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IRF6 AP-2a binding site promoter polymorphism is associated with oral clefts in Latvia

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SUMMARY

Objective. To evaluate the association between *AXIN2, CDH1* and *IRF6* with oral clefts in a cohort from Latvia.

Material and methods. 283 unrelated individuals, 93 born with isolated oral clefts and 190 individuals born without any structural abnormalities were evaluated. Cleft type and dental anomalies outside the cleft area were determined by clinical examination. Four SNPs were selected for this study: rs2240308 and rs11867417 in *AXIN2*; rs9929218 in *CDH1*; and rs642961 in *IRF6*. Genotypes were determined by polymerase chain reaction using the Taqman assay method from a genomic DNA sample extracted from whole blood. Allele and genotype frequencies were compared between individuals born with or without oral clefts using the PLINK program.

Results. Tooth agenesis was the most frequent dental anomaly found among individuals born with oral clefts (N=10; frequency 10.8%). The allele A in the *IRF6* marker rs642961 was associated with all combined types of oral clefts (OR=1.74; CI 95% 1.07-2.82) and with cases with cleft lip with or without cleft palate (OR=1.88, CI 95% 1.15-3.01; p=0.007).

Conclusions. The *IRF6* AP-2a binding site promoter polymorphism is associated with isolated oral clefts in Latvia.

Key words: axis inhibition protein 2; E-cadherin; interferon regulatory factor 6; dental anomalies; cleft lip with or without palate.

INTRODUCTION

Oral clefts are common developmental craniofacial deformities seen worldwide and are complex malformations resulting from multiple genetic and environmental factors (1). Dental anomalies are also craniofacial developmental alterations that can affect the position, form or number of teeth. The etiology of dental anomalies includes a genetic component (2-4).

Numerous studies have reported that individuals born with clefts have considerably more dental anomalies than individuals born without clefts (5-10). Individuals born with clefts have a higher incidence of abnormal crown morphology, supernumerary teeth, taurodontism, and tooth agenesis (3, 4, 6, 8, 11-13) outside the cleft area (contralateral side of the cleft area, posterior maxillary teeth, and mandibular teeth). Studies have indicated that dental anomalies may represent an additional clinical marker for oral clefts, suggesting that isolated oral clefts can be subphenotyped on the basis of dental development (3, 4, 9).

Previously we reported associations between *AXIN2* (axis inhibition protein 2), *CDH1* (E-cadherin), and *IRF6* (interferon regulatory factor 6) with oral clefts and these studies suggested an important role of these genes during craniofacial morphogenesis (14-20). The aim of the present study was to replicate these findings in a population from Latvia.

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MATERIAL AND METHODS

Subjects

The study sample consisted of 93 individuals aged 5 to 17 years (average age 12.3 years) born with cleft lip with or without cleft palate or cleft palate only. Twenty-two had cleft lip (CL), sixty-one had cleft lip and palate (CLP), and ten had isolated cleft palate (CP). All individuals were recruited at the Riga Cleft Lip and Palate Centre of Institute of Stomatology, Riga Stradins University, the only referral unit for cleft children in Latvia. A group of 190 unrelated individuals born without any structural anomalies were used for all comparisons. These un-related and unaffected individuals were selected randomly at the Latvian Biomedical Research and Study Center from the Latvian Genome Data Base. Ethical approval for the study were obtained from the Central Medical Ethics Committee of Latvia. Appropriate informed consent was signed from all participants or their parents.

Determination of cleft type

The determination of the cleft type was based on the confirmed description present in the clinical records. Cleft status was based on cleft laterality (left, right, bilateral) and on the presence of dental anomalies outside the cleft area. The cleft types recorded were: cleft lip (CL); cleft lip and palate (CLP), and isolated cleft palate (CP). Clinical and radiographic evaluations were used to determine the presence of dental anomalies.

Tooth agenesis was recorded when at least one developmentaly missing permanent tooth was observed (excluding third molars) (21). Supernumerary teeth were recorded when at least one tooth was found additional to the normal series (8, 22). Microdontia was recorded when a tooth was much smaller than its homolog (13, 23). For cases with clefts involving the lip, only dental anomalies and tooth agenesis outside the cleft area were considered. Dental anomalies adjacent to the cleft area (affecting maxillary central incisors, lateral incisors, or canines) were not included in this study because the absence or alterations of such teeth may be the consequence of developmental anomalies at the cleft site and/or repairing surgery.

DNA Samples and Genotyping

The genomic DNA for the molecular analysis was extracted from venous blood samples that were available from a previous study (24). Four single nucleotide polymorphisms (SNPs) were selected for this study based on previous studies regardingt dental anomalies: rs2240308 and rs11867417 in *AXIN2* (18-20, 25); rs9929218 in *CDH1* (19); and rs642961 in *IRF6* gene (26). Genotyping was carried out by real-time PCR using the Taqman chemistry method (27) and performed on an Applied Biosystems 7900 (Applied Biosystems Inc., Foster City, CA, USA).

Statistical Analysis

Statistical analyses were conducted using the PLINK 1.05 software (http://pngu.mgh.harvard.edu/~purcell/plink/) and Epi Info3.5.3 statistical software package (http://www.cdc.gov/epiinfo/). Odds ratio calculations and chi-square or Fisher's exact tests were used with a level of significance of 0.05 to determine if the distribution of alleles or genotypes was different between individuals born with clefts or born without any structural abnormalities. Chi-square was also used to test for deviation from Hardy-Weinberg equilibrium (28).

RESULTS

Of all 93 individuals born with oral cleft included in this study, 53 (56.0%) were males and 40 (44.0%) were females. Among the individuals born without any structural abnormalities, 83 (43.7%) were males and 107 (56.3%) were females. Males

Table 1. Clinical characteristics of the individuals born with oral clefts

	(n)	(%)
Type of clefts		
Cleft lip (CL)	22	23.7
Cleft lip and palate (CLP)	61	65.5
Isolated Cleft palate (CP)	10	10.8
Cleft lip with or without cleft palate (CL±P)	83	89.2
Affected Side		
Left	46	49.5
Right	21	22.5
Both	16	17.2
Associated dental anomalies outside the cleft area		
Yes	13	14.0
No	80	86.0
Tooth agenesis outside the cleft area		
Yes	10	10.8
No	83	89.2
Supernumerary teeth outside the cleft area		
Yes	1	1.1
No	92	98.9
Microdontia outside the cleft area		
Yes	2	2.2
No	91	97.8

Table 2. Genotype and allele distributions

Cleft Subgroups	Genotype n (%)			p-value§	Allele n (%)		p-value§	OR (CI 95%)
AXIN2 rs2240308								
	AA	**AG**	**GG**		**A**	**G**		
Non-Cleft	50 (27.3)	96 (52.5)	37 (20.2)	-	196 (53.6)	170 (46.4)	-	-
All Cleft	23 (25.8)	42 (47.2)	24 (27.0)	0.451	88 (49.4)	90 (50.6)	0.367	0.85 (0.58-1.23)
CL±P	21 (26.6)	36 (45.6)	22 (27.8)	0.375	78 (49.4)	80 (50.6)	0.378	0.85 (0.57-1.25)
CP	2 (20.0)	6 (60.0)	2 (20.0)	0.864	10 (50.0)	10 (50.0)	0.756	0.87 (0.32-2.32)
Unilateral	19 (29.7)	28 (43.8)	17 (26.6)	0.454	66 (51.6)	62 (48.4)	0.697	0.92 (0.60-1.41)
Bilateral	2 (13.3)	8 (53.3)	5 (33.3)	0.339	12 (40.0)	18 (60.0)	0.152	0.58 (0.25-1.31)
All cleft with dental anomalies	3 (23.1)	6 (46.2)	4 (30.8)	0.663	12 (46.2)	14 (53.8)	0.465	0.74 (0.31-1.76)
All cleft with tooth agenesis	2 (20.0)	5 (50.0)	3 (30.0)	0.727	9 (45.0)	11 (55.0)	0.455	0.71 (0.26-1.90)
AXIN2 rs11867417								
	CC	**CT**	**TT**		**C**	**T**		
Non-Cleft	88 (47.8)	74 (40.2)	22 (12.0)	-	250 (67.9)	118 (32.1)	-	-
All Cleft	41 (44.1)	39 (41.9)	13 (14.0)	0.806	121 (65.1)	65 (34.9)	0.495	0.88 (0.60-1.30)
CL±P	37 (44.6)	36 (43.4)	10 (12.0)	0.874	110 (66.3)	56 (33.7)	0.703	0.93 (0.62-1.39)
CP	4 (40.0)	3 (30.0)	3 (30.0)	0.250	11 (55.0)	9 (45.0)	0.229	0.58 (0.21-1.56)
Unilateral	30 (44.8)	31 (46.3)	6 (9.0)	0.630	91 (67.9)	43 (32.1)	0.995	1.00 (0.64-1.56)
Bilateral	7 (43.8)	5 (31.3)	4 (25.0)	0.320	19 (59.4)	13 (40.6)	0.322	0.69 (0.31-1.54)
All cleft with dental anomalies	4 (30.8)	8 (61.5)	1 (7.7)	0.321	16 (61.5)	10 (38.5)	0.500	0.76 (0.31-1.85)
All cleft with tooth agenesis	3 (30.0)	6 (60.0)	1 (10.0)	0.455	12 (60.0)	8 (40.0)	0.460	0.71 (0.26-1.95)
CDH1 rs9929218								
	AA	**AG**	**GG**		**A**	**G**		
Non-Cleft	16 (8.7)	70 (38.0)	98 (53.3)	-	102 (27.7)	266 (72.3)	-	-
All Cleft	8 (8.6)	40 (43.0)	45 (48.4)	0.715	56 (30.1)	130 (69.9)	0.556	1.12 (0.75-1.69)
CL±P	7 (8.4)	34 (41.0)	42 (50.6)	0.901	48 (28.9)	118 (71.1)	0.775	1.06 (0.69-1.62)
CP	1 (10.0)	6 (60.0)	3 (30.0)	0.337	8 (40.0)	12 (60.0)	0.235	1.74 (0.63-4.73)
Unilateral	4 (6.0)	28 (41.8)	35 (52.2)	0.724	36 (26.9)	98 (73.1)	0.850	0.96 (0.60-1.53)
Bilateral	3 (18.8)	6 (37.5)	7 (43.8)	0.402	12 (37.5)	20 (62.5)	0.239	1.56 (0.69-3.51)
All cleft with dental anomalies	2 (15.4)	4 (30.8)	7 (53.8)	0.681	8 (30.8)	18 (69.2)	0.737	1.16 (0.45-2.93)
All cleft with tooth agenesis	1 (10.0)	3 (30.0)	6 (60.0)	0.877	5 (25.0)	15 (75.0)	0.791	0.87 (0.27-2.64)
IRF6 rs642961								
	AA	**AG**	**GG**		**A**	**G**		
Non-Cleft	1 (0.5)	53 (29.0)	129 (70.5)	-	55 (15.0)	311 (85.0)	-	-
All Cleft	4 (4.8)	31 (37.3)	48 (57.8)	0.016*	39 (23.5)	127 (76.5)	0.017*	1.74 (1.07-2.82)
CL±P	4 (5.4)	29 (39.2)	41 (55.4)	0.006*	37 (25.0)	111 (75.0)	0.007*	1.88 (1.15-3.01)
CP	0	2 (22.2)	7 (77.8)	0.882	2 (11.1)	16 (88.9)	0.483	0.71 (0.11-3.34)
Unilateral	3 (5.1)	24 (40.7)	32 (54.2)	0.009*	30 (25.4)	88 (74.6)	0.009*	1.93 (1.13-3.28)
Bilateral	1 (6.7)	5 (33.3)	9 (60.0)	0.065	7 (23.3)	23 (76.7)	0.228	1.72 (0.64-4.48)
All cleft with dental anomalies	1 (7.7)	3 (23.1)	9 (69.2)	0.045*	5 (19.2)	21 (80.8)	0.365	1.35 (0.42-3.99)
All cleft with tooth agenesis	1 (10.0)	3 (30.0)	6 (60.0)	0.015*	5 (25.0)	15 (75.0)	0.184	1.88 (0.57-5.84)

Note: OR = odds ratio; CI = confidence interval; All cleft groups were compared to the non-cleft group; §qui-square or Fisher's exact were used; *Statistical significant results

were statiscally significant more affected by oral cleft than females (p=0.035). Fourty-one males and 20 females had CLP, seven males and 15 females had CL, and five males and five females had CP.

Dental anomalies outside the cleft area affected 13 (14%) individuals. Tooth agenesis was the most frequent dental anomaly among the individuals born with clefts (N=10; 10.8%). The characteristics of the individuals born with clefts are presented in Table 1.

All SNPs were in Hardy-Weinberg equilibrium (data not show). Table 2 presents the comparison of allele and genotype frequencies among the studied individuals. *AXIN2* (rs2240308 and rs11867417) and *CDH1* (rs9929218) were not associated with oral clefts.

In *IRF6*, the allele A in the marker rs642961, increased the risk for oral cleft, (OR=1.74, CI 95% 1.07-2.82; p=0.017). Similar result was found when only individuals with lip involvement were used in the analysis (OR=1.88, CI 95% 1.15-3.01; p=0.007).

DISCUSSION

Craniofacial development is a very complex phenomenon, which involves many biological processes. Alterations in tooth germ development and the occurence of oral clefts have a close embriological relationship in terms of timing, anatomical position, and genetic involvement (3, 6, 15). The *AXIN2* gene is a negative regulator of the Wnt signaling pathway that regulates embryogenic development and organogenesis (19). Mutations in *AXIN2* have been associated with familial and sporadic tooth agenesis (25, 29), and tooth agenesis itself has been reported in association with oral clefts (3, 15, 16, 19). *CDH1* is involved in cell-cell adhesion. This gene plays a major role in craniofacial morphogenesis and palatal fusion (19, 30). Although *AXIN2* and *CDH1* were previously suggested to be associated with oral clefts and tooth agenesis, we did not find associations in the studied Latvian cohort.

IRF6 gene deletions and point mutations are responsible for Van der Woude syndrome (OMIM 119300), which is one of the most common cleft syndromes. Van der Woude syndrome is an autosomal dominant disorder that lower-lip pits and less frequently tooth agenesis are the only features distinguishing the disorder from non-syndromic oral clefts (31). Moreover, previous studies demonstrated that *IRF6* is associated with isolated oral clefts (14, 32-35) and isolated tooth agenesis (15-17).

Although numerous studies have demonstrated that variants in *IRF6* are associated with oral clefts (14, 26, 32-36) just few have focused in the SNP rs642961. Rahimov et al. (26) first identified this variant as associated with oral clefts, in which the A allele was significantly overtransmitted in families of European background. This polymorphism, in an *IRF6* enhancer located approximately 10,000 base pairs upstream of *IRF6*, was suggested to cause disruption of the binding site of the transcription factor AP-2. This in turn disrupts proper expression of the *IRF6* gene. Here, we found an association between the rs642961 marker and oral clefts, which corroborated previous studies (26, 33, 34, 36, 37).

We previously showed that *IRF6* was associated with isolated tooth agenesis (15, 17). Also, we showed a trend for association between certain palatine rugae patterns and *IRF6* variants (38). In the present study, the *IRF6* rs642961 genotype AA is 20 times more frequent inindividuals presenting oral clefts associated with tooth agenesis when compared to unaffected unrelated individuals.

CONCLUSIONS

AXIN2 and *CDH1* we did not find associations in the studied Latvian cohort. Here we reported the association between *IRF6* AP-2a binding site promoter polymorphism with oral clefts in a Lativian cohort.

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