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Overt Cleft Palate Phenotype and *TBX1* Genotype Correlations in Velo-cardio-facial/DiGeorge/22q11.2 Deletion Syndrome Patients

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Abstract

Velo-cardio-facial syndrome/DiGeorge syndrome, also known as 22q11.2 deletion syndrome (22q11DS) is the most common microdeletion syndrome, with an estimated incidence of 1/2,000 – 1/4,000 live births. Approximately 9–11% of patients with this disorder have an overt cleft palate (CP), but the genetic factors responsible for CP in the 22q11DS subset are unknown. The *TBX1* gene, a member of the T-box transcription factor gene family, lies within the 22q11.2 region that is hemizygous in patients with 22q11DS. Inactivation of one allele of *Tbx1* in the mouse does not result in CP, but inactivation of both alleles does. Based on these data, we hypothesized that DNA variants in the remaining allele of *TBX1* may confer risk to CP in patients with 22q11DS. To test

the hypothesis, we evaluated *TBX1* exon sequencing (n = 360) and genotyping data (n = 737) with respect to presence (n = 54) or absence (n = 683) of CP in patients with 22q11DS. Two upstream SNPs (rs4819835 and rs5748410) showed individual evidence for association but they were not significant after correction for multiple testing. Associations were not identified between DNA variants and haplotypes in 22q11DS patients with CP. Overall, this study indicates that common DNA variants in *TBX1* may be nominally causative for CP in patients with 22q11DS. This raises the possibility that genes elsewhere on the remaining allele of 22q11.2 or in the genome could be relevant.

Keywords

22q11.2 deletion syndrome; *TBX1* sequencing; cleft palate; genomic disorder

INTRODUCTION

Overt cleft palate (CP) is one of the most common birth defects in humans occurring in one per 1,000 live births [Gorlin 2001]. This phenotype occurs commonly in association with many genetic syndromes such as velo-cardio-facial syndrome (OMIM#192430) [Shprintzen et al., 1978] /DiGeorge syndrome (OMIM#188400) [DiGeorge, 1965], or 22q11.2 deletion syndrome (22q11DS). Approximately 9–11% of patients with 22q11DS have an overt cleft of the bony palate, while the rest have mild craniofacial features [Kobrynski and Sullivan 2007; McDonald-McGinn et al., 1999; Hopper et al., 2007].

The *TBX1* gene, a member of the T-box gene family lies within the 22q11.2 region that is hemizygous in patients [Chieffo et al., 1997]. Homozygous *Tbx1* null mutant mice display a spectrum of 22q11DS related phenotypes including CP, but inactivation of one allele of *Tbx1* in the mouse does not result in CP [Lindsay, 2001; Merscher et al., 2001]. Based upon studies of mouse *Tbx1*^{-/-} mutants we hypothesized that DNA variants in *TBX1* on the remaining allele cause CP. The purpose of the study was to analyze the sequencing and genotyping data with respect to presence or absence of CP in a group of patients with 22q11DS.

MATERIALS AND METHODS

Human Subjects

Informed consent was obtained for all participants according to an IRB-approved protocol (1999–201). Blood or saliva samples were obtained from patients with 22q11DS [Guo et al., 2011]. The cohort used for analysis was self-reported as Caucasian. In our 22q11DS cohort, 1,022 patients had DNA samples and 737 of these patients had diagnosed craniofacial findings. Among the 737 Patients, 54 patients had CP (Fig 1).

TBX1 Resequencing and Genotyping

We had DNA sequence results for 360, of whom 328 had craniofacial data and 20 had CP [Guo et al., 2011]. All 737 DNAs were genotyped and in total, 20 patients with CP had *TBX1* DNA sequence data. A detailed description of the DNA sequence analysis of the *TBX1* exons can be found in Guo et al., [2011]. The SNPs (hg18, 2006) were assigned to linkage disequilibrium (LD) blocks using *TBX1* resequencing data from 360 patients (Supplementary Figure 1) [Guo et al., 2011]. Based on the LD structure of the locus, 16 common SNPs and two additional SNPs from the 5'-flanking region were selected because they covered each LD block and thus served as tagSNPs for genotyping the 1,022 subjects. Primer sequences are available on request.

Statistical analyses—Chi-square (χ^2) test was used to evaluate associations between the “genotype” (allele) and individual phenotypes (Supplementary Table II). Since the *TBX1* gene is located within the 22q11.2 deletion region, and all proband DNAs have one copy of the gene, their individual haplotypes were directly observed [Guo et al., 2011]. Variants with a MAF less than 0.01, amounting to 45 were treated as very rare SNP variations [Guo et al., 2011].

Method 1, rare SNP variant analysis: A simple contingency table was constructed to test for differences in the total number of rare SNP variations in the CP group versus the rest of the patients with 22q11DS but without CP. **Method 2, rare SNP variant analysis:** The rare SNP variant allele counts were collapsed mathematically, by assigning each individual a carrier/non-carrier status. This was based on the presence or absence of rare SNP variant minor alleles. A simple contingency table was constructed to test for differences in rare SNP variant minor allele frequency between cases and controls as described [Guo et al., 2011].

RESULTS

Overt cleft palate in our 22q11DS cohort

All of the 737 patients with 22q11DS in this study had a recorded craniofacial anomaly, although not all craniofacial features besides CP were reported at all research sites. Overt cleft palate (CP) occurs in 9–11% of patients with 22q11DS (Table I), which is a frequency of 10 fold over that in the general population. A total of 54 patients (7.3%) in our cohort had CP (Figure 1). This included 29 females and 25 males. There was no significant difference in the prevalence of CP between the two genders ($p=0.89$).

TBX1 resequencing

The hypothesis we tested was whether mutations in the remaining allele of *TBX1* might cause CP. We searched for mutations in exons of *TBX1* in existing DNA sequence data from 20 patients with CP in the cohort of 360 consecutive subjects with 22q11DS that were sequenced [Guo et al., 2011]. We did not find evidence for association with common, or rare SNP variants. Among the 20 patients with CP and 22q11DS that were sequenced, none had predicted pathogenic missense or nonsense mutations. Since the *TBX1* gene is located within the 22q11.2 deletion region, and all patient DNAs have one copy of the gene, their individual haplotypes could be directly observed. Among the 20 individuals with CP, 17 haplotypes were identified (Supplementary eTable I – see Supporting Information online), but we did not find enrichment of specific haplotypes.

TBX1 re-sequencing rare enrichment findings

It has been suggested that enrichment of rare SNP variants, within particular phenotypes could indicate a genetic association with a particular phenotype [Conti et al., 2003; Gong et al., 2001; Griffin et al., 2010; Paylor et al., 2006; Rauch et al., 2004; Torres-Juan et al., 2007; Yagi et al., 2003; Zweier et al., 2007]. We performed a rare SNP variant enrichment analysis on 328 samples with sequence and phenotype data, 20 of which had CP. When analyzed via Method 1 and Method 2, there was no significant difference in the total number of rare SNP variants or rare SNP enrichment in patients with CP versus those with or without any other recorded palatal defect.

Genotype analysis

To determine if common SNPs in the *TBX1* locus were associated with CP, we examined genotype data in 737 subjects, of which 54 had CP, with available data on common SNPs by direct genotyping (Fig 1). Based upon the DNA sequence analysis of the 360 patients, we

identified 16 tagSNPs in three distinct LD blocks (Supplementary eFigure 1 – See Supporting Information online). The SNPs (MAF 0.07–0.44) were used to genotype 737 subjects, 54 of which expressed the CP phenotype [Guo et al., 2011]. We found two nearby SNPs (824 bp apart), rs4819835 and rs5748410, 26 kb upstream of the first exon that showed nominal significance but failed to show significance after correcting for multiple testing (Fig 2; Fig 3). This SNP, rs5748410 was also nominally significant when subjects with CP were compared to those with SCP, OSCP, or BU ($p < 0.04$). Of note, the two SNPs are not in LD ($R^2 = 0.004$, $D' = 0.09$), thus they are independent, and they are not conserved in mammalian species (Fig 4C).

DISCUSSION

In this study, we analyzed DNA sequence and genotype data in our cohort of 737 patients with 22q11DS [Guo et al., 2011] for association with CP. We found that the remaining *TBX1* gene on 22q11.2 is not a major risk factor for CP in 22q11DS. Even though the two SNPs found upstream of *TBX1* (rs5748410; rs4819835) were not statistically significant after correcting for multiple testing, they are interesting biologically, since they lie in the upstream region that could tag potential regulatory SNPs. One possibility is that these SNPs mark DNA variants that directly alter *TBX1* expression levels thus changing risk of having the CP phenotype as secondary to the deletion on chromosome 22. Unfortunately, both SNPs are in a region that does not have well-defined LD blocks (Figure 4). Further functional studies using mouse models need to address if the variants in this region tagged by the SNPs identified could regulate the gene expression of *Tbx1*. The CP phenotype includes a cleft of the bony and soft palates, as compared to a cleft of the soft palate alone (SCP and OSCP), thus representing a more severe abnormality, with developmentally distinct origins [Moore and Persaud, 2003; Gorlin et al, 2001]. Of note, the CP patients represented a diagnostically reliable phenotype when examining this population. Some of the more subtle palatal features (SCP, OSCP, BU; Fig 2) were not always scored and therefore were not a focus of this investigation. Although this may have contributed to the current study's failure to reject the null hypothesis, a relatively small sample size, as well as the complexity of data processing may have also played a role despite attempts at surveillance and statistical correction. Implementing a protocol for phenotype diagnosis and further deep genotype resequencing could also improve the data quality.

In this report, we tested *TBX1*-genotype correlations in patients with CP and 22q11DS. Our results suggest a possible association between two adjacent SNPs upstream of *TBX1* and the CP phenotype, but were not significant after correcting for multiple testing. Over 400 Mendelian disorders in which clefting occurs have been reported and studies have suggested that many candidate genes may be responsible (<http://www.ncbi.nlm.nih.gov/OMIM>). Although many candidate genes have been reported, the identification of clear correlations between genetic mutations and the cleft phenotype has been complicated by genetic and allelic heterogeneity, incomplete penetrance, and phenotypic variability [Jugessur et al., 2009]. Additionally, few reports have focused on CP without cleft lip involvement. Focusing attention to the overlapping pathways between these previously identified loci and *TBX1* may be of interest. By examining the CP phenotype in a larger sample size of patients with 22q11DS, future studies could focus attention on the other allele of 22q11.2 or elsewhere in the genome. Alternatively, investigation of other syndromic and nonsyndromic cases of CP may yield better understanding of the complex genetic pathways that shape the craniofacial complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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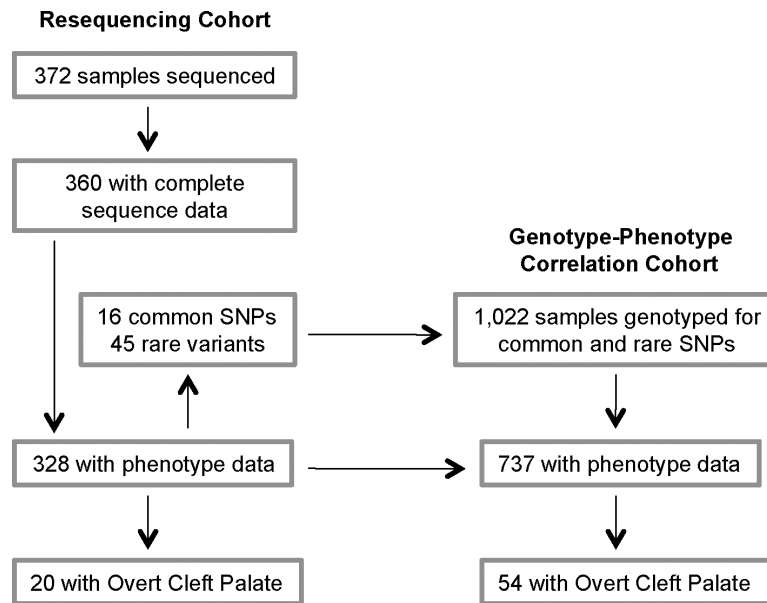


Figure 1. Methods flow chart detailing the resequencing and genotyping cohorts

A total of 372 DNA samples from patients with 22q11DS were used to sequence exons of *TBX1* [Guo et al., 2011]. Among the 372, 20 had CP, complete sequence data, and craniofacial data. A total of 1,022 DNAs from 22q11DS patients were genotyped for common and rare SNPs in the *TBX1* locus. Among those 1,022, 54 had CP. From the resequencing cohort, 71 variants were identified. A total of 16 common SNPs were used in the genotype-phenotype correlations and the 45 rare variants were analyzed within the resequencing cohort samples.

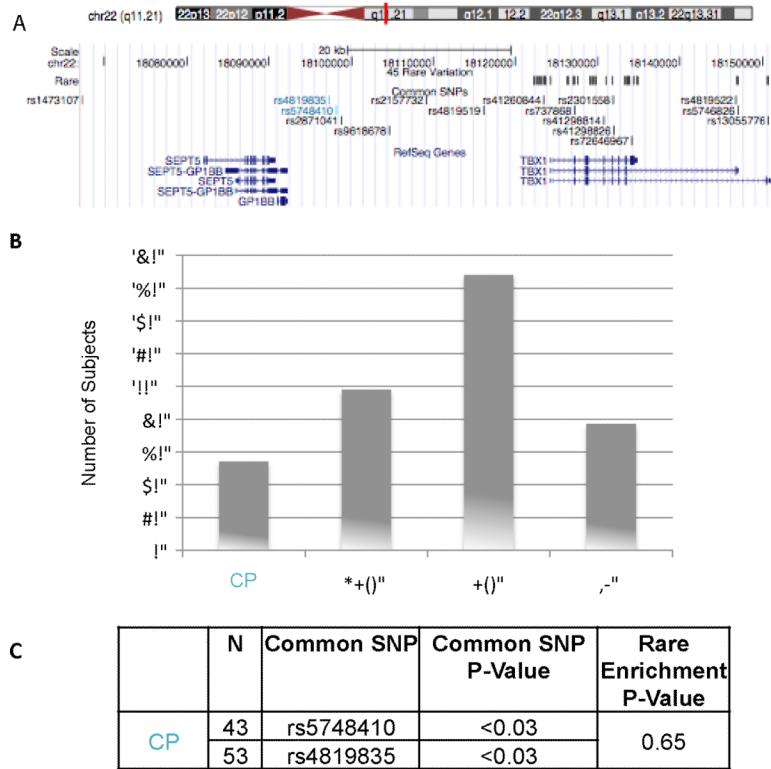


Figure 2. Representative common SNPs in the *TBX1* locus, CP phenotype group in 22q11DS and SNP association values

(A) Snapshot from the UCSC genome browser, hg18, March 2006, of the interval of chromosome 22q11.2 containing the *TBX1* gene. Common SNP markers and rare SNPs (dense mode) within *TBX1* locus and outside the *TBX1* gene region are shown. Two SNPs with nominal association to CP are colored blue (Fig 2A).

(B) Number of subjects with each craniofacial phenotype examined. CP= Overt Cleft Palate, OSCP= Occult Submucous Cleft Palate, SCP= Submucous Cleft Palate, BU= Bifid Uvula.

(C) The number of DNAs evaluated for the two common SNPs showing individual nominal p-values is indicated.

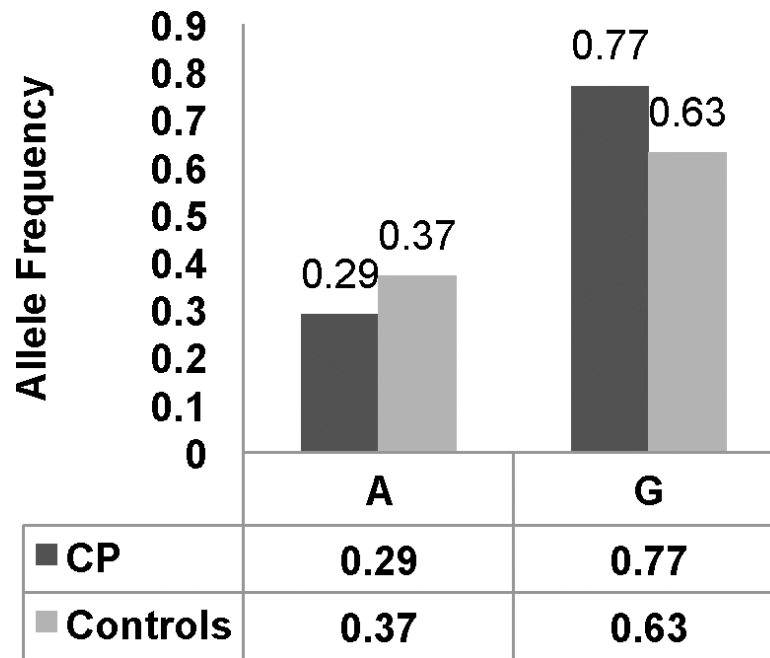


Figure 3. SNP rs4819835: Genotype-phenotype correlations and allele frequencies for the overt cleft palate phenotype

Cases: Patients with overt cleft palate. Controls: All other subjects. Chi-square p-value: 0.03. Odds Ratio: 2.04 (1.02–4.34)

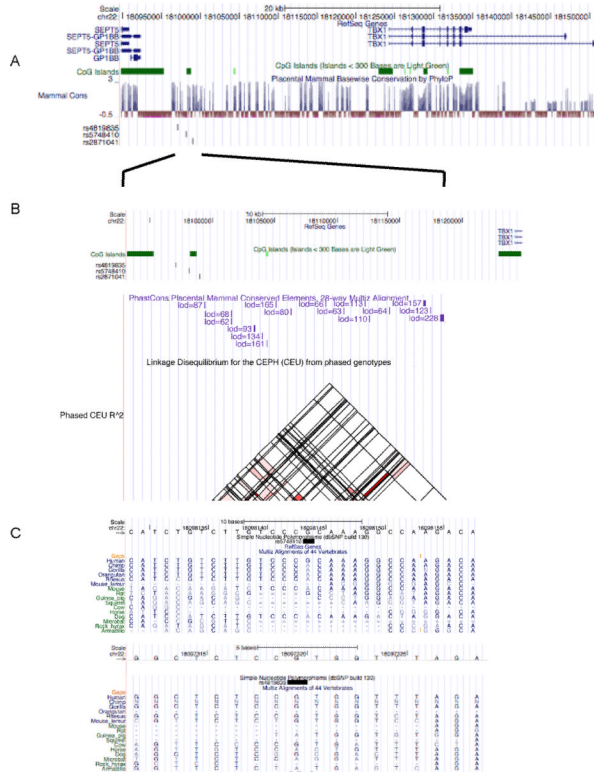


Figure 4. Evaluation of SNPs rs5748410 and rs4819835 upstream of *TBX1*
 (A) Snapshot from the UCSC genome browser showing the location of *TBX1* with respect to SNPs rs5748410 and rs4819835, below. It also includes the mammalian evolutionary conservation track.
 (B) The *GP1BB-TBX1* intergenic interval surrounding SNPs rs5748410 and rs4819835 is shown. Included is the CpG island track identifying high CpG content. We modified the PhastCons Placental Mammalian Conservation Elements, 28-way Multiz Alignment track showing blocks of evolutionary conservation to include only those regions with a lod score greater than 50. The Linkage Disequilibrium track for the CEPH Caucasian population is displayed (r^2 values). The region does not show clearly defined LD blocks.
 (C) Placental mammalian DNA sequence pile-up around SNPs rs5748410 and rs4819835. Part of the Vertebrate Multiz Alignment & Conservation of 44 Species track was included where DNA sequence existed for the mammalian species shown. The SNP rs5748410 has a G or A in that position and rs4819835 has a G or A in the reference sequence of mammalian species indicated in the snapshot from the UCSC Genome Browser. Species without data are excluded from this figure.

Table 1

Craniofacial Phenotype	Observed Prevalence	Previously Reported Prevalence	Reference
Cleft Palate	7.3%	9–11%	(Kobrynski and Sullivan 2007)
Submucous Cleft Palate	22.8%	5–16%	
Bifid Uvula	10.4%	5%	
Occult Submucous Cleft Palate	13.3%	N/A	
Palatal Abnormalities	55%	69–100%	(Kobrynski and Sullivan 2007)