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ORIGINAL ARTICLE

Family based and case–control designs reveal an association of *TFAP2A* in nonsyndromic cleft lip only among Vietnamese population

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Abstract

Aims: Dozens of causative genes and their mechanisms of nonsyndromic cleft lip with or without cleft palate (NSCL/P) were revealed through genome-wide association and linkage studies. Results were, however, not always replicated in different populations or methodologies. This study used case–control and family based approaches to investigate the etiology of NSCL/P and its two subtypes: nonsyndromic cleft lip only (NSCLO) and nonsyndromic cleft lip and palate (NSCLP) among the Vietnamese population.

Methods: Two hundred and seventeen NSCL/P case-parent trios (one affected child and two parents), including 105 NSCLO and 112 NSCLP were involved for a family based design; and 273 ethnic and region-matched healthy controls with no cleft history in their families were recruited for a case–control design. Three SNPs consisting of *TFAP2A* (rs1675414 and rs303048) and 8q24 (rs987525) were genotyped using the TaqMan SNP genotyping assay.

Results: *TFAP2A* rs1675414 was associated with NSCLO, replicated by both case-control and family based tests. Other SNPs yielded no evidence of susceptibility to NSCL/P or two subtypes.

Conclusion: The current investigation suggests an intriguing role of *TFAP2A* in the etiology of NSCLO among the Vietnamese population.

KEYWORDS
cleft, cleft lip palate, nonsyndromic, TFAP2A, Vietnamese

1 | INTRODUCTION

Cleft lip with or without cleft palate (CL/P), the more common of the two groups of orofacial clefts, affects ~3.4–22.9 per 10,000 births (Mossey et al., 2009). CL/P generates an enormous public health burden due to life-long morbidity and multidiscipline interventions (Allam et al., 2014). Even though more than 200 recognized syndromes list CL/P as a feature (Wong & Hägg, 2004), 71% of CL/P cases are isolated (Calzolari et al., 2007). Typically, mutations within a single gene can control a Mendelian syndrome that includes oral clefts as a hallmark, whereas nonsyndromic CL/P (NSCL/P) is considered a complex etiology of multiple genes and environmental factors (Bishop et al., 2020). NSCL/P could be further classified into nonsyndromic cleft lip only (NSCLO) and nonsyndromic cleft lip and palate (NSCLP). Evidence showed that NSCLP and NSCLO might harbor distinct genetic etiologies (Huang et al., 2019; Jia et al., 2015; Ludwig et al., 2012, 2016; Rahimov et al., 2008).

A recent meta-analysis suggested that transcription factors (TFs) are key players in developing NS orofacial clefts subtypes (Huang et al., 2019) Numerous studies revealed the AP2 TFs, with the most notable, the transcription factor activating enhancer-binding protein 2 alpha (*TFAP2A*), are critical factors in neural crest cell development which are involved in facial growth and morphogenesis (Green et al., 2015; Van Otterloo et al., 2018). In animal models, *TFAP2A* knockout mice exhibit malformations including CL/P and prominent mandibular and maxillary dysmorphology (Nottoli et al., 1998). In humans, mutations in *TFAP2A* cause branchio-oculofacial syndrome, an autosomal dominant rare disease that could include CL/P (Milunsky et al., 2008). Previous linkage studies demonstrated evidence of *TFAP2A* in NSCL/P (Moreno et al., 2004; Prescott et al., 2000; Schultz et al., 2004). *TFAP2A* was associated with NSCL/P in a Brazilian population and Italian triads (Araujo et al., 2016; Martinelli et al., 2011). Taken together, these data suggest *TFAP2A* might be a strong candidate for NSCL/P etiology.

8q24 is a gene desert that signifies a major NSCL/P susceptibility locus, which was confirmed in genome-wide association studies (GWASs) and as well as meta-analysis studies (Beaty et al., 2010; Birnbaum et al., 2009; Grant et al., 2009; Leslie et al., 2016; Wang et al., 2012). The locus was found to contain multiple markers highly associated with NSCL/P, including rs987525 polymorphism as the most significant one (Birnbaum et al., 2009). Several enhancers for craniofacial development located in this locus (Hochheiser et al., 2011).

Case–control and family based designs have been used widely in genetic association for complex diseases. Both strategies have, however, key drawbacks. While studies of case–control are prone to confounding due to population stratification, the family based studies can bypass that problem but are susceptible to segregation distortion at loci affecting early life survival (Ackerman et al., 2005). The potential solution is to use both methods and inspect whether a similar estimate is replicated.

Here the current study used case–control and family based approaches to investigate associations of *TFAP2A* gene and 8q24 gene desert in the etiology of NSCL/P and its subtypes among the Vietnamese population.

TABLE 1 NSCLO, NSCLP probands distributed by gender and controls

	NSCLO	NSCLP	NSCL/P	Control
Male	56	69	125	151
Female	49	43	92	122
Total	105	112	217	273

2 | MATERIALS AND METHODS

2.1 | Subjects

The study consisted of 217 NSCL/P case-parent trios (one affected child and two parents), including 105 NSCLO and 112 NSCLP for a family based design; and 273 ethnic and region-matched healthy controls with no cleft history in their families for a case–control design (Table 1). All participants were recruited from the Odonto and Maxillofacial Hospital in Ho Chi Minh City, Vietnam. Syndromic CL/P were ruled out through medical records and examination. Informed consent for participation was collected from all participants and, in cases of children younger than 18 years, their parents. Peripheral blood samples were poured on dried blood cards and stored at World Cleft Gene Banking (Natsume et al., 2013). This study was approved by the Aichi Gakuin University Ethics Committee (Number 78). All the procedures have been performed as per the ethical guidelines laid down by the Declaration of Helsinki (World Medical Association 2013).

2.2 | SNP selection, DNA extraction, and genotyping

Two SNPs of *TFAP2A* rs1675414 (exon 1), rs303048 (intron 6) and one SNP of 8q24 region rs987525 were selected for genotyping based on (a) previous GWASs and association studies (Beaty et al., 2010; Birnbaum et al., 2009; Grant et al., 2009; Leslie et al., 2016; Shi et al., 2011; Wang et al., 2012) and (b) minor allele frequency of KHV population (Kinh in Ho Chi Minh City, Vietnam) above 0.10 from the 1000 genome database (Consortium GP, 2015).

DNA was extracted from dried blood spots using the QIAMP DNA Blood Mini Kit (QIAGEN) following the

manufacturer's protocol. Genotyping was performed with the prestandardized and experimentally validated TaqMan SNP genotyping assay (Applied Biosystems) in the 7900HT Fast-Real Time PCR system (Applied Biosystems). A genotype call rate >99% was considered to be acceptable.

2.3 | Statistical analysis

Hardy–Weinberg equilibrium, pairwise linkage disequilibrium (LD) as both D' and r^2 for each SNPs were computed by the SHEsis application (Shi & He, 2005). Comparison of genotype and allele frequencies between case and control groups were performed by chi-squared test. Odds ratios (ORs) and 95% confidence intervals (CIs) were also calculated. Transmission disequilibrium test (TDT; Spielman et al., 1993) was performed to examine the transmission of target alleles from heterozygous parents to affected offspring. The PLINK software with parent-of-origin effects was applied to screen the parental preference in the allele inheritance (http://zzz.bwh.harvard.edu/plink/). A Bonferroni correction was applied with $\alpha = 0.0125 = 0.05/4$ (2 genes × 2 phenotypes).

3 | RESULTS

3.1 | Hardy–Weinberg equilibrium test and Minor allele frequency

All four SNPs were in Hardy–Weinberg equilibrium. Minor allele frequencies (MAFs) were all above 10% (Table 2), similar to MAFs reported in the KHV population from the 1000 genome database (Consortium GP, 2015).

3.2 | Case–control comparisons

Table 3 shows associations of three SNPs with risk of NSCL/P and two subtypes. There was no evidence of genotypic or allelic association with the susceptibility to NCLO, NSCLP, NSCL/P for *TFAP2A* rs303048, and 8q24 rs987525 in the Vietnamese population. Significant differences compared with controls in genotype frequency and allele frequency were observed in the NSCLO group with *TFAP2A* rs1675414.

Genotype TC and allele C (rs1675414) increased the risk of NSCLO OR = 1.97, (95% CI: 1.22–3.19, $p = .006$) and OR = 1.86 (95% CI: 1.26–2.75, $p = .002$) compared with TT and T, respectively. Further dominant model analysis revealed that combining genotype (TC +CC) was associated with an increased risk for NSCLO (OR = 2.06, 95% CI: 1.29–3.29), $p = .002$ compared with TT.

3.3 | Transmission disequilibrium test

Transmission disequilibrium test showed an overtransmission of allele C at rs1675414 among the NSCLO group (OR = 1.88, $p = .010$), suggesting that patients who were inherited the allele C at rs1675414 had 1.88-fold increased risk of NSCLO compared with those who received the allele T (Table 4).

3.4 | Parent-of-origin effects

There was no significant deviation from expected Mendelian inheritance in paternal transmission. Allele C at *TFAP2A* rs1675414 was overtransmitted from mothers in the NSCLO group ($p = .005$). However, no SNPs showed significant differences between paternal and maternal transmission (Table 5).

3.5 | Linkage disequilibrium tests

Pair-wise LD was performed to test whether the two SNPs at the *TFAP2A* gene were independent. The results showed strong LD between rs1675414 and rs303048 in NSCL/P group (D' = .999, $r^2 = 0.06$).

4 | DISCUSSION

To date, the etiology of CL/P in Vietnamese has been limitedly studied and reported in few genes, consisted of *MSX1* (Suzuki et al., 2004), *IRF6* (Zucchero et al., 2004), *VAX1*, and

TABLE 2 Minor allele frequency and Hardy–Weinberg equilibrium test for each SNPs

				Control		NSCLO		NSCLP		NSCL/P	
Chr	Gene/region	SNP	Allele	MAF	HWE p	MAF	HWE p	MAF	HWE p	MAF	HWE p
6	*TFAP2A*	rs303048	A>T	0.236	0.678	0.195	0.536	0.263	0.708	0.230	0.854
6	*TFAP2A*	rs1675414	T>C	0.150	0.583	0.248	0.451	0.134	0.994	0.189	0.535
8	*8q24*	rs987525	C>A	0.115	0.828	0.138	0.411	0.129	0.347	0.134	0.942

TABLE 3 Associations of SNPs for NSCL/P, its subtypes, and control group

SNP	Gen./alle.	Control (n = 273)	NSCLO (n = 105)	OR	*p*	NSCLP (n = 112)	OR (95% CI)	*p*	NSCL/P (n = 217)	OR	*p*
rs303048	AA	158 (0.579)	69 (0.657)	1.00	—	60 (0.536)	1.00	—	129 (0.594)	1.00	—
	AT	101 (0.370)	31 (0.295)	0.70 (0.43– 1.15)	.160	45 (0.402)	1.17 (0.74–1.86)	.496	76 (0.350)	0.92 (0.63–1.34)	.672
	TT	14 (0.051)	5 (0.048)	0.82 (0.28–2.36)	.710	7 (0.062)	1.32 (0.51–3.42)	.572	12 (0.055)	1.05 (0.47–2.35)	.906
	AT+TT	115 (0.421)	36 (0.343)	0.72 (0.45–1.15)	.164	52 (0.464)	1.19 (0.77–1.85)	.439	88 (0.405)	0.94 (0.65–1.35)	.726
	A	417 (0.764)	169 (0.805)	1.00	—	165 (0.737)	1.00	—	334 (0.770)	1.00	—
	T	129 (0.236)	41 (0.195)	0.78 (0.53–1.16)	.227	59 (0.263)	1.14 (0.80–1.63)	.465	100 (0.230)	0.97 (0.72–1.30)	.830
rs1675414	TT	196 (0.718)	58 (0.552)	1.00	—	84 (0.750)	1.00	—	142 (0.654)	1.00	—
	TC	72 (0.264)	42 (0.400)	**1.97 (1.22–3.19)**	**.006**	26 (0.232)	0.84 (0.50–1.41)	.516	68 (0.313)	1.30 (0.88–1.93)	.189
	CC	5 (0.018)	5 (0.048)	3.38 (0.95–12.08)	.06	2 (0.018)	0.93 (0.18–4.90)	.935	7 (0.032)	1.93 (0.60–6.21)	.269
	TC+CC	77 (0.282)	47 (0.448)	**2.06 (1.29–3.29)**	**.002**	28 (0.25)	0.85 (0.51–1.40)	.522	75 (0.345)	1.34 (0.92–1.97)	.131
	T	464 (0.850)	158 (0.752)	1.00	—	194 (0.866)	1.00	—	352 (0.811)	1.00	—
	C	82 (0.150)	52 (0.248)	**1.86 (1.26–2.75)**	**.002**	30 (0.134)	0.88 (0.56–1.37)	.561	82 (0.189)	1.32 (0.94–1.84)	.107
rs987525	CC	214 (0.784)	77 (0.733)	1.00	—	86 (0.768)	1.00	—	163 (0.751)	1.00	—
	CA	55 (0.201)	27 (0.257)	1.36 (0.80–2.32)	.250	23 (0.205)	1.04 (0.60–1.80)	.887	50 (0.230)	1.19 (0.77–1.84)	.424
	AA	4 (0.015)	1 (0.010)	0.69 (0.08–6.31)	.746	3 (0.027)	1.87 (0.41–8.51)	.420	4 (0.018)	1.31 (0.32–5.33)	.703
	CA+AA	59 (0.985)	28 (0.267)	1.32 (0.78–2.22)	.297	26 (.232)	1.10 (0.65–1.85)	.731	54 (0.248)	1.20 (0.79–1.83)	.393
	C	483 (0.885)	181 (0.862)	1.00	—	195 (0.871)	1.00	—	376 (0.866)	1.00	—
	A	63 (0.115)	29 (0.138)	1.23 (0.77–1.97)	.393	29 (0.129)	1.14 (0.71–1.82)	.585	58 (0.134)	1.18 (0.81–1.73)	.389

Bold texts indicate items with $p < .0125$.

Abbreviations: CI, confidence interval; Gen./alle., genotype/allele; OR, odds ratio; *p*, *p*-value.

TABLE 4 Allelic TDT results of the SNPs for NSCL/P and two subtypes

Phenotype	SNP	A1/A2	T/U	OR (95% CI)	*p*
NSCLO	rs303048	T/A	28/40	0.70 (0.43–1.14)	.146
	rs1675414	**C/T**	**47/25**	**1.88 (1.16–3.05)**	**.010**
	rs987525	A/C	25/20	1.25 (0.69–2.25)	.456
NSCLP	rs303048	T/A	47/33	1.42 (0.91–2.22)	.118
	rs1675414	C/T	25/30	0.83 (0.49–1.42)	.500
	rs987525	A/C	24/35	0.69 (0.41–1.15)	.152
NSCL/P	rs303048	T/A	75/73	1.03 (0.74–1.42)	.869
	rs1675414	C/T	72/55	1.31 (0.92–1.86)	.131
	rs987525	A/C	49/55	0.89 (0.61–1.31)	.556

Bold texts indicate items with $p < .0125$.

Abbreviations: A1/A2, minor allele/major allele; T/U, transmitted/not transmitted.

TABLE 5 Parent-of-origin effects of SNPs

			Paternal		Maternal			
Phenotype	SNP	A1/A2	T/U	*p*	T/U	*p*	z	*p*
NSCLO	rs303048	T/A	15.5/23.5	.200	12.5/16.5	.458	−0.28	.781
	rs1675414	**C/T**	17/13	.465	**30/12**	**.005**	−1.29	.197
	rs987525	A/C	8.5/9.5	.814	16.5/10.5	.248	−0.92	.360
NSCLP	rs303048	T/A	21/14	.237	26/19	.297	0.200	.841
	rs1675414	C/T	10.5/13.5	.540	14.5/16.5	.719	−0.22	.823
	rs987525	A/C	8/16	.103	16/19	.612	−0.95	.343
NSCL/P	rs303048	T/A	36.5/37.5	.908	38.5/35.5	.727	−0.33	.742
	rs1675414	C/T	27.5/26.5	.892	44.5/28.5	.061	−1.13	.260
	rs987525	A/C	16.5/25.5	.165	32.5/29.5	.703	−1.31	.190

Bold texts indicate the items with $p < .008$.

Abbreviations: A1, minor allele; A2, major allele; T/U transmitted/not transmitted; Z, vector of the large sample Z statistic.

NOG (Figueiredo et al., 2014). In this study, we have found strong evidence of susceptibility to NSCLO of *TFAP2A* rs1675414 among the Vietnamese population.

4.1 | TFAP2A

Recently, a study revealed Interferon regulatory factor 6 (*IRF6*) and *TFAP2A* sharing the same genetic pathway as components of a gene regulatory network (GRN) which is made up of transcription factors that regulate each other (Kousa et al., 2019). *IRF6* was first identified as a causative association with van der Woude's syndrome, the most common Mendelian syndrome which includes CL/P; and association studies further showed strong links to isolated forms of CL/P (Kondo et al., 2002; Ludwig et al., 2012; Zucchero et al., 2004). In the Vietnamese population, IRF6 was also associated with NSCL/P (Zucchero et al., 2004. AP-2A, the protein encoded by *TFAP2A*, binds to and regulates MCS9.7, a regulatory enhancer element upstream of *IRF6* in orofacial expression (Fakhouri et al., 2012; McDade et al., 2012; Thomason et al., 2010). Thus, *TFAP2A* through AP-2A protein regulates endogenous expression of *IRF6* in relevant to orofacial development (Kousa et al., 2018; Rahimov et al., 2008). The present study tested two SNPs in *TFAP2A*, rs303048 at intron 6 and rs1675414 at exon 1 in association with NSCL/P. Case–control comparisons showed dominant genotypes (TC + CC) and allele C (rs1675414) increased the risk of NSCLO with OR = 2.06 (95% CI: 1.29–3.29, $p = .002$) and OR = 1.86 (95% CI: 1.26–2.75, $p = .002$) compared with TT and T, respectively. Interestingly, an allele at a common variant (rs642961) disrupting the AP-2A binding site at MCS9.7 was also significantly overtransmitted in the NSCLO group but not in the entire NSCLP group (Rahimov et al., 2008). Growing evidence revealed NSCLO and NSCLP might be separated entities with distinct genetic etiology (Huang et al., 2019; Jia et al., 2015; Jugessur et al., 2011; Ludwig et al., 2012, 2016; Rahimov et al., 2008).

In terms of family based design, a significant overtransmission of allele C at rs1675414 was noted among the

NSCLO group (OR = 1.88, 95% CI: 1.16–3.05, $p = .010$). The TDT does not consider the parental origin of alleles, so it could mask some specific risk alleles. It is necessary to investigate the parent-of-origin effect in congenital anomalies because maternal genotypes control the in-utero environment (Reutter et al., 2008; Sull et al., 2009). Maternal effects for NSCL/P have been reported in several candidate genes, such as *MTHFR*, *TGFA*, or *MSX1* (Prescott et al., 2002; Suazo et al., 2010; Sull et al., 2009). In the present study, analysis of parent-of-origin demonstrated that maternally inherited allele C induced a significantly greater risk of NSCLO than did a maternally inherited allele T at *TFAP2A* rs1675414 ($p = .005$). The maternal effect could be interpreted as either maternal genotyping effects or epigenetic processes like imprinting effects but to discriminate them remains a question (Reutter et al., 2008; Weinberg & Umbach, 2005). There were no epigenetic controls in our study to test if imprinting effects were presented. *TFAP2A* was also reported in association with NSCL/P in Italian triads, however, at rs303050, not rs1675414 (Martinelli et al., 2011).

4.2 | 8q24

Association of 8q24 rs987525 with NSCL/P was replicated in Estonian and Lithuanian, Polish, non-Hispanic White, and Hispanic (Blanton et al., 2010; Mostowska et al., 2010; Nikopensius et al., 2009). However, in our study, both case–control and family based tests yielded no susceptibility of rs987525 to neither NSCL/P nor two subtypes among the Vietnamese population. Beaty et al. found that the association with CL/P for rs987525 was stronger among case-parent trios of European origin (p = 10E-16–10E-24) than among those of Asian origin (p = 10E-3; Beaty et al., 2010). Several studies conducted on Han Chinese (Xu et al., 2012), Brazilian (do Rego Borges et al., 2015) Japanese (Hikida et al., 2012) reported no evidence of this SNP to etiology of NSCL/P. Our result contributed to a hypothesis that 8q24 rs987525 was only associated with NSCL/P among European ancestry. The mechanism underlying the significance of rs987525 in several studies remains a scientific question because no known genes were mapped within the 640 kb region of 8q24.21. The nearest mapped genes are *MYC* and *GSDMC* encode myc proto-oncogene protein and melanoma-derived leucine zipper-containing extranuclear factor (Mostowska et al., 2010).

4.3 | Case–control and family based methods

Typically, case–control design is the more convenient and feasible way in association studies. However, it is difficult to eliminate population stratification in urban or peri-urban areas. In large sample size studies, a modest stratification might lead ta considerable confounding. A review of 93 studies in which used both case–control and family based designs revealed that 28% (26 comparisons) gave opposite ORs (one above 1 and one below 1; Evangelou et al., 2006). The joint analysis used in our study enhanced the accuracy in estimating genetic effects. The TDT and TDT with parent-of-origin analysis confirmed all the case–control results.

In this present work, we found a strong LD between the rs1675414 and rs303048, with a high D' (0.999) but a very low r^2 (0.060). The low r^2 was derived from a rarer allele frequency of rs1675414 compared with rs303048. Even though the study investigated a modest number of SNPs, a Bonferroni correction was required to avoid type I error. In general, physically closed SNPs or strong LD SNPs are likely to have correlated p values, a typical Bonferroni-type correction, therefore, seems to be too conservative. As rs1675414 and rs303048 are in LD, we corrected the empirical p values by the number of genes rather than the number of SNPs (Bailey-Wilson et al., 1995; Silverman & Palmer, 2000).

5 | CONCLUSION

In summary, the present investigation suggests an intriguing role of *TFAP2A* in the etiology of NSCLO among the Vietnamese population. However, further independent studies are required to confirm our hypothesis.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon reasonable request.

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