

# Rare Functional Variants in Genome–Wide Association Identified Candidate Genes for Nonsyndromic Clefts in the African Population

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Nonsyndromic clefts of the lip and palate (NSCLP) are complex genetic traits. Together, they are classified as one of the most common birth defects with a prevalence of 1/700 live births. Genome-wide association studies (GWAS) for nonsyndromic cleft lip with or without cleft palate (NSCL[P]) revealed significant association for common single nucleotide polymorphisms near genes involved in craniofacial development i.e., *MAFB*, *PAX7*, *VAX1*, *ARHGAP29* (*ABCA4* locus), and *IRF6*. Sequencing of protein coding regions of the NSCL(P) GWAS candidate genes or adjacent genes suggest a role for rare functional variants. Replication studies in the African population did not observe any significant association with the GWAS candidate genes. On the other hand, the role of rare functional variants in GWAS candidate genes has not been evaluated in the African population. We obtained saliva samples from case triads in Nigeria and Ethiopia for Sanger sequencing of the GWAS candidate genes (*MAFB*, *PAX7*, *VAX1*, *ARHGAP29*, and *IRF6*) in order to identify rare functional variants. A total of 220 African samples (140 Nigerians and 80 Ethiopians) were sequenced and we found the following new rare variants— p.His165Asn in the *MAFB* gene, p.Asp428Asn in the *PAX7*, a splice-site variant that creates a new donor splice-site in *PAX7*. We also found three previously reported missense variants p.Gly466Ser in *PAX7*; p.Leu913Ser and Arg955His in *ARHGAP29*. No de novo mutations were found. Future genome-wide association and sequencing studies should be conducted using samples from Africa in order to identify new molecular genetic factors that contribute to the etiology of NSCLP.

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## INTRODUCTION

Nonsyndromic clefts of the lip and palate (NSCLP) are complex genetic traits and together, they are classified as one of the most common birth defect with a prevalence of 1/700 live birth [Mossey and Modell, 2012]. These defects are easily recognizable, responsive to surgical correction and benefit from substantial multi-disciplinary team care. This team care will help restore aesthetics, function, and psychosocial stability. However, this comes at a significant lifetime cost to the healthcare system and families [Wehby and Cassell, 2010]. The etiology of these complex traits has been widely studied in order for us to understand the risk factors and to design strategies for prevention. Genetic factors, environmental factors, and the interaction between these two factors have been extensively reported in the literature as prime contributors to cleft outcomes [Dixon et al., 2011].

Genome-wide association studies for nonsyndromic cleft lip with or without palate (NSCL[P]) reported significant association for common variants in intergenic regions close to genes that are involved in craniofacial development i.e., *MAFB*, *VAX1*, *PAX7*, and *IRF6* [Birnbaum et al., 2009; Grant et al., 2009; Beaty et al., 2010; Mangold et al., 2010]. A significant association was also reported in a gene desert in the Chr 8q.24 locus [Birnbaum et al., 2009; Grant et al., 2009; Beaty et al., 2010; Mangold et al., 2010]. This gene desert is enriched for epigenetic markers [Huppi et al., 2012]. A recent meta-analysis by Ludwig et al. [2012] confirmed previous GWAS findings and identified six new additional loci that are genome-wide significant. Additional studies in independent populations replicated the GWAS findings [Nikopensius et al., 2010; Rojas-Martinez et al., 2010; Fontoura et al., 2012; Butali et al., 2013].

Resequencing studies conducted on the NSCL[P] GWAS candidate genes or adjacent genes suggest a role for rare variants [Beaty et al., 2010; Leslie et al., 2012; Nasser et al., 2012; Butali et al., 2013]. Data on European and Asian cleft triads from the Beaty et al., [2010] GWAS study suggest that some genes/loci are population specific (chr8q24.21 for Europeans and *MAFB* for Asians), while other genes play a role in multiple populations (*IRF6* in Europeans and Asians). This conforms to reports from recent population genetics studies showing rare variants to be population specific [Keinan and Clark, 2012; Tennessen et al., 2012].

GWAS replication studies using samples in the African population did not observe any significant association with the GWAS candidate genes [Butali et al., 2011; Weatherley-White et al., 2011]. Nonetheless, the role of rare functional variants in GWAS candidate genes has not been evaluated in the African population. We obtained saliva samples from case triads in Nigeria and Ethiopia for Sanger sequencing of the GWAS candidate genes in order to potential etiologic rare variants.

## MATERIALS AND METHODS

### Samples

The samples used for this genetic study were obtained in Nigeria and Ethiopia following ethical approval by the Institutional Review Boards at the Lagos University Teaching Hospital Idi-Araba, Lagos [IRB approval number: ADM/DCST/HREC/VOL.XV/321], Obafemi Awolowo University Teaching Hospital Ile-Ife (IRB approval

number: ERC/2011/12/01), and the Addis Ababa University (IRB approval number: 003/10/surg). A signed informed consent was obtained from every family recruited after they had read the study information and personal discussions with a member of the research team at the cleft clinic. All patients were examined by maxillofacial surgeons in Nigeria and plastic surgeons in Ethiopia for the presence of other congenital anomalies, features of syndromic forms of clefts, and other major structural anomalies and were excluded if these were present [described in Butali et al., 2011]. We collected samples from 220 affected probands (191 nonsyndromic cleft lip with or without cleft palate (NSCL[P]) and 29 nonsyndromic cleft palate (NSC[P]) and both parents (where possible). In total, samples were collected from 50 complete triads and 170 dyads (these include 47 triads and 93 dyads from Nigeria and 3 triads and 77 dyads from Ethiopia).

The Oragene collection kit [www.dnagenotek.com] was used to collect saliva samples from adults and children who were able to spit into the tube. For the newborn and children who were unable to spit, we used saliva sponges to soak saliva sublingually or in the buccal sulcus and these sponges were cut into the Oragene collection tubes. Blood samples were also collected during surgery for all affected probands.

Samples were shipped to the US for downstream applications that include DNA processing and sequencing. The protocol for DNA processing can be accessed via the Murray Lab website [genetics@uiowa.edu]. We measured the DNA concentration for all the samples using Qubit [http://www.invitrogen.com/site/us/en/home/brands/Product-Brand/Qubit.html] and a 4 ng/ul DNA concentration was used for Sanger sequencing.

### Sequencing

We selected the genes identified from the GWAS reported by Beaty et al. [2010]. These include *MAFB*, *PAX7*, *VAX1*, *ARHGAP29* (*ABCA4* locus), and *IRF6*. We included *ARHGAP29* reported by Leslie et al. [2012] which was identified as the candidate gene following resequencing of the GWAS signal around the *ABCA4*. The method used for sequencing has been previously reported [Leslie et al., 2012; Butali et al., 2013]. In summary, primers for the coding regions of all the candidate genes (*MAFB* NM\_00546; *PAX7* NM\_001135254.1; *VAX1* NM\_001112704.1; *ARHGAP29* NM\_004815.3; and *IRF6* NM\_006147.2) were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and optimized in the Murray lab using a gradient Polymerase Chain Reaction (PCR) to determine the annealing temperature for each primer set. A master mix containing 10xNH<sub>4</sub> buffer, 5% DMSO, 200 μM dNTPs, 50 μM MgCl<sub>2</sub>, water, 20 μM of forward and reverse primers and the 5u/μl Taq polymerase enzyme was prepared. We added 9 μl of the master mix to 1 μl of DNA in a 96-well plate. Two *Centre d'Etude du Polymorphisme Humain* (CEPH) samples and two water samples were added as controls. The primers used and annealing temperatures are available on request. Amplified DNA products were shipped to Functional Biosciences (http://order.functional-bio.com/seq/index) in Wisconsin for sequencing using an ABI 3730XL. Chromatograms were transferred to a Unix workstation, base-called with PHRED (v.0.961028), assembled with PHRAP (v. 0.960731), scanned by POLYPHRED (v. 0.970312), and viewed

with the CONSED program (v. 4). Variants found were compared to the variants that are present in the 1000 genome (1 KG) database (<http://www.1000genomes.org/>) and the Exome Variant Server (EVS) database (<http://snp.gs.washington.edu/EVS/>). We predicted the functional effects of these variants on the protein by using bioinformatics tools, such as polyphen (<http://genetics.bwh.harvard.edu/pph2/>) [Adzhubei et al., 2010], SIFT (<http://sift.jcvi.org/>) [Kumar et al., 2009] and HOPE (<http://www.cmbi.ru.nl/hope>) [Venselaar et al., 2010], and the Human Splice Finder (HSF) [Desmet et al., 2009]. We first sequenced only the probands in order to identify rare functional variants. In probands with new rare functional variants, we sequenced samples from their parents to tease out variants that are de novo or segregating in the family.

## RESULTS

### Sequencing Results

We found three new variants; a missense variant c.493C > G in *MAFB*, c.1282G > A in *PAX7*, and a splice-site variant c.952 + 2T > A that changes the donor site in exon 5 of *PAX7*. Two new variants were also found: c.493C > G in one unaffected parent and c.952 + 2T > A in another unaffected parent. For the proband with the c.1282G > A variant, we only have a sample from the father (who did not have the variant) and cannot confirm if the variant segregates or not. The other variants c.2864G > A and c.2738C > A in *ARHGAP29* and c.1396G > A in *PAX7* have been previously identified. We did not identify any rare variant in *VAX1*.

The mutant residue (Serine) is bigger than the wild-type residue (Glycine) at position 466 of the *PAX7* protein (Fig. 1) and will probably not be the fit core of the protein occupied by the wild type. Though the glycine residue is conserved at this position, a few other residue types have been observed at this position too. This includes the mutant Serine residue. This mutation is possibly not damaging to the protein based on conservation since homologous proteins exist with the same residue type as Serine mutant at this position.

The wild-type residue (Aspartic acid) is negatively charged and the mutant residue (Asparagine) is neutral at position 428 of the *PAX7* protein (Fig. 2). The negative charge of the buried wild-type residue in the core domain is lost when replaced by neutral mutation. The loss of negative charge might disturb the core structure of this domain.

The wild-type residue (Histidine) is not conserved at position 165 of the *MAFB* protein (Fig. 3). The mutant residue (Asparagine)

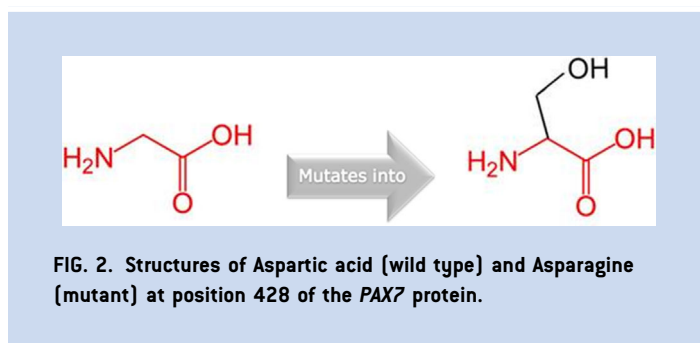


FIG. 2. Structures of Aspartic acid (wild type) and Asparagine (mutant) at position 428 of the *PAX7* protein.

is among the observed residue types at this position in other homologous sequences. This suggests that this variant is not damaging to the protein's structure and function.

## DISCUSSION

We found three new variants (p.Asp428Asn in *PAX7*; p.His165Asn in *MAFB*, and a donor splice site mutation in *PAX7*) following resequencing of GWAS candidate genes in the African population. The wild-type residue (Aspartic acid) in the variant p.Asp428Asn in *PAX7* is highly conserved. Nonetheless, other residue types have been identified at this position too in homologous sequences. Neither the Asparagine residue nor another residue with similar properties was observed at this position in other homologous sequences. Therefore, based on conservation scores and amino acid properties, the variant in *PAX7* was predicted to be probably damaging and deleterious. Glycine, the wild-type residue in the variant p.Gly466Ser in *PAX7*, is the most flexible of all residues and this unique property might be necessary for the protein's function. This flexibility may be required at this position to make a greater supporting structure or to ease movement of the protein. Therefore, mutation of glycine can eliminate these molecular properties by affecting the structure and movement of the protein leading to a less flexible residue. We previously reported this mutation in a Filipino case with CLP [Butali et al., 2013].

The variant p.His165Asn in *MAFB* was predicted to be benign by polyphen (<http://genetics.bwh.harvard.edu/pph2/>) [Adzhubei et al., 2010] and tolerated by SIFT (<http://sift.jcvi.org/>) [Kumar et al., 2009]. HOPE (<http://www.cmbi.ru.nl/hope>) [Venselaar et al., 2010] shows that the mutant residue is smaller than the wild type and this

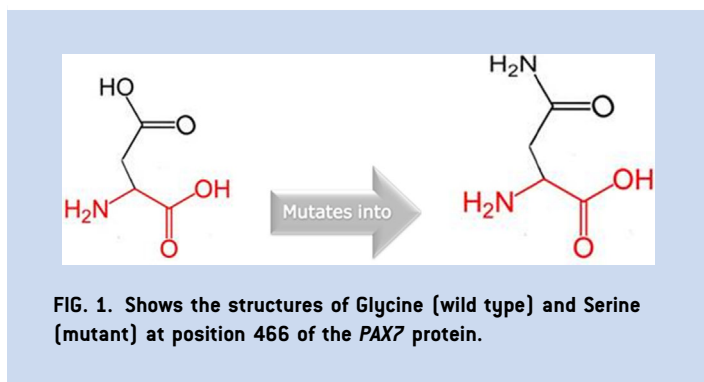


FIG. 1. Shows the structures of Glycine (wild type) and Serine (mutant) at position 466 of the *PAX7* protein.

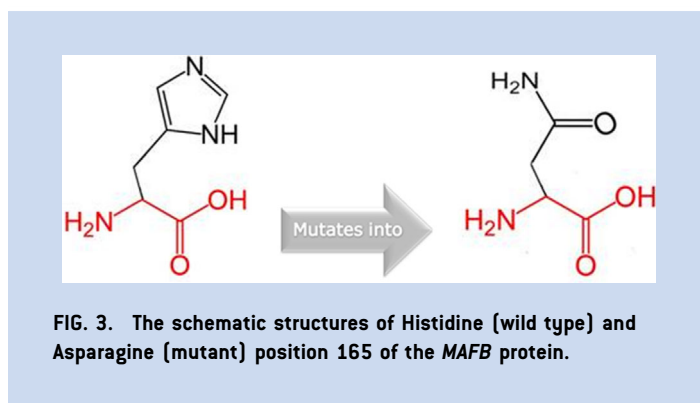


FIG. 3. The schematic structures of Histidine (wild type) and Asparagine (mutant) position 165 of the *MAFB* protein.

TABLE I. Rare functional Variants in GWAS candidate Genes

Genes	HGVS <sup>a</sup>	Amino acid change	Polyphen/SIFT predictions	Cleft details	Nigerian Cases [N = 140]	Ethiopian Cases [N = 80]	1KG	EVS
<i>ARHGAP29</i>	c.2864G > A	p.Arg955His	Benign/tolerated	CLP	1	0	1	0
	c.2738C > A	p.Ser913Leu	Stop retained	CL	1	0	3	11
<i>PAX7</i>	c.1396G > A	p.Gly466Ser	Benign/tolerated	CL	0	1	0	0
	c.952 + 2T > A**		Donor splice site variant	CL	0	1	0	0
<i>MAFB</i>	c.1282G > A**	p.Asp428Asn	Possibly damaging/deleterious	CL	1	0	0	0
	c.493C > G**	p.His165Asn	Benign/Tolerated	CP	1	0	0	0

1KG = 1000 genomes, EVS = Exome variant server \*\* New variants.

CL = Cleft lip CP = Cleft palate CLP = Cleft lip and palate.

<sup>a</sup>Ref Seq [*ARHGAP29* NM\_004815.3; *PAX7* NM\_001135254.1; *MAFB* NM\_00546.

change in size can be detrimental to the molecular interactions and deleterious to the protein. A previous study reported a rare damaging variant at position p.His131Glu in a Filipino case [Beaty et al., 2010]. The variants in *ARHGAP29* are known variants and previously reported in NSCL[P] by Leslie et al. [2012]. The *ARHGAP29* variants were also found in the 1000 genome database and Exome Variant Server (EVS). The individuals in the 1000 genome database have limited clinical information and family history; and the individuals in the Exome Variant Server were selected for specific heart, lung, and blood diseases. Therefore, it is possible that these variants may be rare normal variants, but because the cleft status of individuals in the EVS database is unknown, we cannot be sure. There are Nigerians and Kenyans (from East Africa – sharing the same border and similar geographical location as Ethiopia) in the 1 KG database. In the EVS, there are African Americans who are historically from West Africa (the geographical region where Nigeria is). Individuals in both databases serve as population controls for our cases. We observed that two of the new mutations [c.493C > G and c.952 + 2T > A] are in unaffected parents suggesting that there is incomplete penetrance of the phenotype in these individuals.

Previous studies have argued that it is important to investigate diverse populations in the study of complex diseases [Ramos et al., 2012]. A recent GWAS clearly showed the existence of population heterogeneity for some candidate genes [Beaty et al., 2010]. In addition, rare coding variants have been reported to play a role in the etiology of NSCLP [Leslie and Murray, 2012]. The data on the new rare variants in *PAX7* and *MAFB* (conservation score, amino acid properties and disruption of core domain) in the cases and not in controls suggest they are etiologic and specific to the African population. Our study thus provides additional evidence that studies in diverse populations may identify rare variants that are population specific.

The etiology of NSCL[P] is complex and it is possible that there are new candidate genes that may be specific to the African population – but this requires further verification. The findings also support the notion that rare variants may be population-specific or individual family specific. Therefore, future genome-wide studies in an independent African population should be conducted in order to unravel these additional molecular

genetic etiologies in addition to considering the role of common and unique environmental etiologies as co-variate in NSCLP risk.

## WEB RESOURCES

genetics@uiowa.edu

<http://order.functionalbio.com/seq/index>

<http://www.umd.be/HSF/>

<http://www.1000genomes.org/>

<http://snp.gs.washington.edu/EVS/>

<http://genetics.bwh.harvard.edu/pph2/>

<http://sift.jcvi.org/>

<http://www.cmbi.ru.nl/hope>

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