University of Dundee

Association between a common missense variant in LOXL3 gene and the risk of non-syndromic cleft palate

Khan, Mohammad Faisal Jamal; Little, Julian; Mossey, Peter; Steegers-Theunissen, Regine P. M.; Bonsi, Martina; Andreasi, Rita Bassi

1 **Article type.** Original article

2 **Full Title.** Association between a common missense variant in lysyl oxidase like 3

3 (*LOXL3*) gene and the risk of non-syndromic cleft palate.

4 **First author's surname.** Khan

5 **Short title.** LOXL3 variant in ns-cleft palate

6 **Full name of all authors.** Mohammad Faisal J Khan[1], Julian Little[2], Peter A Mossey[3],

7 Régine PM Steegers-Theunissen[4], Martina Bonsi[5], Rita Bassi Andreasi[6], Michele

8 Rubini[7].

9 **Address at which the work was carried out.** Department of Biomedical and Specialty

10 Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics,

11 University of Ferrara, Ferrara, Italy.

12 1. Department of Biomedical and Specialty Surgical Sciences, Section of Medical

13 Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.

14 Email: khnmmm@unife.it

15 2. School of Epidemiology and Public Health, University of Ottawa, Ottawa, Ontario,

16 Canada. Email: jlittle@uottawa.ca

17 3. Craniofacial Development at the World Health Organization–collaborating Centre for

18 Oral and Craniofacial Research, Dental Hospital and School, University of Dundee,

19 Dundee, Scotland. Email: p.a.mossey@dundee.ac.uk

20   4. Department of Obstetrics and Gynaecology, Department of Pediatrics, Division

21   Neonatology Erasmus MC, University Medical Center, Rotterdam, The Netherlands.

22   Email: r.steegers@erasmusmc.nl.

23   5. Department of Biomedical and Specialty Surgical Sciences, Section of Medical

24   Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.

25   Email: martina.bonsi@student.unife.it

26   6. Department of Biomedical and Specialty Surgical Sciences, Section of Medical

27   Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.

28   Email: bssrti@unife.it

29   7. Department of Biomedical and Specialty Surgical Sciences, Section of Medical

30   Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.

31   Email: rub@unife.it

32   **Corresponding author.**

33   **Michele Rubini**, Department of Biomedical and Specialty Surgical Sciences, Section

34   of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Via

35   Fossato di Mortara 74 I-44121, Ferrara, Italy. Fax No: +39.0532.236157 Telephone

36   No:

37   Email: rub@unife.it

38

ABSTRACT

To investigate possible association between functional common variants in the lysyl oxidase like 3 (*LOXL3*) gene and non-syndromic cleft palate (nsCP) we selected a common missense variant p.Ile615Phe (rs17010021), which was predicted to have a probably damaging effect on the *LOXL3* enzyme. We genotyped 258 nsCP case-parent triads of European origin and tested genetic association using the transmission disequilibrium test (TDT) and log-linear regression analyses of genotypic relative risks (RR) and of parent-of-origin effects. The observed genotype frequency in parents was in Hardy-Weinberg equilibrium. Compared with wild-type Ile/Ile homozygotes, the RR for Phe/Phe homozygote infants was 6.87 (p-value 3.0×10^{-3}), while that for Ile/Phe heterozygotes was not significant. Assuming an autosomal recessive model, the RR for Phe/Phe genotype resulted 10.54 (p-value 2.9×10^{-5}), with a 3.6% population attributable risk. No parental-of-origin effect was observed. The identification in *LOXL3* of a missense variant which under a recessive model associates with ten-fold increased risk of nsCP supports the hypothesis that the genetic etiology of this congenital anomaly includes relatively uncommon recessive variants with moderate penetrance and located in genes which are also involved in syndromes that include CP as part of the phenotype. Our findings require functional validation and replication in a larger independent genetic association study.

Key Words: lysyl oxidase like 3, non-syndromic, cleft palate, missense variant.

INTRODUCTION

The programming of palatal development starts early in the 4th week, with the formation of facial primordia that involves a complex series of closely coordinated events that includes proliferation, differentiation and morphogenetic movement (Dixon et al. 2011; Mossey et al. 2009). By the end of the 6th week, the primary palate is formed by the fusion of the medial nasal process with the maxillary process. The secondary palate arises as bilateral, medially directed outgrowths of the maxillary processes (palatal shelves) that initially grows vertically on either side of the tongue but later elevate to a horizontal position above the tongue. This horizontal growth of the adjacent palatal shelves leads to their contact with one another and fusion to form the secondary palate (Dixon et al. 2011). Disruption or perturbations at any step during this process that includes elevation, migration or fusion is likely to induce cleft palate.

Cleft palate (CP) is a common congenital orofacial malformation. Its prevalence at birth varies with geography and ethnicity between 1 and 25 per 10,000 live births, highest in non-Hispanic Whites and lowest in Africans (Burg et al. 2016). The sex ratio (male:female) of CP is 1:2. This might possibly be explained by differential gene expression as observed between sexes in animal models (Suazo et al. 2011), differential effects of female hormones (Miura et al. 1990), or delayed fusion of palate in females (Burg et al. 2016). Approximately 50% of CP cases are non-syndromic (nsCP), (Mai et al. 2014; Watkins et al. 2014), and are generally considered multifactorial conditions, due to interplay between genetic and environmental factors (Dixon et al. 2011; Mangold et al. 2011).

83   nsCP shows strong familial aggregation, which suggests a genetic component to

84   etiology (Marazita & Leslie 2016). Analyses of nationwide records from Norway and

85   Denmark show an increase in risk of recurrence among first-degree relatives of affected

86   individuals (Sivertsen et al. 2008; Grosen et al. 2010). The environmental factors

87   contributing to CP etiology so far identified are largely the same as those for cleft of the

88   lip and palate (CL/P) including tobacco smoke and alcohol (Little et al. 2004; Sabbagh

89   et al. 2015; Bell et al. 2014) and an inverse association with reported maternal use of

90   vitamin supplements (Butali et al. 2013).

91   A number of genetic factors associated with non-syndromic oral clefts have been

92   identified, mainly for CL/P rather than CP. Based on these genetic findings, non-

93   syndromic CL/P and non-syndromic CP are considered to have only very limited

94   overlap in terms of their genetic etiology (Cura et al. 2016). This is further supported by

95   genome-wide association studies (GWASs) or meta-analyses of GWAS data in different

96   populations that have identified 37 risk loci for non-syndromic CL/P (Birnbaum et al.

97   2009; Mangold et al. 2010; Beaty et al. 2010, 2013; Leslie et al. 2015, 2017; Yu et al.

98   2017; Ludwig et al. 2017; Ludwig et al. 2017; Ishorst et al., 2018), but just one

99   replicated finding for non-syndromic CP (Leslie et al. 2016; Mangold et al. 2016).

100   Although GWAS have helped detect and replicate associations between common gene

101   variants and orofacial clefts, the proportion of heritability accounted for by these

102   variants is relatively low, with inconsistencies across studies (Beaty et al. 2016). GWAS

103   have typically been designed to minimize the risk of false positive genetic associations

104   in common chronic disease, but it is known that there is a substantial risk of false

105   negatives (Ioannidis et al. 2011). Moreover, it has been observed that common variants

106 of genes that are involved in Mendelian disorders have been associated with non-

107 Mendelian forms of the same disorders (Blair et al. 2013). In addition, variants of some

108 genes involved in syndromic clefts have been found to have replicated associations with

109 non-syndromic clefts, reflecting the two forms of clefting as parts of a single spectrum

110 (Stanier & Moore 2004; Dixon et al. 2011). An excellent example is *GRHL3*, the second

111 gene associated with Van der Woude syndrome and its recent identification as

112 associated with nsCP (Leslie et al. 2016; Mangold et al. 2016).

113 The human *LOXL3* gene located on chromosome 2p13.1 has been associated with

114 Stickler syndrome (MIM #108300), which includes CP as a phenotype (Alzahrani et al.

115 2015). Additionally, deletion of this gene impairs collagen assembly and crosslinking

116 during palate development in mouse model (Zhang et al. 2015). However, no evidence

117 of linkage or association with nsCP was reported for this gene in a recent GWAS

118 (Leslie et al. 2016) or an imputation based meta-analysis of GWAS data (Ludwig et al.

119 2017). Of note, the ability to detect common variants with very weak effects, or less

120 common variants with small to modest effects, is strongly dependent on assumptions

121 concerning linkage disequilibrium, allele frequency and genotype certainty (Bomba et

122 al. 2017).

123 We therefore examined the potential association between putative functional variants of

124 *LOXL3* and nsCP, a disorder that is less common than other nsCL/P in humans, and less

125 investigated, in European case-parent triads.

126

127 **MATERIALS AND METHODS**

128 **Participants**

129   The study includes 258 nuclear families of infants with nsCP identified through the

130   EUROCRAN and ITALCLEFT biobanks, which include case-parent trios from 9

131   European countries (Mossey et al. 2017; Ghassibe-Sabbagh et al. 2011), including the

132   United Kingdom, Netherlands, Italy, Spain, Slovenia, Slovakia, Hungary, Estonia and

133   Bulgaria. The case-parent trio design of the present study makes it less vulnerable to

134   population stratification, a particular concern of multi-centre studies [Mossey et al.

135   2017]. Ethical permission was sought and obtained at surgical centres in each

136   participating countries at the time of first surgical intervention on the index infant.

137   Infants with recognized syndromic clefts or Pierre Robin sequence were excluded.

138   Peripheral blood or buccal cell samples were used to obtain genomic DNA from infants

139   and their parents. The use of data and DNA samples from EUROCRAN and

140   ITALCLEFT biobanks was approved by MREC Scotland (Dec 7th 2011, #MREC/1/0/7)

141   and S. Paolo Hosp. E.C. (Mar 2nd, 2012, #3503) respectively.

142   **Exposure information**

143   In both the EUROCRAN and ITALCLEFT studies mothers were asked to respond to a

144   specific questionnaire that was administered by personal interview when the index

145   affected infant was brought in to the surgical centre to undergo the primary surgery.

146   Major areas about which information was sought included use of nutritional

147   supplements and tobacco smoking. Folic acid supplementation was defined as having

148   taken folic acid or folic acid-containing supplements (at least 0.4 mg/day) for at least

149   one month during the periconceptional period (3 months before to 3 months after

150   conception). Maternal smoking during pregnancy was defined as having smoked at least

151   one cigarette per day during the periconceptional period (Mossey et al. 2017).

Selection of putative functional single nucleotide variants in *LOXL3*

We screened the exons of the *LOXL3* gene for nucleotide substitutions and insertions and deletions using the UCSC Genome browser GRCh38/hg38 assembly (https://genome-euro.ucsc.edu/) and identified 336 missense and 139 synonymous variants. Of these, according to dbSNP build 150 (www.ncbi.nlm.nih.gov/projects/SNP), only three are polymorphic, with minor allele frequency (MAF) >1%: rs17010022, rs17010021, and rs77706750. The first SNP, rs17010022, is a synonymous p.Leu371Leu variant located in exon 7 of *LOXL3* gene, with putative no effect on conformation of the encoded peptide, and therefore was discarded. The other two variants, rs77706750 in exon 7, and rs17010021 in exon 11, cause substitutions (p.Arg375His and p.Ile615Phe, respectively) both predicted to be "probably damaging" by PolyPhen-2 (Adzhubei et al. 2013). However, considering the available sample size, the MAF of rs77706750 was too low (1.46%) to provide enough power (0.80) under dominant or recessive genetic models (Quanto 1.2.4, biostats.usc.edu), and hence was not included in the present study. However, the MAF of rs17010021 was much higher (8.23% reported in dbSNP), granting sufficient power for a genetic association study.

Genotyping

For most individuals included in the study, genomic DNA (gDNA) was extracted from peripheral blood specimens using the Nucleon BACC1 kit (Amersham Biosciences, part of GE Healthcare Europe, CH). For around 5% of participants, gDNA was extracted from buccal swab specimens using QIAamp DNA Blood Mini Kit (Qiagen, Hilden DE) according to the manufacturer's instructions. All gDNA samples were quantified using Qubit® dsDNA BR Assay Kit (Life technologies Oregon, USA).

176   Genotypes of p.Ile615Phe variant were obtained by TaqMan allelic discrimination assay

177   using an ABI 7300 real-time thermocycler according to the standard protocol of

178   manufacturer (Applied BioSystems, Foster City, CA). In 15% of samples, genotyping

179   was repeated for quality testing.

180   **Statistical analysis**

181   The $\chi 2$ test for the Hardy-Weinberg equilibrium (HWE) were computed for genotypes

182   of parents and case-infants. The genetic association of the missense variant in nsCP

183   case-parent triads was calculated using the transmission disequilibrium test (TDT),

184   (Spielman et al. 1993). We estimated relative risk (RR) and 95% of confidence interval

185   (CI) for the independent effects of mother and infant genotypes using a log-linear

186   regression model that incorporates an expectation-maximization algorithm to allow

187   inclusion of triads for which both parent genotype were missing (Weinberg et al. 1998;

188   Wilcox et al. 1998). The analyses were implemented using the Stata package

189   (http://www.biostat-resources.com, StataCorp LP, College Station, TX). As exploratory

190   analyses, we carried out subgroup analyses stratifying on the sex of the infant and

191   maternal smoking and use of supplements containing folic acid.

192   We further investigated a possible parent-of-origin effect, by assessing the risk

193   increment (I_M) in the offspring associated with receiving the allele transmitted from the

194   mother as compared to the father in log-linear regression analysis (Weinberg et al. 1998;

195   Wilcox et al. 1998).

196

197   **RESULTS**

198   The study included 258 nsCP case-parent trios from 9 European countries. As expected,

199   female cases outnumbered the males, and male:female sex ratio was 0.78 (95% C.I.

200   0.74-0.84).

201   The allele and genotype frequency of the triads included in the study is shown in Table

202   1. Among the 516 parents included in the study the frequency of Phe allele was 4.7%

203   (95%CI 2.9-6.5%), a value lower than the 8.23% reported in dbSNP. Genotype

204   frequency among cases was significantly out of Hardy-Weinberg equilibrium (p-value =

205   2.27×10^{-6}), while both parents resulted not in disequilibrium (p-value = 0.68).

206   Remarkably, the frequency of Phe/Phe homozygotes, predicted to be only 0.22% on the

207   basis of allele frequency in the parents, was 7-fold higher (1.55%) than predicted among

208   nsCP cases.

209   Application of TDT showed no significant evidence of asymmetric segregation of Phe

210   allele from parents (Transmitted:Non-transmitted = 21:23, p-value = 0.673).

211   Considering the observed low frequency of Ile/Phe genotype among parents (Table 1),

212   and being the power of the TDT heavily dependent on the number of heterozygous

213   parents (Sebro, Rogus, 2010), we performed the calculation of genotype-associated RR

214   using a log-linear regression model (Weinberg et al. 1998; Wilcox et al. 1998).

215   Calculation of genotype-associated RR showed significant association between Phe/Phe

216   homozygous infant genotype and nsCP risk (RR = 6.9, p-value = 0.003), whereas there

217   was no significant association with the heterozygous genotype. Mother's genotype was

218   not associated with increased risk of nsCP in the offspring (Table 2).

219   Considering that the Ile/Phe genotype provided no increased risk of nsCP compared to

220   wild type Ile/Ile homozygotes, while Phe/Phe genotype associated with increased risk,

221 we assumed a recessive genetic model. Under this model, log-linear regression analysis

222 showed that infant's Phe/Phe genotype associated with a significant ten-fold increased

223 risk of nsCP (RR = 10.54 (95% C.I. 3.34-33.30, p-value = 2.85×10^{-5}). No parental of

224 origin effect was observed (I^M = 0.58, p-value = 0.455).

225 Considering the genotypic frequencies of parents as reference and a birth prevalence of

226 nsCP of 1:2216 among Europeans (Calzolari et al., 2004), the population attributable

227 risk of Phe/Phe genotype was 3.6%, whereas the penetrance was 0.48%.

228 Although we are aware of the limited sample size of our study, we conducted subgroup

229 analyses and report these. Among the four Phe/Phe infants, three females and one male,

230 only one girl was born from a mother exposed to folic acid supplementation during the

231 periconceptional period. As regards periconceptional exposure to tobacco smoking, all

232 four Phe/Phe infants were born from non-smoking mothers. RR did not significantly

233 differed between male and female cases.

234

235 **DISCUSSION**

236 In the present study, we investigated a potential association between functional common

237 variants in lysyl oxidase like 3 (*LOXL3)* gene and the risk of developing nsCP. Rare

238 variants in *LOXL3* have been detected in patients with Stickler syndrome, which may

239 present with CP (Alzahrani et al. 2015), and in mouse model a crucial role of Loxl3

240 gene in palate development has been demonstrated (Zhang et al. 2015). Among the

241 hundreds of missense variants annotated in *LOXL3* gene we selected p.Ile615Phe, which

242 is the only one that is predicted to be probably damaging and has relatively high MAF,

243 sufficient to provide enough statistical power considering the sample size of the study.

244   Although Phe/Phe homozygotes are very uncommon, we identified four Phe/Phe

245   homozygotes among the 258 cases included in the study, and detected a significant

246   association between infant's homozygote Phe/Phe genotype and the risk of nsCP,

247   compared to common Ile/Ile homozygotes. Heterozygous Ile/Phe genotype was not

248   significantly associate with nsCP. Therefore, assuming an autosomal recessive model,

249   the Phe/Phe genotype turned out to associate with around ten-fold increased risk of

250   nsCP (p-value = 2.85×10^{-5}). Autosomal recessive genetic model is typical for enzyme-

251   encoding genes, and fits well with the nature of *LOXL3*. As the p.615Phe enzyme is

252   predicted to have lost most or all catalytic activity, we presume that Phe/Phe

253   homozygotes are severely deficient of the amine oxidase activity of *LOXL3* enzyme,

254   and consequently have impaired collagen fiber assembly in palatal mesenchyme. We

255   hypothesize that this impairment could have played a role in determining the failure of

256   fusion of palatal shelves during embryogenesis, and ultimately caused CP. The lack of

257   efficient catalysis of collagen crosslinking associated with p.615Phe enzyme may

258   resemble the effect of LOX's inhibitor β-aminopropionitrile, which determine reduced

259   collagen fibres density and development of CP in animal model (Pratt & King 1972).

260   Functional studies using animal models are awaited to confirm the phenotypic effect of

261   p.615Phe enzyme.

262   The failure of TDT to detect association could rely on the fact that, due to the relative

263   low MAF of the studied *LOXL3* variant, among the cases most of p.615Phe alleles are

264   carried by heterozygotes, which are not at risk of nsCP, and therefore distortion of

265   transmission from parents would not be expected.

266 As might be anticipated for a gene expressed in palate shelves during embryonic

267 development, maternal p.Ile615Phe genotype was not associated with the infant's risk

268 of nsCP. Moreover, no preferential transmission of minor allele from one of two parents

269 was observed. Due to the low frequency of Phe/Phe homozygotes, the statistical power

270 of the study was not sufficient to detect interaction with infant sex, periconceptional

271 folic acid supplementation, or exposure to tobacco smoking.

272 The infant's Phe/Phe genotype seems to strongly increase the risk of nsCP, but its actual

273 weight among the multiple genetic and environmental factors as part of the

274 multifactorial etiology of nsCP is relatively small. Due to the relatively low MAF of

275 p.Ile615Phe, the calculated population attributable risk was only 3.6%, and the

276 penetrance modest (0.48%). We hypothesize that other functional variants of the *LOXL3*

277 gene, mainly classified as rare variants and less frequent than the p.Ile615Phe variant,

278 might be associated with nsCP risk.

279 The impact of rare or less common variants associated with increased risk of nsCP has

280 begun to emerge from recent exome-wide and genome-wide sequencing studies

281 (Mangold et al. 2016). In particular, a low frequency missense p.Thr454Met variant in

282 *GRHL3* (rs41268753) was significantly associated with nsCP risk (Mangold et al. 2016;

283 Leslie et al. 2016). From the latest genetic investigations on nsCP, a difference in terms

284 of frequency spectrum of susceptibility variants compared to nsCL/P, is becoming

285 evident. While GWAS of nsCL/P identified a number of common polymorphic variants

286 (Birnbaum et al. 2009; Beaty et al. 2010; Mangold et al. 2010), GWAS of nsCP have

287 detected only one genome-wide significant variant (Leslie et al. 2016), even though

288 sample size were comparable. This evidence suggests that the genetic aetiology of nsCP

289 may mainly rely on relatively rare variants, or less common variants that act under

290 recessive model, which may present moderate penetrance, tending to escape detection

291 by genome-wide studies and to be located within genes involved in syndromes that

292 include CP as part of the phenotype. *LOXL3* p.Ile615Phe may be one of these variants.

293 In conclusion, using a candidate gene approach, we identified a missense variant in

294 *LOXL3* gene, p.Ile615Phe, which under a recessive model is associated with a

295 significant ten-fold increased risk of nsCP. This finding should be replicated in a larger

296 cohort of case-parent trios, and joint effects with environmental exposure factors

297 investigated. We suggest that *LOXL3* p.Ile615Phe, along with *GRHL3* p.Thr454Met, are

298 part of a constellation of low frequency variants that compose the genetic background of

299 nsCP.

300

310

311 **DISCLOSURE**   None.

312

REFERENCES

Adzhubei I, Jordan DM, Sunyaev SR. 2013. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet Chapter 7: Unit7 20.

Alzahrani F, Al Hazzaa SA, Tayeb H, Alkuraya FS. 2015. LOXL3, encoding lysyl oxidase-like 3, is mutated in a family with autosomal recessive Stickler syndrome. Hum Genet 134: 451-3.

Beaty TH, Murray JC, Marazita ML et al. 2010. A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. Nat Genet 42: 525-9.

Beaty TH, Taub MA, Scott AF et al. 2013. Confirming genes influencing risk to cleft lip with/without cleft palate in a case-parent trio study. Hum Genet 132: 771-81.

Beaty TH, Marazita ML, Leslie EJ. 2016. Genetic factors influencing risk to orofacial clefts: today's challenges and tomorrow's opportunities. F1000Res 5: 2800.

Bell JC, Raynes-Greenow C, Turner RM, Bower C, Nassar N, O'Leary CM. 2014. Maternal alcohol consumption during pregnancy and the risk of orofacial clefts in infants: a systematic review and meta-analysis. Paediatr Perinat Epidemiol 28: 322-32.

Birnbaum S, Ludwig KU, Reutter H et al. 2009. Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. Nat Genet 41: 473-7.

Blair DR, Lyttle CS, Mortensen JM et al. 2013. A nondegenerate code of deleterious variants in Mendelian loci contributes to complex disease risk. Cell 155: 70-80.

336 Bomba L, Walter K, Soranzo N. 2017. The impact of rare and low-frequency genetic

337   variants in common disease. Genome Biol 18: 77.

338 Burg ML, Chai Y, Yao CA, Magee W, 3rd, Figueiredo JC. 2016. Epidemiology,

339   Etiology, and Treatment of Isolated Cleft Palate. Front Physiol 7: 67.

340 Butali A, Little J, Chevrier C et al. 2013. Folic acid supplementation use and the

341   MTHFR C677T polymorphism in orofacial clefts etiology: An individual

342   participant data pooled-analysis. Birth Defects Res A Clin Mol Teratol 97: 509-14.

343 Calzolari E, Bianchi F, Rubini M, Ritvanen A, Neville AJ, EUROCAT Working group.

344   2004. Epidemiology of cleft palate in Europe: implications for genetic research.

345   Cleft Palate Craniofac J 41: 244-9.

346 Cura F, Bohmer AC, Klamt J et al. 2016. Replication analysis of 15 susceptibility loci

347   for nonsyndromic cleft lip with or without cleft palate in an italian population. Birth

348   Defects Res A Clin Mol Teratol 106: 81-7.

349 Dixon MJ, Marazita ML, Beaty TH, Murray JC. 2011. Cleft lip and palate:

350   understanding genetic and environmental influences. Nat Rev Genet 12: 167-78.

351 Ghassibe-Sabbagh M, Desmyter L, Langenberg T et al. 2011. FAF1, a gene that is

352   disrupted in cleft palate and has conserved function in zebrafish. Am J Hum Genet

353   88: 150-61.

354 Grosen D, Chevrier C, Skytthe A et al. 2010. A cohort study of recurrence patterns

355   among more than 54,000 relatives of oral cleft cases in Denmark: support for the

356   multifactorial threshold model of inheritance. J Med Genet 47: 162-168.

357 Ioannidis JP, Tarone R, McLaughlin JK. 2011. The false-positive to false-negative ratio

358   in epidemiologic studies. Epidemiology 22: 450-6.

359   Ishorst N, Franceschelli P, Böhmer AC et al. 2018. Nonsyndromic cleft palate: An

360   association study at GWAS candidate loci in a multi-ethnic sample. Birth Def. Res

361   Part A.

362   Leslie EJ, Taub MA, Liu H et al. 2015. Identification of functional variants for cleft lip

363   with or without cleft palate in or near PAX7, FGFR2, and NOG by targeted

364   sequencing of GWAS loci. Am J Hum Genet 96: 397-411.

365   Leslie EJ, Liu H, Carlson JC et al. 2016. A Genome-wide Association Study of

366   Nonsyndromic Cleft Palate Identifies an Etiologic Missense Variant in GRHL3.

367   Am J Hum Genet 98: 744-754.

368   Leslie EJ, Carlson JC, Shaffer JR et al. 2017. Genome-wide meta-analyses of

369   nonsyndromic orofacial clefts identify novel associations between FOXE1 and all

370   orofacial clefts, and TP63 and cleft lip with or without cleft palate. Hum Genet 136:

371   275-286.

372   Little J, Cardy A, Munger RG. 2004. Tobacco smoking and oral clefts: a meta-analysis.

373   Bull World Health Organ 82: 213-8.

374   Ludwig KU, Ahmed ST, Böhmer AC, et al. 2017. Meta-analysis reveals genome-wide

375   significance at 15q13 for nonsyndromic clefting of both the lip and the palate, and

376   functional analyses implicate GREM1 as a plausible causative gene. PLoS Genet

377   12: e1005914.

378   Ludwig KU, Böhmer AC, Bowes J et al. 2017. Imputation of orofacial clefting data

379   identifies novel risk loci and sheds light on the genetic background of cleft lip +/-

380   cleft palate and cleft palate only. Hum Mol Genet 26: 829-842.

381   Mai CT, Cassell CH, Meyer RE et al. 2014. Birth defects data from population-based

382   birth defects surveillance programs in the United States, 2007 to 2011: highlighting

383   orofacial clefts. Birth Defects Res A Clin Mol Teratol 100: 895-904.

384   Mangold E, Ludwig KU, Birnbaum S et al. 2010. Genome-wide association study

385   identifies two susceptibility loci for nonsyndromic cleft lip with or without cleft

386   palate. Nat Genet 42: 24-6.

387   Mangold E, Ludwig KU, Nothen MM. 2011. Breakthroughs in the genetics of orofacial

388   clefting. Trends Mol Med 17: 725-33.

389   Mangold E, Bohmer AC, Ishorst N et al. 2016. Sequencing the GRHL3 Coding Region

390   Reveals Rare Truncating Mutations and a Common Susceptibility Variant for

391   Nonsyndromic Cleft Palate. Am J Hum Genet 98: 755-62.

392   Marazita ML, Leslie EJ. 2016. Genetics of Nonsyndromic Clefting. In: Losee J,

393   Kirschner R, editors. *Comprehensive Cleft Care*. Boca Roton FL: CRC Press,

394   Taylor & Francis Group. p 207–24.

395   Miura S, Natsume N, Horiuchi R. 1990. Experimental study on cleft lip and palate.

396   Preventive effects of estradiol on cleft lip and/or palate in A/J mice. J Jpn Cleft

397   Palate Assoc 15:122–131.

398   Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. 2009. Cleft lip and palate.

399   Lancet 374: 1773-85.

400   Mossey PA, Little J, Steegers-Theunissen R et al. 2017. Genetic Interactions in

401   Nonsyndromic Orofacial Clefts in Europe-EUROCRAN Study. Cleft Palate

402   Craniofac J 54: 623-630.

403   Pratt RM, Jr., King CT. 1972. Inhibition of collagen cross-linking associated with beta-

404   aminopropionitrile-induced cleft palate in the rat. Dev Biol 27: 322-8.

405 Sabbagh HJ, Hassan MH, Innes NP, Elkodary HM, Little J, Mossey PA. 2015. Passive

406   smoking in the etiology of non-syndromic orofacial clefts: a systematic review and

407   meta-analysis. PLoS One 10: e0116963.

408 Sebro R, Rogus JJ. 2010. The power of the Transmission Disequilibrium Test in the

409   presence of population stratification. Eur J Hum Genet 18:1032-8.

410 Sivertsen A, Wilcox AJ, Skjaerven R, Vindenes HA, Abyholm F, Harville E, Lie RT.

411   2008. Familial risk of oral clefts by morphological type and severity: population

412   based cohort study of first degree relatives. BMJ 336: 432-4.

413 Spielman RS, McGinnis RE, Ewens WJ. 1993. Transmission test for linkage

414   disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus

415   (IDDM). Am J Hum Genet 52: 506-16.

416 Stanier P, Moore GE. 2004. Genetics of cleft lip and palate: syndromic genes contribute

417   to the incidence of non-syndromic clefts. Hum Mol Genet 13 Spec No 1: R73-81.

418 Suazo J, Tapia JC, Santos JL, Castro VG, Colombo A, Blanco R. 2011. Risk variants in

419   BMP4 promoters for nonsyndromic cleft lip/palate in a Chilean population. BMC

420   Med Genet 12: 163.

421 Watkins SE, Meyer RE, Strauss RP, Aylsworth AS. 2014. Classification, epidemiology,

422   and genetics of orofacial clefts. Clin Plast Surg 41: 149-63.

423 Weinberg CR, Wilcox AJ, Lie RT. 1998. A log-linear approach to case-parent-triad

424   data: assessing effects of disease genes that act either directly or through maternal

425   effects and that may be subject to parental imprinting. Am J Hum Genet 62: 969-

426   78.

427   Wilcox AJ, Weinberg CR, Lie RT. 1998. Distinguishing the effects of maternal and

428   offspring genes through studies of "case-parent triads". Am J Epidemiol 148: 893-

429   901.

430   Yu Y, Zuo X, He M et al. 2017. Genome-wide analyses of non-syndromic cleft lip with

431   palate identify 14 novel loci and genetic heterogeneity. Nat Commun 8: 14364.

432   Zhang J, Yang R, Liu Z et al. 2015. Loss of lysyl oxidase-like 3 causes cleft palate and

433   spinal deformity in mice. Hum Mol Genet 24: 6174-85.

434

435   **Table 1** Allele and genotype frequencies of p.Ile615Phe (rs17010021) in 258

436   nsCP case-parent triads, and p-value of difference from Hardy-Weinberg (H-W)

437   equilibrium.

438

Alleles/Genotypes	Cases n (%)	Mothers n (%)	Fathers n (%)
Ile	493 (95.5)	498 (96.5)	486 (94.2)
Phe	23 (4.5)	18 (3.5)	30 (5.8)
Ile/Ile	239 (92.6)	240 (93.0)	230 (89.1)
Ile/Phe	15 (5.8)	18 (7.0)	26 (10.1)
Phe/Phe	4 (1.6)	0 (0.0)	2 (0.8)
H-W p-value	2.27×10^{-6}	0.85	0.44

439

440　　　**Table 2** Genotype-associated relative risk of p.Ile615Phe (rs17010021) in 258

441　　　nsCP case-parent triads assuming the common Ile/Ile homozygous genotype as

442　　　reference.

443

Mother's genotypes	RR (95% C.I.)	p-value
Ile/Phe	0.54 (0.28-1.05)	0.071
Phe/Phe	n.c.	-
Infant's genotypes	RR (95% C.I.)	p-value
Ile/Phe	0.61 (0.31-1.17)	0.136
Phe/Phe	6.87 (1.97-23.98)	0.003

444