Genetic-epigenetic interactions (meQTLs) in orofacial clefts etiology

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ABSTRACT

Objectives: Nonsyndromic orofacial clefts (OFCs) etiology involves multiple genetic and environmental factors with over 60 identified risk loci; however, they account for only a minority of the estimated risk. Epigenetic factors such as differential DNA methylation (DNAm) are also associated with OFCs risk and can alter risk for different cleft types and modify OFCs penetrance. DNAm is a covalent addition of a methyl (CH3) group to the nucleotide cytosine that can lead to changes in expression of the targeted gene. DNAm can be affected by environmental influences and genetic variation via methylation quantitative loci (meQTLs). We hypothesize that aberrant DNAm and the resulting alterations in gene expression play a key role in the etiology of OFCs, and that certain common genetic variants that affect OFCs risk do so by influencing DNAm. **Methods:** We used genotype from 10 cleft-associated SNPs and genome-wide DNA methylation data (Illumina 450K array) for 409 cases with OFCs and 456 controls and identified 23 cleft-associated meQTLs. We then used an independent cohort of 362 cleft-discordant sib pairs for replication. We used methylation-specific qPCR to measure methylation levels of each CpG site and combined genotypic and methylation data for an interaction analysis of each SNP-CpG pair using the R package MatrixeQTL in a linear model. We also performed a Paired T-test to analyze differences in DNA methylation between each member of the sibling pairs.
Results: We replicated 9 meQTLs, showing interactions between rs13041247 (*MAFB*) - cg18347630 (*PLCG1*) (P=0.04); rs227731 (*NOG*) - cg08592707 *(PPM1E)* (P=0.01); rs227731 (*NOG*) - cg10303698 (*CUEDC1*) (P=0.001); rs3758249 (*FOXE1*) - cg20308679 (*FRZB*) (P=0.04); rs8001641 (*SPRY2*) - cg19191560 (*LGR4*) (P=0.04); rs987525(8q24) - cg16561172(*MYC*) (P=0.00000963); rs7590268(*THADA*) - cg06873343 (*TTYH3*) (P=0.04); rs7078160 (*VAX1*) - cg09487139 (P=0.05); rs560426 (*ABCA4/ARHGAP29*) - cg25196715 (*ABCA4/ARHGAP29*) (P=0,03). Paired T-test showed significant differences for cg06873343 (*TTYH3*) (P=0.04); cg17103269 (*LPIN3*) (P=0.002), and cg19191560 (*LGR4*) (P=0.05). **Conclusions:** Our results confirm previous evidence that some of the common non-coding variants detected through GWAS studies can influence the risk of OFCs via epigenetic mechanisms, such as DNAm, which can ultimately affect and regulate gene expression. Given the large prevalence of non-coding SNPs in most OFCs genome wide association studies, our findings can potentially address major knowledge gaps, like missing heritability, reduced penetrance, and variable expressivity associated with OFCs phenotypes.

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Introduction

Nonsyndromic orofacial clefts (OFCs) are the most common craniofacial birth defects in humans, affecting approximately 1 in 700 individuals worldwide [1]. OFCs are sub-divided into cleft lip occurring with or without cleft palate (CL/P) and isolated cleft palate (CPO) [2, 3]. The identification of causal factors for OFCs has been a challenge due to its complex etiology arising from multiple genetic and environmental contributions. Family history is observed in about 23% of cases showing a strong genetic component; however, monozygotic twins have a concordance rate of about 50% [4] and amongst those that are concordant there are often discernable differences in OFCs phenotypic expression indicating that other factors are at play beyond genetic risk.

Together, GWAS [5-12], GWAS meta-analyses [13, 14], linkage [15] and replications studies have identified over 60 risk loci for OFCs; however, they account for a minority of the estimated risk, and many of them reside in non-coding regions with unclear functional relevance so far. Thus, the challenge of translating statistical associations into biological mechanisms still limits our knowledge on OFCs etiology, and thus to gain further insight it is necessary to expand beyond the single-omics analyses.

Epigenetic modification, such as DNA methylation (DNAm) is a likely mechanism through which environmental factors or genetic variation can modify gene expression. DNAm consists of the covalent addition of a methyl (CH3) group to a nucleotide cytosine followed by a guanine, a CpG site, by a family of enzymes called methyltransferases (DNMTs) [16-18]. Changes in DNAm can lead to differential transcriptional activity of a target gene and have important roles in multiple processes during human development and throughout life such as genomic imprinting, maintenance of X-inactivation and genomic stability [18-22].

Multiple studies have shown that genetic variants can impact a significant proportion of the human DNA methylome[23-33]. Some studies indicate that epigenetic factors such as differential DNA methylation (DNAm) are associated with OFCs risk [34-43], also that differentially methylated regions tend to cluster around gene pathways previously linked to palatogenesis, and that some of these differential DNAm regions seem to be influenced by genetic variation [34, 37, 44, 45]. However, the overall role of epigenetics in OFCs etiology remains underexplored[46].

Individual genotypes at methylation quantitative trait loci (meQTLs) can modify DNA methylation patterns. meQTLs can be divided into *cis* or *trans* based on the proximity of the genetic variant to the CpG site. Cis-meQTLs are genetic variants near to or proximal to the target CpG site, and trans-meQTLs are separated by one or more Mbp from the target CpG or located on different chromosomes [47-49]. Studies on blood DNA and commercial methylation arrays (Illumina Infinium MethylationEPIC array) show that 34% to 45% of the CpGs [24, 50-53] are influenced by SNPs, with 98% of the effects cis-acting or within 1Mb of the tested CpG sites[33]. Additionally, both meQTLS SNPs and target CpGs are frequently found in enhancers [33, 54-56]. Literature has shown meQTL SNPs in various tissues like brain[57, 58], adipose[59], and buccal tissue samples[60]. Further, studies have shown that there is a significant overlap of meQTLs between ancestral groups, developmental stages and tissue types[61].

Differential DNAm may alter risk for different cleft types and modify OFCs penetrance [34]. Our group recently showed that differential methylation can contribute to phenotypic variability observed in patients with Van der Woude syndrome, including monozygotic twins with discordant phenotypes despite carrying the same causal mutation in the *IRF6* gene[27, 29]. These findings support the role of DNA methylation as a modifier influencing phenotypic severity.

Therefore, analyses that combine known genetic risk factors with epigenetic data to explore methylation quantitative loci (meQTLs) function significantly contribute to understanding such loci by addressing the causal alleles, their pathogenic consequences, and the biological mechanisms through which they influence OFCs risk.

Discordant sib pairs are valuable cohorts for mapping complex human traits. The use of discordant sibling pairs in methylation studies offers several advantages: siblings share a significant portion of their genetic makeup, which helps control for genetic variation and isolates environmental influences on methylation. Additionally, siblings often experience similar early-life environments, aiding in the study of

environmental impacts on DNA methylation[62]. Comparing affected and unaffected siblings (discordant pairs) can reveal disease-related methylation changes, and the reduced variability from genetic and environmental similarities enhances the statistical power and reliability of the study.

Based on an analysis of subset of cases and controls from previous studies [63, 64], we found that genotypes of 10 cleft-associated SNPs were highly correlated with the methylation levels of certain CpG sites, indicating that these SNPs function as methylation quantitative trait loci (meQTLs). This genetic-epigenetic association was replicated using a unique sample of same-sex siblings discordant for OFCs.

Methods

Samples

The first cohort was composed of a subset of subset of unrelated cases with cleft lip with or without cleft palate and unaffected controls from a previous published studies [63, 64]. We used genotype from 10 cleft-associated SNPs and genome-wide DNA methylation data (obtained with Illumina 450K array) for 409 cases with OFCs and 456 controls, all with DNA from blood. The second independent cohort was composed of 362 pairs of same-sex siblings, discordant for cleft lip with or without cleft palate (one sibling affected-case, one unaffected-control); of these, 166 pairs had DNA extracted from blood and 196 pairs had DNA extracted from saliva from saliva. All samples were obtained in accordance with prior study protocols, following their respective approval by the University of Iowa and locals Institutional Review Boards (IRBs). Informed consent was provided by patients, parents or guardians prior obtaining samples and clinical information.

Genotyping

As mentioned above, for cohort 1, we extracted the genetic data for the selected 10 SNPs (top loci for OFCs from previous GWAS and candidate gene studies) (Table 1) from a previous published studies [63 64]. All the details of the data collection, quality control, and preprocessing are available in the Appendix of the cited work. For cohort 2, we genotyped the 10 SNPs in the discordant sib-pairs using Taqman assays on a Fluidigm (Fluidigm Corp., South San Francisco, CA, USA) nanofluidic platform. Genotype calling was done using the Fluidigm SNP genotyping software, version 4.1.2, with the default settings. The quality check included setting the confidence threshold to 65% for the genotype calling algorithm and then visually checking all the genotyping plots.

Bisulfite conversion and DNA methylation profiling

All samples were bisulfite converted prior measuring DNA methylation levels. Briefly, DNA quality was assessed QubitTM dsDNA High Sensitivity Range Assay Kit (Thermo Fisher Scientific) and 1.5% agarose gel. After quantification of each sample, 500 ng of each genomic DNA sample was submitted to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research) according to manufacturer's protocol.

For cohort 1, DNA methylation levels were obtained using the Illumina Human Methylation 450K BeadChip. DNA methylation level was then estimated at 485,577 CpG sites and the R package illuminaio (ver. 0.18)3 was used to extract the raw probe intensity values. These raw values were preprocessed using ENmix R package4. The following criteria were used to identify low-quality samples: (1) average intensity value across internal control probes less than 5500, (2) more than 5% of CpG probes having low-quality data (Illumina detection P value > 10^{-6}, read from less than 3 beads, or outlier value for the probe in the dataset), and (3) clear outliers based on visual inspection of a density plot of total intensity. Next, the low quality CpG probes were defined as follows: (1) more than 5% low-quality data; (2) common SNP within the probe's sequence (minor allele frequency $\geq$ 0:05 in Europeans based on 1000 Genomes Project data), or probes mapping to multiple genomic locations, or CpGs on X or Y chromosomes; (3) CpGs with multiple mode distributions identified with ENmix. More details about the filtering steps can be found in the cited work[63].

For cohort 2, after bisulfite conversion, genomic DNA samples were amplified by fluorescence-based, real time quantitative PCR (Methylight) [65]. Quantitative MethyLight assays (e.g., EpiTect

MethyLight Assays), consists of two probes, one methylation specific, and the other nonmethylation specific, which be used in a single real-time PCR reaction. This enables highly accurate quantitative methylation analysis, due to the simultaneous detection of methylated and unmethylated DNA. We used locus-specific PCR primers flanking an oligonucleotide probe with a 5' fluorescent reporter dye to detect methylated (SUN) and unmethylated (FAM) alleles and a 3' quencher dye (3IABkFQ). The primers and fluorescent probes were designed against bisulfite-converted DNA sequence and quantitative information was obtained in real time. Serial dilutions of the EpiTect Control DNAs (Qiagen) were included on each plate to generate a standard curve and to verify plate to plate consistency. The PCR amplification was performed in a 384-well plate format and each sample was ran in duplicate. EpiTect MethyLight assays enable the direct quantification of the methylation degree in a sample by taking the threshold cycles (C_T) determined in the SUN channel with the probe detecting methylated DNA or in the FAM channel with the probe detecting unmethylated DNA. Ten nanograms of bisulfite converted, methylated and unmethylated human control DNA, or defined mixtures of both DNAs were used for methylation quantification. The methylation degree of each sample was calculated from the average of C_T values in SUN ($C_{T(CG)}$) and FAM ($C_{T(TG)}$) channel, obtained in quantitative real-time PCR using the formula described in [66]: $C_{meth} = 100/[1+2^{(C_{T(CG)} - C_{T(TG)})}]\%$; where $C_{T(CG)}$ is the methylated signal obtained by the threshold cycle of the CG reporter (SUN channel), and $C_{T(TG)}$ is the unmethylated signal obtained by the threshold cycle of the CG reporter (FAM channel).

Statistical analysis

The meQTL analyses were performed using the R package MatrixEQTL version 2.1.1 [67], which was designed to perform ultra-fast calculations of associations between SNPs and gene expression data (eQTLs). Due to similarity between the gene expression and methylation data (both are numeric big matrices), the method has been widely applied to methylation quantitative loci as well [48, 68-74]. In addition to replicating the meQTLs, we used data obtained from GeneHancer [75]— a regulatory element database containing genomic coordinates of known enhancer and promoter elements, including those active during craniofacial development [76, 77]—to (1) investigate whether the validated CpG sites overlapped with any regulatory elements and (2) prioritize the most likely CpG-target genes in epithelial and mesenchymal tissues.

For cohort 1, all 10 SNPs were analyzed against the 407,513 CpGs that passed QC. Linear regression was used to assess the relationship between each CpG methylation and each SNP genotype (0, 1, or 2 copies of the minor allele) using cleft status (affected or unaffected) as covariate. The linear model analyzes the effect that the genotype (additive effect) has on the methylation based on the cleft status (case or control). It tests for the significance of the genotype and the cleft status over the methylation level. The linear model was tested separately for each of the 3 groups, blood, saliva and combined.

Matrix eQTL performs a separate test for each SNP-CpG pair and corrects for multiple comparisons by

Table 1: Most significant meQTLs resulting from case-control study

SNP	SNP gene	CpG	CpG target gene	p-value
rs227731	*NOG*	cg08592707	*SKA2*	1.59E-05
rs4752028	*VAX1*	cg08319991	*UCHL1*	2.83E-08
rs7590268	*THADA*	cg06873343	*N/A*	7.72E-08
rs7078160	*VAX1*	cg08319991	*UCHL1*	1.11E-07
rs3758249	*FOXE1*	cg20308679	*FRZB*	1.29E-07
rs4752028	*VAX1*	cg11876012	*AFAP1L2*	1.41E-07
rs8001641	*SPRY2*	cg19191560	*LGR4*	2.88E-08
rs1873147	*TPM1*	cg11936410	*TPM1*	5.41E-04
rs560426	*ABCA4/ARHGAP29*	cg25196715	*BCAR3/MIR760*	6.72E-04
rs987525	*8q24*	cg16561172	*MYC*	7.04E-04
rs560426	*ABCA4/ARHGAP29*	cg00405232	*ABCA4*	7.93E-04
rs227731	*NOG*	cg01964121	*PPM1E*	8.11E-04
rs227731	*NOG*	cg10303698	*CUEDC1*	8.74E-04
rs1873147	*TPM1*	cg24483493	*HERC1*	9.14E-04
rs4752028	*VAX1*	cg08329473	*POLR3K*	9.29E-04
rs1304124	*MAFB*	cg18347630	*CHD6*	1.02E-03
rs7078160	*VAX1*	cg08329473	*AFAP1L2*	1.06E-03
rs1873147	*TPM1*	cg16659880	*TPM1*	1.11E-03
rs7078160	*VAX1*	cg09487139	*N/A*	1.38E-03
rs1304124	*MAFB*	cg00514723	*LPIN3*	1.39E-03
rs742071	*PAX7*	cg20940024	*N/A*	1.56E-03
rs1873147	*TPM1*	cg19631779	*TPM1*	1.67E-03
rs1304124	*MAFB*	cg17103269	*PLCG1*	1.78E-03

calculating Benjamini-Hochberg FDR. From this first analysis, we selected 23 SNP-CPG associations with p≤ 0.002 and FDR<0.4 for replication with an independent cohort (cohort 2).

For cohort 2, we used MatrixEQTL to run each SNP-CpG pair in the 362 pairs of siblings discordant for OFCs, following the same parameters above. Further, to test for associations between DNA methylation levels independent of SNP effect within sibling pairs, we used paired Student's T-test analyses for each tissue subgroups (blood, saliva and combined).

Bioinformatic analysis

We used bioinformatic methods to prior regions and select the most likely target gene for each CpG site. We obtained data from GeneHancer—a regulatory element database containing genomic coordinates of known enhancer and promoter elements [75], including those active during craniofacial development [76, 77]— and compared the coordinates to our CpG sites to (1) investigate whether the validated CpG sites overlapped with any regulatory elements and (2) prioritize the most likely CpG-target genes in relevant tissues (e.g., epithelial and mesenchymal tissues) based on GeneHancer *in silico* prediction tools.

Results

The analysis with the first cohort (409 cases, 456 controls) correlated the genotype of 10 cleft-associated SNPs [5, 8, 63, 64, 78-82] with genome-wide DNAm patterns, identifying 23 significant SNP-CpG interactions (meQTLs). All 10 SNPs are non-coding (and may be located near/in regulatory elements)—supporting the need for novel approaches to elucidate their pathological consequence—and several have been identified as meQTLs in other tissues and phenotypes (Table 1, SNPs in *Italics*) [83]. We then followed-up with an independent cohort of 362 discordant sib pairs and replicated 9 out of the 23 cleft-associated SNP-CpG associations. In Table 1, we show the result of the first analysis for the case-control cohort and the best replication result obtained with the sib pairs cohort for each pair of SNP-CpG associations.

Table 2 shows results for the SNP-CpG linear model for each tissue group. For blood analysis we detected interaction between

Table 2: Replicated SNP-CpG associations (meQTLs) from linear analysis in all groups

Sample	Affected /Unaffected	SNPs	SNP gene	CpG	CpG gene	pvalue
All	353/353	rs13041247	*MAFB*	cg18347630	*PLCG1*	2.13E-01
Blood	160/160	rs13041247		cg18347630		**3.93E-02**
Saliva	193/193	rs13041247		cg18347630		3.38E-01
All	354/354	rs227731	*NOG*	cg08592707	*PPM1E*	**1.54E-02**
Blood	163/163	rs227731		cg08592707		3.66E-01
Saliva	191/191	rs227731		cg08592707		5.34E-01
All	300/300	rs227731	*NOG*	cg10303698	*CUEDC1*	**1.50E-03**
Blood	110/110	rs227731		cg10303698		5.08E-01
Saliva	190/190	rs227731		cg10303698		**5.95E-02**
All	318/318	rs3758249	*FOXE1*	cg20308679	*FRZB*	1.10E-01
Blood	131/131	rs3758249		cg20308679		2.92E-01
Saliva	187/187	rs3758249		cg20308679		**3.80E-02**
All	340/340	rs8001641	*SRPY2*	cg19191560	*LGR4*	**4.70E-02**
Blood	158/158	rs8001641		cg19191560		**9.33E-02**
Saliva	182/182	rs8001641		cg19191560		**3.72E-02**
All	305/305	rs987525	*8q24*	cg16561172	*MYC*	**2.78E-05**
Blood	138/138	rs987525		cg16561172		6.12E-01
Saliva	167/167	rs987525		cg16561172		**9.63E-06**
All	356/356	rs7590268	*THADA*	cg06873343	*TTYH3*	3.08E-01
Blood	165/165	rs7590268		cg06873343		**3.91E-02**
Saliva	191/191	rs7590268		cg06873343		9.14E-01
All	333/333	rs7078160	*VAX1*	cg09487139	N/A	7.30E-01
Blood	157/157	rs7078160		cg09487139		**4.60E-02**
Saliva	176/176	rs7078160		cg09487139		2.75E-01
All	297/297	rs560426	*ABCA4/ ARHGAP29*	cg25196715	*ABCA4/ ARHGAP29*	**3.16E-02**
Blood	126/126	rs560426		cg25196715		3.88E-01
Saliva	171/171	rs560426		cg25196715		**3.81E-02**

p-values in bold are boderline significant (p<0.09); FDR=False Discovery Rate

Beta value ranges: 0-0.2 = fully unmethylated; 0.21-0.60 = partially methylated; 0.61-1.00 = fully methylated

rs13041247/*MAFB* - cg18347630/*PLCG1* (P=0.04); rs7590268/*THADA* - cg06873343/*TTYH3* (P=0.04); rs7078160/*VAX1* - cg09487139 (P=0.05). Analysis for saliva revealed significant associations between rs3758249/*FOXE1* - cg20308679/*FRZB/* (P=0.04). The combined analysis (blood and saliva together) showed associations between rs227731/*NOG* - cg08592707/*PPM1E* (P=0.01) and rs227731/*NOG* - cg10303698/*CUEDC1* (P=0.001). Three meQTLs had significant associations in both saliva and combined analysis (blood and saliva), being rs8001641/*SPRY2* - cg19191560/*LGR4* (P=0.04-saliva, P=0.05-all); rs987525/8q24 - cg16561172/*MYC* (P= P=9.6E-06-saliva, P= 2.8E-05-all); rs560426*ABCA4/ARHGAP29* - cg25196715/*ABCA4/ARHGAP29* (P=0.04-saliva, P=0.03-all).

For the 10 CpG sites analyzed, the differential methylation analysis (independent of SNP effect) between sibling pairs and subdivided by groups through Paired T-test (Table 3) showed significant results for cg06873343 (*TTYH3*) for blood (P=0.04), saliva (P=0.05) and all (P= 0.004); cg17103269 (*LPIN3*) (P=0.002 – blood) and cg19191560 (*LGR4*) (P=0.05 – all). The most significant result was found for the cg17103269 (*LPIN3*), followed by cg06873343 (*TTYH3*).We had borderline results for cg19191560 (*LGR4*) (P=0.06-blood) and cg25196715 (*ABCA4/ARHGAP29*) (P=0.07-blood).

Table 3: Differential methylation levels between sibling pairs and subdivided by groups

Paired T-Test					
CpG site	**Chr position**	**Closest Gene**	**# of sib pairs**	**Group**	**p-value**
cg06873343	chr7:2,667,608	*TTYH3*	358	All	**0.004**
			165	Blood	**0.04**
			193	Saliva	**0.05**
cg17103269	chr20:39,972,305	*LPIN3*	360	All	0.25
			166	Blood	**0.0017**
			194	Saliva	0.87
cg19191560	chr11:27,492,758	*LGR4*	340	All	**0.05**
			158	Blood	**0.06**
			182	Saliva	0.26
cg25196715	chr1:94,489,792	*ABCA4/ARHGAP29*	297	All	0.13
			126	Blood	**0.07**
			171	Saliva	0.56

p-values in bold are borderline significant (p<0.09)

Discussion

The integration of emerging epigenetic data for orofacial clefts with existing GWAS hits may elucidate functional mechanisms that underlie statistical significance. Moreover, enrichment analysis of epigenetic data also has the potential to reveal pathways not implicated by genetic findings. Our hypothesis was that certain common genetic variants that affect OFCs risk do so by influencing DNAm which in turn can modulate gene expression of target genes that are key to palatogenesis. Using discordant sibling pairs, we were able to refine our results and control for heterogeneity, population stratification and environmental exposures while still being able to detect differences in genetic and epigenetic influences

Our top SNP-CpG association after replication included rs987525 ($p=9.63E^{-06}$), known to be the lead SNP in the OFCs-associated 8q24 region [5, 10, 13, 14, 78, 84-87]. While OFCs-associated variants continue to be identified at the 8q24 locus (one of them, rs72728755, is in high linkage disequilibrium [$r^2>0.81$ in Caucasians] with our meQTL SNP rs987525) the mechanism behind the significant association remains unclear [88-90]. Our analysis shows that rs987525 interacts with a CpG site, cg16561172, located upstream of the *MYC* gene. Thus, our data suggests genetically driven abnormal DNAm in 8q24 as a potential pathogenic mechanism contributing to increased OFCs risk. A second replicated association was rs227731 and cg08592707 ($p=2.64E^{-02}$). While rs227731 was one of the 9 SNPs in our meQTL analysis—owing to its identification as a lead SNP in a previous GWAS study— there is prior evidence that rs227731 likely serves as a sentinel for a cleft-associated allele in complete LD with rs227731, rs227727. Importantly, the cleft-associated allele in rs227727 disrupts an epithelial enhancer located near the *NOG* transcription start site in GMSM-K cells [82]. Intriguingly, *PPM1E,* the gene nearest the associated CpG (cg08592707) has been shown to be epigenetically regulated and controls osteoblast proliferation [91-95], providing rationale for its influence on OFCs. While biologically compelling, functional studies are a prerequisite and a necessary next step for mechanistically link these associations.

We also found significative association between rs7078160 (*VAX1*) and cg09487139 (N/A gene) (p=0.05-blood). A study carried out by Zhang et al. (2018) [96], which included 302 nonsyndromic cleft lip with or without palate (OFCs) in Western Han Chinese population, confirmed that allele A at rs7078160 at *VAX1* gene was a risk factor for OFCS and was over-transmitted among OFCs group.

Research using an animal model with *Vax1* knockout mice demonstrated that this gene is related to palate development, showing its role in the etiology of OFCs[97]. We know that this gene contains a homeobox and has significant expression in brain development.[98] Some independent GWAS studies conducted in different populations have shown that SNPs in VAX1 are associated with OFCs [97 8 99 100 101]. The CpG site cg09487139 maps to a long intergenic non-protein coding RNA 2626 (LINC002626) which has been associated to deficit hyperactivity disorder (ADHD) & bipolar disorder[102]. However, neither a craniofacial candidate gene nor enhancer region proximal to cg09487139 has been characterized, thus precluding any assertions regarding its association with target genes or enhancers implicated in craniofacial development.

Another significant association was found between rs13041247/*MAFB* and cg18347630 (*PLCG1*) (p=0,04-blood). *MAFB* is a basic leucine zipper transcription factor that acts as a transcriptional activator or repressor [103]. It plays a crucial role in regulating lineage-specific hematopoiesis [104] and is important for the differentiation of monocytes, macrophages, osteoclasts, podocytes, and islet beta cells [105-107].

SNPs near *MAFB* were first associated with OFCs in a cleft GWAS using case-parent trios. The authors also demonstrated through an expression study conducted in mouse that the *Mafb* gene plays a role in palatal development. Asian families showed stronger evidence for association with *MAFB* and *ABCA4* [5]. These results were confirmed in different populations, with Asian families showing a strong association of these genes with CL/P, although some results were conflicting [5, 108-111].

Although the relationship between *MAFB* and *PLCG1* is unclear, it is known that PLCG1 is a gene related to cell growth and migration, as well as cancer[112]. In addition to being expressed in immune cells, it is also expressed in various organs and tissues, such as the skin, brain, kidneys, gastrointestinal tract, and muscles [113]. *PLCG1* also plays critical roles in maintaining immune homeostasis[114].

Additionally, our replication study has identified other associations such as rs7590268/*THADA* and cg06873343/*TTYH3*, and rs560426/*ABCA4/ARHGAP29* with cg25196715/*ABCA4/ARHGAP29*. *THADA* and *ABCA4-ARHGAP29*, show a strong link to CLP but not CLO [115]and *ARHGAP29* is recognized as causative for nonsyndromic OFCs[116, 117].

Regarding the *TTYH3* gene found near cg06873342, it is known that this gene encodes a large-conductance chloride channel [118]. A study conducted with *ttyh3 gene in* Xenopus laevis, showed expression during embryonic development in the nervous system and in somites, suggesting important role both in early proliferation and differentiation[119]. However, it is still uncertain whether this gene is similarly expressed in humans [120]. Research related to cancer shows its high expressivity in cervical cancer [121]. At present, its relationship with orofacial clefts is unknown. As for *ARHGAP29,* Liu et al. (2017) [122] applied an experimental pipeline to test the hypothesis that functional SNPs in the 1p22 regions lie within enhancers that drive the expression of *ARHGAP29* in one or more oral tissues, and that the risk alleles alter *ARHGAP29* expression. The study included the application of *in vitro* and *in vivo* enhancer assays, chromatin conformation capture, and genome-editing to provide evidence of functional SNPs. In addition, chromatin immunoprecipitation analysis indicated that the risk associated SNPs affect the activity of *ARHGAP29* enhancers. A recent study found four new pathogenic variants in *ARHGAP29*, which lead to loss of function, such as the loss of the important RhoGap functional domain, showing strong evidence of its relationship with oral clefts [123].

Finally, we also present association between rs3758249 located in *FOXE1* and cg20308679 close to *FRZB* (p=0.04) in the saliva group. Both genes are known to be associated with orofacial clefts. According to a study conducted by Conte et al. (2016), patients with OFCs presented deletions in *FRZB* and *SPRY1* genes[124]. Animal models showed that *FRZB* is expressed in oral tissues, has an important role in the ossification of craniofacial regions by interacting with the WNT pathway. Activation of the

Wnt signaling pathway is necessary for development of many organs of the body [125-129] and also for palatogenesis[130].

We also conducted a differential DNA methylation analysis via paired T tests (Table 3), which shows significant results for three CpG sites, cg006873343 near *TTYH3* gene, cg17103269 near *LPIN3* and cg19191560 *LGR4*. These three genes have unknow function on craniofacial development as of yet. Although the cg25196715 has shown borderline result, it is of particular interest, as it is located close to genes highly associated with clefting in Asian population (*ABCA4/ARHGAP29)* [10]. Mice animal models showed *Arghgap29* gene expression in craniofacial development[117].

Methylome analysis is a valuable post-GWAS resource, giving insights into regulatory genomic potential of GWAS signals, especially the non-coding variants, and helping to prioritize loci to further follow-up. Analyses that combine known genetic risk factors with epigenetic data to explore methylation quantitative loci (meQTLs) function, significantly contribute to understanding such loci by addressing the causal alleles, their pathogenic consequences, and the biological mechanisms through which they influence disease.

Our study indicates the potential for the identification of new cleft related gene networks that can be disrupted via aberrant DNA methylation. A better understanding of genetic-epigenetic interactions underlying OFCs etiology has significant implications in risk prediction and can uncover biological mechanisms that could potentially be targeted for prevention strategies for OFCs and other craniofacial conditions. Given the large prevalence of non-coding SNPs in most OFCs genome wide association studies, our findings can potentially address major knowledge gaps, like missing heritability, reduced penetrance, and variable expressivity associated with OFCs phenotypes.

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