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Candidate Gene Studies in Hypodontia Suggest Role for *FGF3*

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Abstract

The majority of tooth agenesis cases are mild (hypodontia) and typically not associated with the gene mutations linked to oligodontia. From this, we hypothesize that most cases of tooth agenesis fit a polygenic mode of inheritance, where several genes with small effects cause a variety of varying phenotypes. In this study, we looked at 18 not typically studied genes in this condition, to ascertain their contribution to hypodontia. Our study subjects consisted of 167 patients with hypodontia and their parents from two cohorts (one from Brazil and one from Turkey). An additional 465 DNA samples (93 cases with hypodontia and 372 controls without family history for tooth agenesis or oral clefts) from Brazil were also available for this study. 93 single nucleotide polymorphisms that maximally represent the linkage disequilibrium structure of the genes for the 18 genes were selected and genotyped using Taqman chemistry. Chi-square was used to test if genotype distributions were in Hardy-Weinberg equilibrium, and 24 markers that were in Hardy-Weinberg equilibrium and had allele frequencies higher than 5% in a panel of 50 CEPH samples were further tested. Association between hypodontia and genetic variants was tested with the transmission disequilibrium test within the program Family-Based Association Test (FBAT) and by using chi-square and Fisher's exact tests. Alpha at a level of 0.05 was used to report results. Results suggest possible associations between several genes and hypodontia in the three populations. In the Turkish cohort (n=51 parent-affected child trios) the most significant results were as follows*: FGF3* rs1893047, p=0.08; *GLI3* rs929387, p=0.03; *GLI3* haplotype rs929387-rs846266, p=0.002; and *PAX9* rs2073242, p=0.03. In the Brazilian cohort (n=116 parent-affected child trios), the results were as follows: *DLX1* rs788173, p=0.07; *FGF3* rs12574452, p=0.03; *GLI2* rs1992901, p=0.03; and *PITX2* rs2595110, p=0.01. The second Brazilian cohort also suggested that *FGF3* (rs12574452, p=0.01) is associated with hypodontia

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and added *EDAR* (rs17269487, p=0.04), *LHX6* (rs989798, p=0.02), and *MSX1* (rs12532, p=0.003). Our results suggest that several genes are potentially associated with hypodontia and their individual contributions may be modest. Hence, these cases may not be explained by inactivating mutations such as many oligodontia cases segregating in a Mendelian fashion but rather are influenced by one or more susceptibility alleles in multiple small effect genes.

Introduction

Severe cases of tooth agenesis (oligodontia or absence of six or more teeth) have a prevalence of 0.3% and comprise just a small percentage of the total instances of the condition (Celikoglu et al., 2010). A subset of these cases can be linked to mutations in *MSX1*, *PAX9*, *AXIN2*, *EDA*, *EDAR*, and *EDARADD* (reviewed by Vieira, 2012) and many appear to have mutations in *WNT10A* (van den Boogaard et al., 2012). However, the vast majority of tooth agenesis cases involve absence of just one or two (hypodontia) teeth and they are typically not associated with deleterious mutations in the genes described above. Furthermore, we have shown statistical evidence for association between genetic variation in *MSX1*, *PAX9*, *TGFA*, *IRF6*, *FGFR1*, *AXIN2*, *MMP1*, and *MMP20* and hypodontia suggesting that these cases fit a polygenic mode of inheritance rather than a single gene disorder (Vieira et al., 2004; Vieira et al., 2007; Vieira et al., 2008; Callahan et al., 2009a and b; Küchler et al, 2011).

A few hundred genes have been associated with dental development and can potentially contribute to tooth agenesis. These genes code for signaling molecules, transcription factors, and factors controlling cell proliferation and differentiation (Thesleff, 2003). Much of the work aiming to understand the genetics of tooth agenesis has focused on families with multiple affected individuals being analyzed by linkage and/or direct sequencing with the goal of identifying a major gene effect. Our group is one of the few that focuses on the hypothesis that most of the cases of tooth agenesis fit a multifactorial or polygenic mode of inheritance and may be influenced by several genes with small effects. The aim of the present study was to expand our investigations to less studied genes searching for evidence that they contribute to hypodontia.

Materials and Methods

Hypodontia Subjects

Our study group consisted of 167 patients with tooth agenesis and their parents. The patients were from two different cohorts, 116 were from Rio de Janeiro, Brazil, which is an admixed population of Europeans (from Portugal) and Africans, with a very small percentage of Native South Americans. This population contains 71 sporadic cases and 41 familial cases (which includes four instances of affected siblings) and samples were collected between January 2001 and June 2002. Seventy-four were females and 42 were males. The second cohort consisted of 51 parent-child trios from Turkey. Twenty-six of the affected subjects were females and 25 were males. All Turkish cases with the exception of one were of sporadic origin and were collected between June 2004 and June 2006. Tooth agenesis was the sole disorder affecting these patients and second premolars were the teeth most commonly absent, followed by lateral incisors. None of the families reported history for clefts. One family from Brazil was segregating isolated oligodontia and was excluded from further analysis (Vieira et al., 2004; Vieira et al., 2008).

An additional 465 DNA samples (93 cases with hypodontia and 372 controls without family history for tooth agenesis or oral clefts) from Rio de Janeiro were also available for this study. These samples were collected between January 2010 and July 2011 and do not

overlap with the familial cohort collected almost a decade earlier. Details of the three study groups are described on Table 1.

The study was approved by the University of Pittsburgh Institutional Review Board (IRB), as well as the appropriate ethics committee at the Federal University of Rio de Janeiro and Istanbul University, and/or appropriate informed consent was obtained from human subjects. After informed consent was obtained, cheek swabs, saliva, or whole blood were collected from each individual. Clinical analysis, sample collection, and DNA extraction were performed using a consolidated protocol described elsewhere (Jezewski et al., 2003; Vieira et al., 2004; Vieira et al., 2008).

Genetic Association Studies

Besides *MSX1* and *PAX9*, we selected 16 genes for this study: *BARX1*, *BMP4*, *CARTPT*, *DLX1*, *DLX2*, *EDA*, *EDAR*, *FGF3*, *FGF10*, *GLI2*, *GLI3*, *INHBA*, *LEF1*, *LHX6*, *MSX2*, and *PITX2*. We selected 93 single nucleotide polymorphisms (SNPs) for the 18 genes studied (Supplemental Material 1) from the International HapMap Project database (http://www.hapmap.org). We used the function *"Download tag SNP data"* and selected polymorphisms as representative of the polymorphisms in the region. We selected 93 polymorphisms that maximally represent the linkage disequilibrium structure of the genes to avoid redundant information (Carlsson et al., 2004). Preference was given to polymorphisms with high heterozygosity levels in Whites.

The 93 selected SNPs were tested first in a panel of 50 unrelated CEPH (Foundation Jean Dausset-Centre d'Etude du Polymorphisme Humain) DNA samples. These 50 samples were genotyped using Taqman chemistry (Ranade et al., 2001) on an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA, USA). Assays and reagents were supplied by Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Chi-square was used to test if genotype distributions were in Hardy-Weinberg equilibrium. Based on information content (heterozygosity) and Hardy-Weinberg equilibrium, 24 markers (rs1893047, rs35620964, rs6866864, rs4365796, rs900379, rs1042484, rs17563, rs929387, rs846266, rs989798, rs2237435, rs788173, rs2595110, rs12574452, rs1992901, rs1955734, rs4904155, rs12532, rs13029834, rs7585138, rs12992554, rs899259, rs17269487, rs2073242) were further tested. Association between hypodontia and genetic variants was tested with the transmission disequilibrium test (TDT) within the programs Family-Based Association Test (FBAT) (Horvath et al., 2001). When possible, a haplotype analysis was also performed in FBAT. Also, the differences in genotype and allele frequencies between cases and controls from Brazil were tested by chi-square or Fisher's exact tests. The case-control cohort was analyzed taking into consideration cases having specific types of teeth missing: at least one maxillary lateral incisor, at least one mandibular incisor, at least one premolar, and at least one canine or molar. We used logistic regression to test if variation in *MSX1* or *PAX9* influenced the association found for *FGF3* variants in the case-control cohort.

Results

Table 2 summarizes the most significant results in the family data and Table 3 in the case-control analysis. Complete summary of the results are presented as supplemental tables. The most significant results include an association between the *GLI3* haplotype rs929387-rs846266 and hypodontia in the Turkish families (p=0.002) and *MSX1* rs12532 and hypodontia in the Brazilian case-control sample missing at least one premolar (p=0.003). *FGF3* was the only gene that had suggestive association results for the three populations studied. Logistic regression showed a trend for association of the genotype GG of *MSX1*

rs12532 (p=0.09) when analysis was adjusted by variation in *FGF3* and *PAX9* in the case-control sample (Table 4).

Discussion

Genes such as *MSX1* and *PAX9*, which when mutated are associated with autosomal dominant forms of oligodontia showed suggestive association with less severe forms of tooth agenesis at least in one of the studied groups. The most interesting result is the suggestive association of *FGF3* with hypodontia in all three comparisons, including the same allele (the most common one in the population) being over-represented in both Brazilian cohorts. Both *FGF3* SNPs (rs12574452 and rs1893047) are located in introns of the gene and their potential functional roles are unexplored. Mutations in *FGF3* are associated with variable inner ear malformations, microtia, and microdontia (Tekin et al., 2007). *FGF3* has been previously studied in seven Finish families with autosomal dominant incisor-premolar hypodontia and linkage analysis suggested no etiologic role of *FGF3* (Arte et al., 1996). Since our results suggest that markers of *FGF3* are associated with hypodontia, these contradictory results may be explained by the inherited differences of the two approaches. Whereas linkage is well suited for the identification of rare genetic variants with strong genetic effects, association will detect more common variants with weaker effect sizes that may increase individual susceptibility to the defect or be in linkage disequilibrium with other variants that may have functional roles. Another possibility is that *FGF3* is not primarily involved with incisor-premolar hypodontia and this clinical phenotype is possibly a distinct clinical entity.

Although we found a suggestive association between *FGF3* and hypodontia in three independent analyses, these results should be considered hypothesis generating and taken cautiously. If a stricter threshold for association were considered (*i.e.*, Bonferroni correction) in the attempt to correct for multiple tests, these association signals would be missed. The best approach is continuing to expand the study population to replicate these findings. Another limitation of our study is that a large number of SNPs could not be further tested and many of the genes we pre-selected to study could not be completely interrogated. This fact implies that our study cannot exclude the possibility of a role of any of the 18 genes we proposed to study in hypodontia.

Mitogenic activities of *Fgf3* stimulate epithelial growth during tooth morphogenesis (Wang et al., 2007). $Pax9^{+/-};Msx1^{+/-}$ mouse mutants form a smaller lower incisor dental papilla, indicating that these two genes interact prior to the bud stage, that leads to smaller lower incisors in the animals. But it is remarkable that the expression of *Fgf3* is strongly affected in these mutants at all stages of dental development and becomes undetectable after day E14.5 (Nakatomi et al., 2010). We have previously suggested that *MSX1* and *PAX9* interactions may be involved in hypodontia (Vieira et al., 2004; Ogawa et al., 2006) and the results from the mouse models confirm this hypothesis and implicate *FGF3* in this pathway. Our samples include at least 40% of cases with incisor agenesis and the suggestive associations with *FGF3* are possibly related to those cases. We propose that sporadic incisor agenesis in humans may result from the imbalance of *MSX1* and *PAX9* interactions that may lead to *FGF3* under-expression. Our preliminary analysis in the case-control sample provided suggestive evidence that the association of genetic variation in *MSX1* with hypodontia is influenced by variation in *FGF3* and *PAX9*.

Conclusion

We suggest based on our data that genetic variation affecting the expression of *FGF3* could increase individual susceptibility to hypodontia, particularly agenesis of the lateral incisors.

In addition to a role of *FGF3*, our data suggest that *DLX1*, *EDAR*, *GLI2*, *GLI3*, *LHX6*, *MSX1*, *PAX9*, and *PITX2* may have a role in isolated hypodontia. Whereas *MSX1* and *PAX9* are genes that for years have been implicated in tooth agenesis (Vieira, 2012), the other genes are less studied. *EDAR* and *PITX2* are examples of genes linked to syndromic forms of tooth agenesis that may play a role in isolated cases. Using what has been learned from the study of single gene syndromes that include tooth agenesis has been a successful tool in determining the presence of association between genetic variation and isolated tooth agenesis (Vieira et al., 2007). The other genes were selected based on evidence they are involved in dental development and are part of common pathways, which demonstrates that future genetic association studies should take into consideration genetic pathways rather than studying unrelated genes only.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Demographic data of the studied groups.

Characteristic	Brazilian (Families)	Turkish (Families)	Brazilian (Cases)
	N(%)	N(%)	N(%)
Gender distribution			
Males	42(36.2)	24(47.0)	37(39.8)
Females	74(63.8)	27(53.0)	56(60.2)
Number of teeth missing			
1	44(37.9)	7(13.7)	47(50.5)
2	46(39.7)	17(33.3)	32(34.4)
3 to 5	26(22.4)	27 (53.0)	14(15.1)
Type of teeth more often missing			
(total teeth missing)	274	207	175
Premolars	130 (47.4)	83(40.1)	88(70.3)
Incisors	98 (35.8)	86(41.5)	73(41.7)
Canines and molars	46(16.8)	38(18.4)	14(8.0)
Associated dental anomalies	16 (13.8)	None	10 (10.8)
Positive family history	41 (35.3)	1 (2.0)	8 (8.6)

Table 2

Summary of the most significant association results in the family-based analysis.

Study Group/Gene	Marker/Haplotype	p-value	Number of Informative Families	Minor Allele Frequency
Turkey: 51 affected child/father/mother trios				
FGF3	rs1893047	0.08	26	0.49
GLI3	rs929387	0.03	26	0.41
GLI3 haplotype	rs929387-rs846266	0.002	26	0.48
PAX9	rs2073242	0.03	26	0.4
Brazil: 116 affected child/father/mother trios				
DLX1	rs788173	0.07	55	0.41
FGF3	rs12574452	0.03	50	0.35
GLI2	rs1992901	0.03	50	0.25
PITX2	rs2595110	0.01	25	0.31

Table 3

Summary of the results of the case-control analysis.

Brazil: 93 cases and 372 controls		p-value	Number of Cases	Allele Frequency Controls	Cases
EDAR	rs17269487	0.04 (all cases)	38	0.42	0.53
		0.02 (only when cases of maxillary lateral incisor agenesis were considered)	38	0.42	0.57
	rs12992554	0.05 (only when cases of maxillary lateral incisor agenesis were considered)	38	0.44	0.57
FGF3	rs12574452	0.01 (only when cases of maxillary lateral incisor agenesis were considered)	10	0.31	0.6
GLI3	rs929387	0.08 (only when cases of mandibular incisor agenesis were considered)	44	0.33	0.15
LHX6	rs989798	0.02 (only when cases of premolar agenesis were considered)	44	0.24	0.63
MSX1	rs12532	0.003 (only when cases of premolar agenesis were considered)	44	0.37	0.47

Table 4

Regression adjusted for genotypes in markers of *FGF3, MSX1* and *PAX9* in the case-control cohort.

Gene	rs#	Variables	Univariate analysis		Multivariate analysis	
		P-value	OR (95%CI)	P-value	OR (95%CI)	
FGF3	rs12574452	AA	Reference		Reference	
		AG	0.320	1.67(0.60–4.62)	0.137	2.62(0.73–9.35)
		GG	0.553	1.36(0.49–3.78)	0.223	2.21(0.61–7.95)
MSX1	rs12532	AA	Reference		Reference	
		AG	0.917	1.02(0.59–1.76)	0.992	1.00(0.52–1.90)
		GG	0.121	1.68(0.87–3.24)	0.091*	2.05(0.89–4.76)
PAX9	rs4904155	CC	Reference		Reference	
		CG	0.646	0.88(0.53–1.47)	0.388	0.71(0.33–1.52)
		GG	0.455		0.335	0.59(0.20–1.70)
PAX9	rs2073242	AA	Reference		Reference	
		AC	0.833	1.08(0.50–2.31)	0.700	1.22(0.44–3.37)
		CC	0.939	1.09(0.48–2.17)	0.771	1.17(0.39–3.49)

Note: The analyses were adjusted for genotypes in *FGF3, MSX1* and *PAX9*. OR (95% C.I.)= Odds ratios; 95% confidence intervals.