Rethinking isolated cleft lip and palate as a syndrome

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Objective. The goal of the present work was to use dental conditions that have been independently associated with cleft lip and palate (CL/P) as a tool to identify a broader collection of individuals to be used for gene identification that lead to clefts.
Study design. We studied 1573 DNA samples combining individuals that were born with CL/P or had tooth agenesis, supernumerary teeth, molar incisor hypomineralization, or dental caries with the goal to identify genetic associations. We tested 2 single-nucleotide polymorphisms that were located in the vicinity of regions suggested to contribute to supernumerary teeth. Overrepresentation of alleles were determined for combinations of individuals as well as for each individual phenotypic group with an α of .05.
Results. We determined that the allele C of rs622260 was overrepresented in all individuals studied compared with a group of unrelated individuals who did not present any of the conditions described earlier. When subgroups were tested, associations were found for individuals with hypomineralization.
Conclusions. Although we did not test this hypothesis directly in the present study, based on associations reported previously, we believe that CL/P is actually a syndrome of alterations of the dentition, and considering it that way may allow for the identification of genotype-phenotype correlations that may be useful for clinical care. (Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:307–312)

The endeavor of identifying genes responsible for the etiology of common complex oral and craniofacial conditions has been the focus of many research groups for the last 3 decades. For isolated forms of cleft lip and palate, it started with *TGFA* in 1989[1] and continued more recently with hypothesis-free work scanning the whole genome continues to suggest new associations. A concept our group has proposed is that isolated forms of cleft lip and palate are often accompanied by other minor anomalies of the dentition. Individuals born with cleft lip and palate are at least 4 times more likely to present dental anomalies, such as tooth agenesis and supernumerary teeth.[2] We also know that individuals born with clefts have more enamel defects.[3] Individuals born with cleft lip and palate are also historically suggested to have more dental caries,[4,5] even though our own data from populations that have no or very little access to dental care suggest otherwise.[6,7] Furthermore, tooth agenesis on occasion is associated with supernumerary teeth,[8,9] and individuals with enamel defects such as molar incisor hypomineralization are suggested to have more dental caries[10] or tooth agenesis of second premolars[11] or co-occurrence of tooth agenesis and supernumerary teeth.[12] Based on these correlations (Figure 1), we designed an experiment that included individuals born with clefts or who have tooth agenesis, supernumerary teeth, molar incisor hypomineralization, or dental caries, and compared them with individuals ascertained as having none of these conditions, to test for association with markers in the vicinity of 2 genes that have been suggested as possibly contributing to supernumerary teeth (*HMCN1* and *IGSF9 B*)[12] with the assumption that isolated forms of clefts are actually syndromes that combine 1 or more alterations of the dentition that share genetic contributors. The goal of the present work was to use dental conditions that have been independently associated with cleft lip and palate as a tool to identify a broader collection of individuals to be used for gene identification that lead to clefts. The combined analysis would yield the identification of genes that contribute to the occurrence of multiple signs (syndrome) or a particular one. Here we report these analyses and continue to provide evidence that isolated forms of cleft lip and palate are likely syndromes that potentially include multiple alterations of the dentition.

Statement of Clinical Relevance

We believe that isolated cleft lip and palate is actually a syndrome of alterations of the dentition and considering it that way may allow for the identification of genotype-phenotype correlations that may be useful for clinical care.

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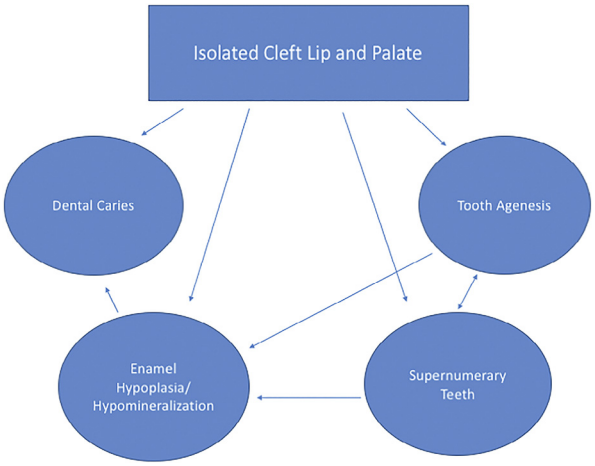

Fig. 1. Schematic representation of oral and craniofacial phenotypes that occur in association.

PATIENTS AND METHODS

We used DNA samples from 1573 individuals who were ascertained to have distinct oral and craniofacial alterations as part of our decade-long studies in oral clefts and other dental abnormalities. These individuals are summarized in Table I and described in this section. All participants signed an informed consent document before entering into this study. Parents consented for their offspring, and age-appropriate consent was obtained from all children older than 7 years. This protocol was approved by both the Istanbul University and University of Pittsburgh Institutional Review Boards. Genomic DNA was obtained from whole saliva.

The first study group[14] was composed of 573 individuals, 158 with clefts (59 with bilateral cleft lip and cleft palate, 10 with cleft palate only, 3 with left cleft lip only, 67 with left cleft lip and cleft palate, and 23 with right cleft lip and cleft palate), 254 unaffected family members, and 161 nonrelated individuals with no history of syndromic clefting. Seventy-three individuals were the product of consanguineous marriages (41 with clefts, 32 from the nonrelated individual pool).[14] All participants were recruited at the Department of Pedodontics clinics, Istanbul University, Turkey. Individuals born with isolated forms of cleft of the lip with or without cleft palate or cleft palate only and all of their available first-degree relatives were invited to participate in this study between October 2007 and October 2009. We also invited at least 1 unrelated individual for each cleft case recruited of the same age and sex during the same period. All study participants were examined in a dental office by the same professional (M.K.), and panoramic radiographs were available for all participants. Dental anomalies outside the cleft area (tooth agenesis, supernumerary teeth, macrodontia, microdontia, malocclusion, and enamel hypoplasia) were recorded. Tooth agenesis was defined based on the age of the individual and when initial tooth formation would be visible radiographically. As expected,[2] participants with clefts had more dental anomalies outside the cleft area than controls (tooth agenesis in 31 cases vs in 7 controls, supernumerary teeth in 34 cases vs in 1 control, macrodontia and microdontia in 40 cases vs in 9 controls, enamel hypoplasia in 30 cases vs in 21 controls).[14]

The second study group[15] consisted of 52 unrelated patients with sporadic tooth agenesis (29 participants had missing incisors, 44 had missing premolars, 8 had missing molars, and 10 had missing canines; 7 participants were missing just 1 tooth, 17 were missing 2 teeth, and 27 were missing 3 teeth or more) and their parents and siblings (total of 170 individuals) who were recruited in the metropolitan area of Istanbul. No one was the result of a consanguineous marriage. No one reported to have another relative affected by tooth agenesis, oral clefts, or anosmia. Probands had at least 1 developmentally missing tooth, excluding third molars. Twenty-seven participants in this group were female and 25 were male. All cases were of sporadic origin. 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 a history of clefts.[15]

The third study group included unrelated individuals who were enrolled in the Pedodontics Clinics of Istanbul University and in daycare facilities in Istanbul, Turkey (n = 245) and were ascertained to have molar incisor hypomineralization (MIH).[16] No one was the result of

Table I. Summary of the individuals studied (N = 1573)

	Phenotype			
Condition originally ascertained	*Affected (N)*	*Unaffected (N)*	*Consanguinity (N)*	*Total (N)*
Cleft lip and palate	158	415	73	573
Tooth agenesis	52	118	0	170
Supernumerary teeth	83	129	21	212
Molar-incisor hypomineralization	163	82	0	245
Dental caries	170	203	Unknown	373
Total individuals included in analyses	508*	88†	—	596

*Individuals with associated asthma from Ergöz et al.[13] were excluded from further analyses.
†Individuals confirmed to be not affected by any of the phenotypes listed here.

a consanguineous marriage. The exclusion criteria included having evidence of a syndrome, fluorosis, or use of a fixed appliance. Calibrated examiners carried out the clinical examination (E.B.T. calibrated M.B.). Examination calibrations were performed according to the following protocol: First, the calibrator presented to the examiner the criteria for MIH detection, showing pictures of several situations to be noted in the examination and discussing each of these situations in a session that lasted 1 to 2 hours. Next, the calibrator and examiner(s) examined 10 to 20 patients and discussed each case. E.B.T. and M.K. prescreened patients, and M.B. performed the full examination, with a κ of 1.0. The MIH diagnosis was performed according to the European Association of Paediatric Dentistry criteria.[17] The 245 individuals were defined as either a participant with the MIH phenotype or as patients with no evidence of MIH (including no evidence of fluorosis).

The fourth study group was composed of 373 individuals with or without caries experience.[13,18,19] These patients were recruited at the Pedodontics Clinics of Istanbul University and daycare facilities in the city of Istanbul. One of the authors (A.P., N.E., M.B.) carried out the clinical examination after being calibrated by an experienced specialist (F.S.). The intraexaminer agreement was assessed by a second clinical examination in 10% of the sample after 2 weeks, with a κ of 1.0. Presence of caries was defined as individuals having decayed or filled tooth surfaces or teeth extracted due to caries, and individuals with no evidence of caries (including no evidence of white-spot lesions) and no history of caries were called caries free. Examinations were done with the use of a flashlight and mouth mirror. Caries experience was scored by the decayed, missing, and filled teeth (dmft/DMFT) and decayed, missing, and filled surfaces (dmfs/DMFS) indexes according to World Health Organization guidelines. We had no information about consanguinity in this group.

The final study group consisted of 212 individuals, in which 83 were ascertained as having supernumerary teeth and their parents and siblings were included in the study when possible. Supernumerary teeth were confirmed by radiographic examination. Twenty-one individuals were the result of a consanguineous marriage. Most of cases had mesiodens (N = 74), with 5 cases having supernumerary maxillary premolars or molars and 4 cases having supernumerary mandibular premolars or molars.

Because we had both families (parents with between 1 and 5 offspring) and unrelated unaffected individuals, we could perform family-based and case-control analyses. Genomic DNA samples were obtained from saliva. Genotyping was performed by the TaqMan method,[20] with a QuantStudio 6 Flex instrument and predesigned probes (Applied Biosystems, Foster City, CA, USA). The markers rs10798049 and rs622260 were genotyped (supplementary file). For all the comparisons, we used Fisher exact test for both the allelic transmission and logistic regression to determine the genotypic association between case (as defined by presence of a dental or craniofacial alteration) and the single-nucleotide polymorphism genotypes, as implemented in PLINK.[21] The primary comparison group consisted of 88 individuals that we could confirm were not born with oral clefts, did not have dental anomalies, and were caries free. This group includes everyone in the sample who were ascertained for all phenotypes we included in this studied and confirmed as being unaffected. No one was the result of consanguineous marriage. This was a rigorously defined group to allow for effectively testing our hypothesis. In the first pass, we compared these 88 unaffected individuals with all individuals affected by at least 1 of the previously described conditions (N = 508). We performed just 2 tests, 1 for each genetic marker. In the second pass, we compared unaffected individuals with subsets of affected conditions and to just 1 condition at a time (or composite of conditions). We did that for both markers, for the one that had an association to see if a particular phenotype or certain combinations of these phenotypes were associated with the genetic variants, and for the other that did not have an association with the total sample to determine that a lack of evidence for association would remain when the subsets were tested. We decided not to test for additional subgroups within each phenotype (ie, clefts based on laterality or anatomic structure affected, dental anomalies based on teeth affected) to not increase further the number of tests to be performed.

RESULTS

In the analysis of the group that combined individuals affected by isolated cleft lip and palate, tooth agenesis, supernumerary teeth, molar incisor hypomineralization, or dental caries, we found an association with rs622260 but not with the rs10798049 marker. We determined that the allele C of rs622260 was overrepresented in all individuals studied compared with a group of unrelated individuals who did not present any of these conditions ($P = .05$). To determine if this association was related to a particular subgroup, we then tested each phenotype individually. We found evidence that the association with the marker rs622260 was driven by the subset of samples with molar incisor hypomineralization (Table II; Figure 2). When subgroups were tested, associations were identified for individuals with molar incisor hypomineralization ($P = .05$), isolated cleft lip and palate or molar incisor hypomineralization ($P = .0003$), and dental caries or molar incisor hypomineralization ($P = .005$). No other combinations had suggestive association. No further analyses for the marker rs10798049 had an association. Family-based analysis did not reveal any associations (data not shown).

Table II. Summary of the association results

Gene marker	Phenotype	Minor allele frequency in affected individuals	Minor allele frequency in unaffected individuals (N = 88)	P
IGSF9 B rs10798049	All cases combined (N = 508)	0.27	0.31	.37
	Isolated cleft lip and palate (N = 195)	0.25	0.31	.21
	Tooth agenesis (N = 52)	0.24	0.31	.35
	Supernumerary teeth (N = 122)	0.27	0.31	.47
	Dental caries (N = 134)	0.29	0.31	.77
	Molar incisor hypomineralization (N-140)	0.28	0.31	.52
	Isolated cleft lip and palate + tooth agenesis + supernumerary teeth (N = 369)	0.26	0.31	.22
	Dental caries + molar incisor hypomineralization (N = 274)	0.28	0.31	.69
	Isolated cleft lip and palate + molar incisor hypomineralization (N = 335)	0.26	0.31	.31
	Isolated cleft lip and palate + dental caries (N = 229)	0.27	0.31	.41
	Isolated cleft lip and palate + tooth agenesis (N = 247)	0.25	0.31	.25
	Isolated cleft lip and palate + supernumerary teeth (N = 317)	0.26	0.31	.25
	Tooth agenesis + supernumerary teeth (N = 174)	0.26	0.31	.35
HCMN1 rs622260	All cases combined (N = 508)	0.41	0.32	.05
	Isolated cleft lip and palate (N = 195)	0.39	0.32	.16
	Tooth agenesis (N = 52)	0.35	0.32	.58
	Supernumerary teeth (N = 122)	0.25	0.32	.18
	Dental caries (N = 134)	0.35	0.32	.58
	Molar incisor hypomineralization (N-140)	0.41	0.32	.05
	Isolated cleft lip and palate + tooth agenesis + supernumerary teeth (N = 369)	0.34	0.32	.72
	Dental caries + molar incisor hypomineralization (N = 274)	0.45	0.32	.005
	Isolated cleft lip and palate + molar incisor hypomineralization (N = 335)	0.5	0.32	.0003
	Isolated cleft lip and palate + dental caries (N = 229)	0.37	0.32	.29
	Isolated cleft lip and palate + tooth agenesis (N = 247)	0.39	0.32	.18
	Isolated cleft lip and palate + supernumerary teeth (N = 317)	0.34	0.32	.75
	Tooth agenesis + supernumerary teeth (N = 174)	0.28	0.32	.35

DISCUSSION

Our group has approached the challenge of identifying genes contributing to isolated cleft lip and palate by exploring the presence of concomitant dental anomalies and the occurrence of cancer in the families that had children born with oral clefts (summarized by Vieira[22,23]). We have proposed the concept that isolated forms of cleft lip and palate are quite rare and that the majority of cases actually have accompanying signs, particularly in the dentition. Evidence indicates that incisor development is intrinsically linked with that of the upper lip and primary palate, such that cleft lip and hypodontia or supernumerarism of the lateral incisor are likely in some cases to arise from shared pathogenesis.[24] In fact, "supernumerary" lateral incisors may actually reflect a failure of the normal fusion of 2 independent dental epithelial thickenings to form a single incisor—the same basic mechanism that results in a typical cleft lip. This exemplifies that the coincidence of phenotypes occurring in the same individual or these clinical presentations occurring individually but segregating in the same family, at least in some cases, have a common anatomic development component. Descriptions such as "isolated" vs "syndromic" may hinder gene discovery, and more complete clinical descriptions, such as "right cleft lip with cleft palate and bilateral mandibular second premolar and left maxillary lateral incisor agenesis with family history of breast cancer" may be more suited to provide genotype-phenotype correlations. With that in mind, we designed an experiment in which we combined individuals with conditions that we know are reported to be associated with each other (Figure 1). This criterion fits the definition of "syndrome," which is a term that defines a group of signs and symptoms that consistently occur together (or a condition characterized by a set of associated signs or symptoms). This is a clinically defined term that does not necessarily relate to the etiology of the signs and symptoms but rather how consistently they associate with each other. One limitation of our work is that we did not test for subtypes of clefts (bilateral, unilateral left or right, cleft lip only, cleft palate only) or types of teeth affected by agenesis or having a supernumerary in the vicinity, and this should be done in future analyses with more substantial sampling.

For our experiment, we purposely chose markers that have not been previously associated directly with isolated

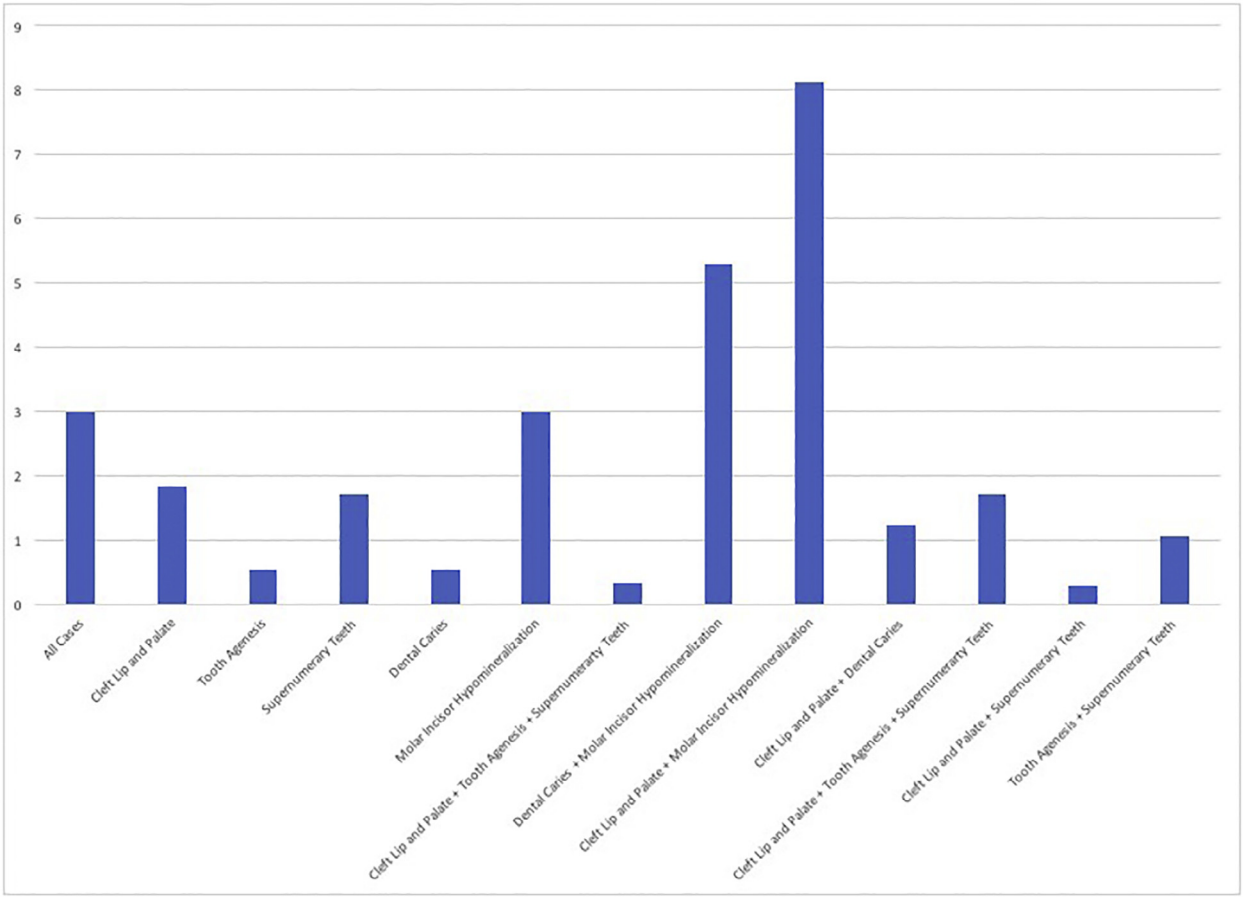

Fig. 2. Summary of *P* values of the association between the phenotypes tested and rs622260. *P* values were transformed in the negative of their logarithm of base 10; 3 in the x-axis corresponds to $P = .05$; 5 in the x-axis corresponds to $P = .0067$.

cleft lip and palate but are in the vicinity of genes recently suggested to contribute to supernumerary teeth. The single-nucleotide polymorphism rs10798049 is located in 1 q31.1 and rs622260 is located in 11 q25. A duplication of 1 q31.1 was reported to lead to hemifacial macrosomia, anophthalmia, anotia, macrostomia, and cleft lip and palate in a 2-year old boy.[25]

The analysis yielded results that suggest rs622260 is associated with molar incisor hypomineralization, and when this condition was studied in combination with individuals born with isolated cleft lip and palate. The single-nucleotide polymorphism rs622260 is located in the intron of *NCAPD3*, which is a gene that forms a complex that establishes mitotic chromosome architecture. Interestingly, 30 individuals (15.4%) in the present study group of 195 born with clefts had associated enamel hypoplasia, and 17 of them (57%) carried 1 copy of the associated allele, a higher frequency than found for the overall group of cases with molar incisor hypomineralization or any combinations (Table I). The association was also identified when the analysis combined individuals with caries experience and individuals with molar incisor hypomineralization. We have proposed that minor alterations of the developing enamel may lead to an enamel surface more susceptible to demineralization and hence to a higher caries experience.[19,26]

Being concerned about multiple testing, we avoided applying the strict Bonferroni correction and increasing the type II error. If we had used Bonferroni correction, we would have lowered the α to .00027 (.05/180). We have reported before[27] that known true associations are missed when correction for multiple testing is implemented. The results of our work should be considered with caution and serve to generate hypotheses to be directly tested in larger and more homogeneous samples. On the other hand, simply disregarding the nominal associations presented here may delay discovery by misleading the field to believe that no true biological relationships exist. We believe that the approach presented here, if implemented in a larger scale, can lead to the determination of more homogeneous study groups and the identification of genotype-phenotype correlations that may aid in the future to personalized clinical management of individuals born with cleft lip and palate.

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