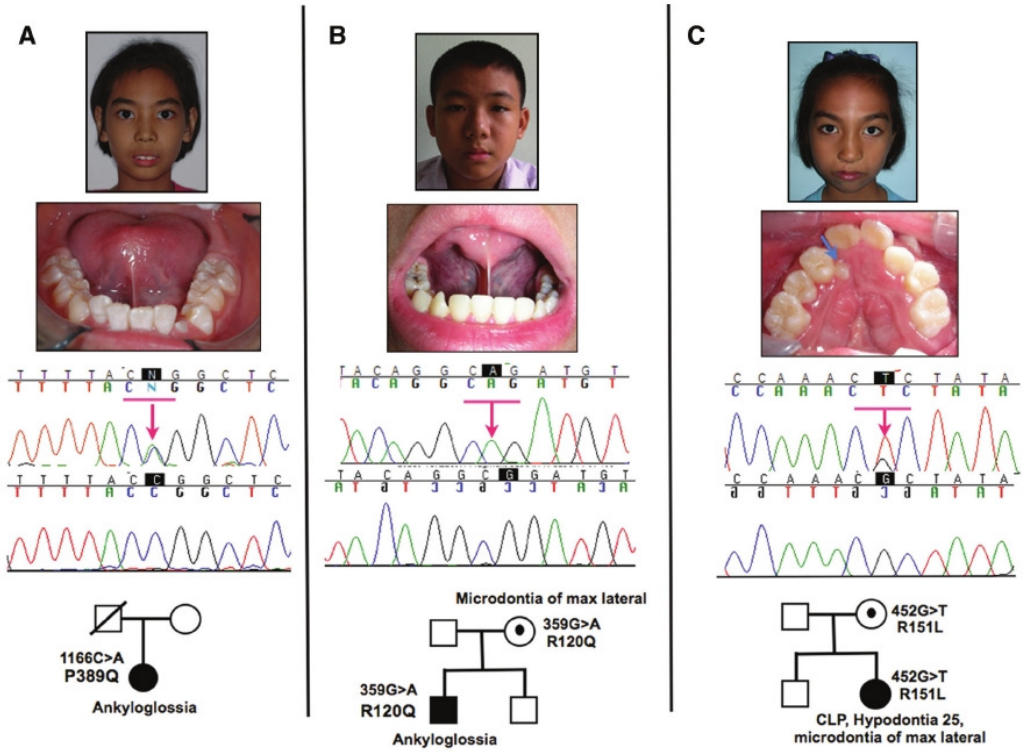

Figure 1. TBX22 sequence variants in patients with different phenotypes. **(A)** 1166C>A (P389Q) and **(B)** 359G>A (R120Q) were present in patients with isolated ankyloglossia. The carrier mother in (B) has microdontia. **(C)** 452G>T (R151L) was found in a patient with CLP and tooth anomalies.

A heterozygous missense mutation was detected in exon 8 (1199C>A; S400Y) in a Thai family (Figs. 2A-2I), initially recruited due to the presence of CPA in the 2.5-year-old daughter. The mutation was also present in the mother, who has a bifid tongue. Her father has CP, ankyloglossia, and microdontia of the maxillary permanent lateral incisor but does not carry the mutation, or any other *TBX22* variant. This change was not found in controls or the SNP database and is conserved across species. The inheritance indicates that the S400Y variant may be related to the tongue defect but is unlikely to be the cause of the girl's palate phenotype.

Next we investigated 90 Thai patients with non-syndromic CLP. A heterozygous missense variant in exon 3 (452G>T; R151L) was identified in an 11-year-old Thai girl (Fig. 1C). This residue is highly conserved across species and in the TBX1 subfamily of T-box proteins. This variant was previously reported in an unrelated Thai male with non-syndromic CP (Suphapeetiporn *et al.*, 2007). Closer inspection of this patient revealed that the maxillary left second premolar was congenitally missing, and the maxillary right lateral incisor was peg-shaped. The mutation was inherited from her unaffected mother.

To further investigate the prevalence of *TBX22* mutations in non-syndromic CLP patients, we sequenced a UK cohort of 192 affected individuals (Prescott *et al.*, 2000). Although numerous previously described (Marçano *et al.*, 2004) polymorphic intronic and exonic variants were detected, no novel or potentially pathogenic variants were identified.

Finally, we report the analysis of a multi-generation UK family with mixed clefting phenotypes. An affected male presented with typical CPX, while his daughter has CLP (Figs. 2H-2J). We identified a novel missense variant in exon 3 (376C>T; R126W), which was not found in the SNP database or controls. It is located within the T-box domain andis highly conserved in different species and other TBX1 sub-family members (TBX15, TBX18, and TBX20).

To discriminate between functional and non-functional mutations, we investigated each variant using a series of bioinformatics tools and biochemical assays. The 5 missense variants were analyzed with PANTHER, PolyPhen, and SIFT prediction algorithms as previously described (Won *et al.*, 2008). For controls, we included several mutations that have previously been validated as non-functional (E187K) or functional (R120W, N264Y) (Andreou *et al.*, 2007). Although there are some differences between the algorithms, predictions are consistent with previous data. The E187K polymorphism has low PANTHER and PolyPhen scores, while the mutations N264Y and R120W are among the highest scores (Table). The 3 mutations located in the T-box domain (R120Q, R151L, R126W) show high scores and are predicted to have a functional effect. Interestingly, R120Q has a lower score than R120W, indicating a potentially qualitative difference. The 2 mutations in the C-terminal region (P389Q, S400Y) also have low scores and are comparable with the E187K polymorphism. However, since these mutations

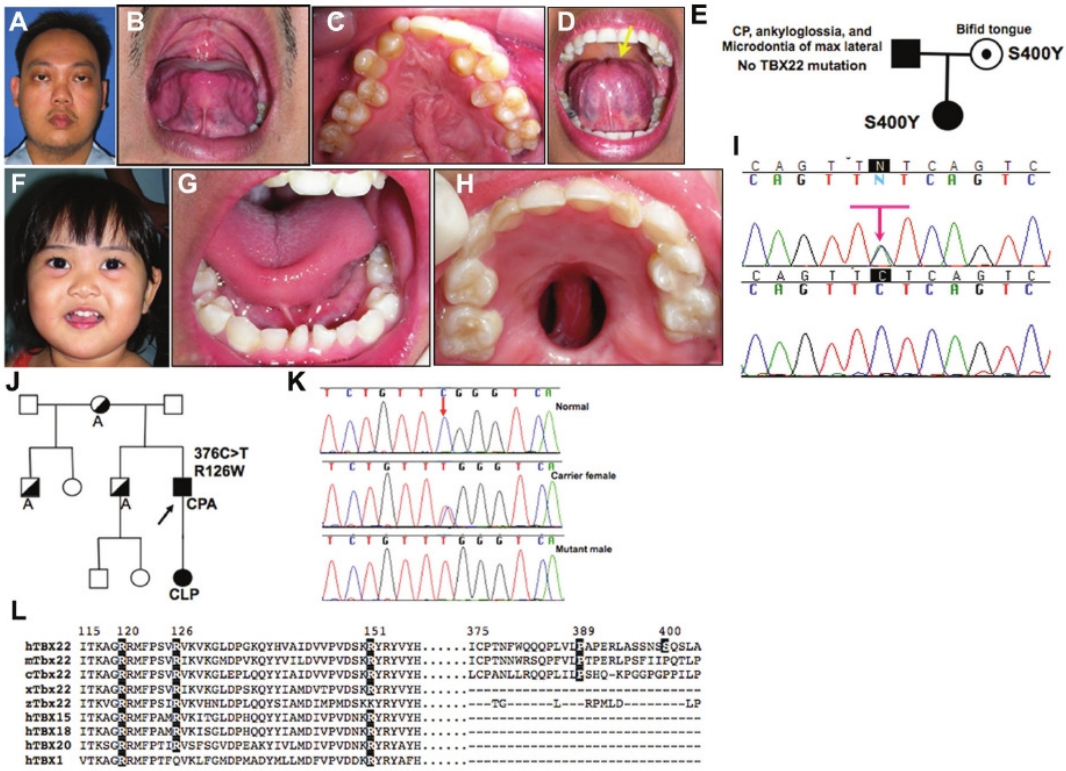

Figure 2. Sequence analysis in two unusual families. **(A-I)** The sequence variant 1199C>A (S400Y) was identified in a family with cleft palate, ankyloglossia, and microdontia. **(A-D)** show the father with repaired cleft palate, ankyloglossia, and dental anomalies. **(F-H)** show the daughter with ankyloglossia and repaired cleft palate. **(J)** Pedigree of a family containing both classic CPX and CLP patients. **(K)** The sequence traces show wild-type, heterozygous, and hemizygous examples of the 376C>T (R126W) variant. **(L)** Clustal multispecies sequence alignment showing the location and conservation of the various mutations in TBX22 and other TBX1 protein family members. The first block is within the highly conserved T-box domain; the second is the poorly conserved C-terminal region.

Table. *In silico* Prediction of the Effects of TBX22 Missense Mutations

Substitution	PANTHER Score	$P_{deleterious}$	PolyPhen Score	Prediction	SIFT Score (P)	Prediction
R120W[a]	−8.80	0.99699	2.65	Probably damaging	0.00	Deleterious
R120Q	−5.94	0.94994	1.75	Possibly damaging	0.00	Deleterious
R126W	−7.09	0.98361	2.65	Probably damaging	0.00	Deleterious
R151L	−7.14	0.98432	2.23	Probably damaging	0.00	Deleterious
E187K[a]	−3.71	0.67057	1.65	Possibly damaging	0.01	Deleterious
N264Y[a]	−8.07	0.99379	2.51	Probably damaging	0.00	Deleterious
P389Q	−3.57	0.63788	2.27	Probably damaging	0.25	Tolerated
S400Y[b]	−3.21	0.55156	1.80	Possibly damaging	0.06	Tolerated

Scores indicate the probability (P value) or prediction that a missense mutation is functionally damaging. Value ranges are as follows: PANTHER, [-10—0], cut off = -3; PolyPhen, possibly damaging when > 1.5, probably damaging when > 2.0; SIFT, [0—1], deleterious when $p < 0.05$.
[a]Previously described polymorphism (E187K) and functional mutations (R120W, N264Y) (Andreou *et al.*, 2007). This mutation is most likely a non-synonymous polymorphism.

occur in a poorly conserved protein region, it is possible that the programs underestimate the effect.

Next, we evaluated the missense variants using *in vitro* methods. We recently identified the *MyoD* promoter as a direct target of *TBX22* (unpublished observations). Using a *MyoD* promoter construct expressing luciferase in 293T cells, we detected approximately 40% repression of promoter activity when co-transfected with full-length *TBX22* (Fig. 3A). The effect of each missense change on this repression can then be measured. The T-box domain mutations (R120Q, R126W, R151L) all show impaired repression of *MyoD* promoter activity (Fig. 3A). The R120Q mutation has less effect compared with the R120W

mutation. This correlates well with the *in silico* prediction (Table) and may explain the differences in phenotypic severity among the patients. The putative C-terminal mutations (P389Q, S400Y) did not significantly alter *MyoD* promoter activity.

To investigate whether the effect on repression was DNA-binding-dependent, we used a reporter containing only GAL4 and VP16 domains, where luciferase expression requires DNA binding *via* the GAL4 binding site. In the presence of *TBX22* fused to GAL4, repression is maintained (*i.e.*, independent of binding *via* the T-box domain) (Fig. 3B). We detected no loss of repression for any of the missense mutations (Fig. 3B), suggesting that they most likely exert their effect directly through specific DNA binding of the T-box domain. Mutations in the C-terminal region also did not alter repression. Alternative studies will be required to adequately assess the function of this part of the protein.

Finally, we investigated the missense changes for effects on direct interaction between *TBX22* and the *MyoD* promoter. The *MyoD* promoter was reproducibly recovered from immunoprecipitated chromatin isolated from cells transfected with the *TBX22*.V5 expression construct (Fig. 3C). This interaction was completely abolished for the N264Y missense construct, previously shown to abrogate DNA binding (Andreou *et al.*, 2007). Similarly, R126W and R151L failed to interact with the *MyoD* promoter in 4 independent assays. The R120Q was variable between assays (bound in 3/4), and although the method is not designed to be quantitative, we interpret this as suggesting a weak interaction. The C-terminal variants, P389Q and S400Y, consistently retained interaction (Fig. 3C).

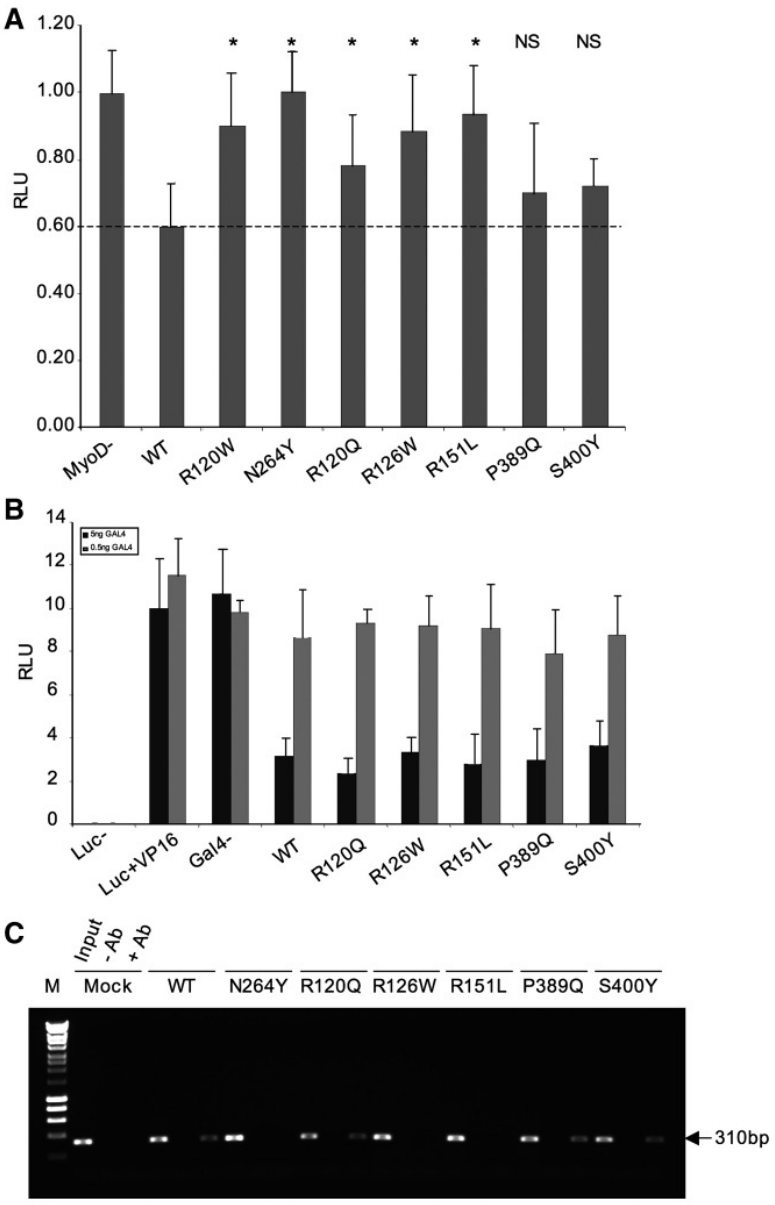

Figure 3. Functional analysis of TBX22 missense changes. **(A)** Promoter repression assay with wild-type (WT) and mutant TBX22 proteins co-expressed with the *MyoD*-Luc promoter reporter construct (*MyoD*-). The data are normalized to present average promoter activity measured by fluorescence in 4 independent experiments, each in quadruplicate. RLU = relative light units, NS = not significant, *$p < 0.01$ (ANOVA). **(B)** DNA-binding independent repression assay for TBX22 mutants with LexA/GAL4 promoter-driven luciferase construct (Luc-). Activity is generated when the LexA/VP16 expression construct is present (Luc+). The addition of mutant GAL4-TBX22 constructs inhibits promoter activity and does not statistically differ from wild-type (WT). **(C)** PCR amplification of the *MyoD* promoter region from immunoprecipitated chromatin recovered from 293T cells transfected with mock (empty pCDNA3.1 vector), wild-type (pCDNA3.1.TBX22.V5/His), or mutant constructs. The previously described N264Y pathogenic mutant is used as a positive control. Each assay consists of total input and immunoprecipitated without (- Ab) or with (+ Ab) anti-V5 antibody. Input lanes are loaded with one-quarter of the starting volume used for immunoprecipitated lanes. M = size marker (BIOLINE).

DISCUSSION

Development of the secondary palate is a complex process influenced by a large number of genes. In mouse mutants, many of these are known to result in a partial or complete CP (Wilkie and Morriss-Kay, 2001). Nevertheless, comparatively little is known about the molecular pathology underlying human

palatal defects. One of the most common known genetic causes of human CP involves *TBX22* (Braybrook *et al.*, 2001; Marçano *et al.*, 2004; Suphapeetiporn *et al.*, 2007). In this study, we have identified mutations in patients with isolated ankyloglossia, dental anomalies, and CLP to expand the phenotypic spectrum. With the exception of the P389Q, the likely pathogenic missense mutations detected are all located in the T-box domain. A high proportion of mutations alter an arginine residue, 3 of which are described in this report. This correlates with the importance of hydrophobic residues in establishing contact with the DNA strand (Muller and Herrmann, 1997). Analysis of our data also supports the underlying mechanism of DNA-binding-dependent transcriptional repression. However, the role of the C-terminal mutation remains enigmatic. While several functional domains have been described for the N-terminal part of *TBX22* (Andreou *et al.*, 2007; Farin*et al.*, 2007), little is known about the C-terminal domain, although we can conclude that it is not involved in DNA binding or activation of the repression domain.

The hypodontia and cleft lip phenotype found in our study support the idea that *TBX22* may also play a significant role in both tooth and upper lip development. However, its role is not yet sufficiently understood to explain why some patients (and some mice) develop an overt cleft, while the majority develop a closed but submucous cleft (Pauws *et al.*, 2009a). Abnormalities of the upper lip and secondary palate are often considered to be etiologically distinct, since they are only rarely found within the same family and have distinct spatio-temporal development and epidemiology (Fogh-Andersen, 1942; Wyszynski *et al.*, 1996; Stanier and Moore, 2004). In contrast, there are several examples indicating that a common molecular mechanism does exist, including *MSX1*, *P63*, and *IRF6* (van den Boogaard *et al.*, 2000; van Bokhoven *et al.*, 2001; Kondo *et al.*, 2002). A broader range of phenotypes can now also be associated with *TBX22*.

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