The Cleft Palate–Craniofacial Journal 00(00) pp. 000–000 Month 0000

ORIGINAL ARTICLE

Genetic Interactions in Nonsyndromic Orofacial Clefts in Europe—EUROCRAN Study

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?1 ***Background:*** **Nonsyndromic cleft lip with or without cleft palate (nsCL±P) and nonsyndromic cleft palate (nsCP) are caused by a combination of genetic and environmental risk factors. We investigated gene-environment and gene-gene joint effects in a large multicenter study of case-parent triads.**

Methods: **The nsCL±P or nsCP triads were recruited in 11 European countries between 2001 and 2005. We collected DNA samples from infants and from their mothers and fathers, and mothers completed a questionnaire on exposures, including smoking and folic acid supplement use during pregnancy. We used log-linear regression to estimate relative risks (RRs) and 95% confidence intervals (CIs) for associations between nsCL±P or nsCP and variants in MTHFR, MTHFD1, TGFA, SATB2, and MSX1, stratifying by environmental or genetic factors.**

Results: **We obtained genotype and exposure data for 728 nsCL±P triads and 292 nsCP triads. In male infants, there was no association between the mother's homozygous MSX1 p(CA) *4/*4 genotype and nsCL±P (RR, 0.98; 95% CI, 0.63–1.54), but this maternal genotype resulted in a doubling of risk for female infants (RR, 2.21; 95% CI, 1.13–4.34). There was evidence suggestive of gene-gene joint-effects between MTHFR-TGFA for nsCP but not for nsCL±P.**

Conclusion: **Although we chose the genes and their variants and putative joint effects based on associations previously reported in the literature, we replicated few associations. These results do not provide evidence supporting associations between these genes and oral clefts in European populations, although gene-environment and gene-gene interactions could play a role in oral cleft etiology.**

KEY WORDS: *cleft lip, cleft palate, genetics, gene-environment interaction, gene-gene interaction*

Cleft lip with or without cleft palate (CL±P) and cleft palate (CP) are some of the most common craniofacial malformations. Their etiologies remain poorly understood, but both nonsyndromic CL±P (nsCL±P) and nonsyndromic CP (nsCP) are thought to be multifactorial traits, with genetic and environmental risk factors contributing to risk (Dixon et al., 2011).

Genome-wide association studies (GWASs) have identified several loci associated with nsCL±P (Birnbaum et al., 2009; Mangold et al., 2010; Beaty et al., 2010; Ludwig et al., 2012; Leslie et al., 2016a) and a locus associated with nsCP (Leslie et al., 2016b). The case-parent trio design can provide greater statistical power to detect genetic associations compared with case-unrelated control studies, and it is less vulnerable to the effects of population stratification (Beaty et al., 2010). The European Collaboration on Craniofacial Anomalies (EUROCRAN) case-parent trio resource has been

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The study was supported by EU Framework V grant number QLG1-2000-01019 EUROCRAN project (European Collaboration on Craniofacial Anomalies). Dr. Little holds a Canada Research Chair in Human Genome Epidemiology.

Submitted February 2016; Revised August 2016; Accepted September 2016.Month 2016

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DOI: 10.1597/16-037

valuable in replicating GWASs in European populations and extending them in meta-analyses. The first case-control GWAS on nsCL±P (224 cases, 383 controls) identified a 640-kb region in 8q24 as strongly associated with cleft, with the single nucleotide polymorphism (SNP) rs987525 the most significant marker, and replicated in 101 EUROCRAN trios (Birnbaum et al., 2009). Also in this analysis, the previously reported association of rs642961 (near the *IRF6* gene) with nsCL±P in which EUROCRAN samples had been used (Rahimov et al., 2008), was confirmed. The next European case-control GWAS (Mangold et al., 2010) identified two new nsCL±P susceptibility loci with genome-wide significance (10q25.3 and 17q22) and three further suggestive loci (2p21, 13q31.1, and 15q13.3) with EUROCRAN samples included. At the same time, the Baltimore GWAS in two independent sets of populations (Asian and of European origin) identified another two loci associated with nsCL±P (1p22 and 20q12) and some loci with signals near genome-wide significance (1p36, 10q25.3, and 17p13) (Beaty et al., 2010). The data from these two GWASs were combined in the first meta-analysis for nsCL±P (Ludwig et al., 2012). All the loci previously identified as having genome-wide significant association were confirmed, and six additional loci reached genome-wide significance and were thereafter classified as new susceptibility loci (1p36, 2p21, 3p11.1, 8q21.3, 13q31.1, and 15q22). The nsCL±P risk loci identified in this series of European GWASs were replicated using the EUROCRAN samples that had not been included in any GWAAS, and thus could be considered as truly independent: some loci (2p21, 10q25.3, 13q31.1, 15q13.3, 17q22) were confirmed in a replication assay performed on 793 EUROCRAN trios in the Bonn-II study (Mangold et al., 2010), while 467 EUROCRAN trios were included in the replication samples used for the remaining six loci (1p22, 1p36, 3p11.1, 8q21.3, 15q22.2, and 20q12) (Böhmer et al., 2013).

However, these loci have not included the candidate genes commonly studied in previous epidemiologic studies, such as genes involved in folate metabolism (*MTHFR* and *MTHFD1)*, or encoding a mammalian growth factor (*TGFA*), a DNA binding-protein (*SATB2*), and a component of the muscle segment homeobox family (*MSX1)* (Kohli and Kohli, 2012). Candidate gene studies themselves have not achieved consensus on the roles of these genes, if any, in oral cleft etiology, with studies producing inconsistent results. More consistent evidence exists for roles of environmental and lifestyle risk factors, including maternal smoking during pregnancy (positive association with clefts) (Little et al., 2004) and periconceptional use of multivitamins/folic acid–containing supplements (inverse association) (Johnson and Little, 2008; Butali et al., 2013).

One possible explanation for inconsistent results between studies is the existence of gene-environment or gene-gene joint effects that, when not taken into account, make marginal genetic effects difficult to compare (Greene et al., 2009). We use the term "joint effect" in preference to "interaction" because our primary interest is identifying whether there are subgroups of the population in which efforts to promote health might best be targeted and because of the ongoing debate about statistical and biological concepts of interaction (Greenland, 2009). Similarly, GWASs might not be able to identify susceptibility genes if their effects are observable only in the presence or absence of other factors (Shi and Weinberg, 2011). Possible gene-environment and gene-gene joint effects have been reported in both candidate gene studies and GWASs of oral clefts (Beaty et al., 2011; Kohli and Kohli, 2012), although the interpretation has been tentative because of limited statistical power to detect such effects, especially since most studies have been confined to single populations with limited exposure variability.

In this study, we investigate genetic associations, gene-environment joint effects, and gene-gene joint effects in oral cleft etiology using a large dataset of case-parent triads from European populations with diverse exposure patterns, focusing on replicating associations with candidate genes and joint effects previously reported in the literature.

Methods

Families of infants with nsCL±P or nsCP were identified through EUROCRAN, a collaboration between investigators in 11 European countries: Bulgaria, Denmark, England, Estonia, Hungary, Italy, the Netherlands, Scotland, Slovakia, Slovenia, and Spain. This collaboration was established through a consortium agreement. Ethical permission was sought and obtained in all participating countries prior to the commencement of the study, and later ethical approval was sought and obtained for the analyses reported in this article from the Ottawa Hospital Research Ethics Board. Between 2001 and 2005, consecutive families were invited to enroll in the study at participating surgical centers at the time of the first surgical intervention on the index child. Diagnosis of nsCL±P or nsCP was confirmed at the surgical centers; infants with recognized syndromic clefts or the Pierre Robin sequence were excluded. Infants in the first year of life were recruited just prior to the primary surgery. The protocols in place for cleft repair specified that cleft lip repair be carried out around 3–4 months of age and cleft palate repair around 6–9 months for these patients.

Mothers were asked to respond to a questionnaire that was specifically designed for the EUROCRAN study and was administered by personal interview when the index affected child was brought to the surgical center to undergo the primary surgery. The major areas about which information was sought included educational status of mother; ethnic group and family history of both parents; maternal medical and reproductive history; use of supplements, drugs, and medications; exposure

?9 **Table 1 Investigated Genetic Variants**

Gene Variants	*N. Genotyped Trios*	*Alleles*	
		Variant	*Reference*
MTHFR:c.677C>T (rs1801133)	1020	T	C
MTHFD1:c.1958G>A (rs2236225)	907	A	G
TGFA:c.IVS5+739_742delTAAT (rs11466267) (TaqI)	680	Deletion	TAAT
SATB2:c.IVS4+35G>C (rs1348813)	745	C	G
MSX1:c.IVS1+1529CA(9_12) (pCA)	710	$(CA)^{9}$	$(CA)^{10-12}$

to pesticides, herbicides, and solvents: consumption of foods that are fortified in some countries in Europe (bread, cereals, and milk); active and passive smoking; and alcohol use. The lifestyle factors of particular interest in the gene-environment interaction analyses were periconceptional folic acid supplementation and smoking during pregnancy. Folic acid supplementation was defined as having taken folic acid or folic acid–containing supplements (at least 0.4 mg/day) for at least ‘ month during the periconceptional period (3 months before to 3 months after conception). Maternal smoking during pregnancy was defined as having smoked at least one cigarette per day during the periconceptional period.

Peripheral blood or buccal cell samples were collected from the mother, father, and infant to obtain DNA. A protocol concerning the method to be used for collection and transport was developed in a consensus EUROCRAN meeting and distributed to all participating centers. The DNA extraction and storage, according to a common protocol developed in the same consensus meeting, was carried out in two sites: Dublin, Ireland, and Ljubljana, Slovenia.

We selected five genetic variants that have previously been reported in association with either nsCL±P or ?2 nsCP in the literature: three SNPs, that is, *MTHFR*:c.677C>T (rs1801133); *MTHFD1*:c.1958G>A (rs2236225); and *SATB2*:cIVS4+35G>C (rs1348813); *TGFA*:cIVS5+739_742delTAAT (rs11466267), a 4nt deletion (TaqI) in exon 6 of the *TGFA* gene, also known as A2 allele (Jugessur et al., 2003b); and *MSX1*:c.IVS+1529CA(9_12) (pCA), a short tandem repeat polymorphism (STRP) in exon 1 of the *MSX1* gene. For this latter, the CA(9) allele, also referred as *4 allele (Jugessur et al., 2003b), was considered a variant allele. Genotyping of SNPs in *MTHFR, MTHFD1*, and *SATB2* was carried out using polymerase chain reaction (PCR) amplification, amplicon digestion with restriction enzymes, and agarose gel electrophoresis (Mostowska et al., 2006; Chevrier et al., 2007), while *TGFA* TaqI deletion was detected by PCR amplification and agarose gel electrophoresis (Shaw et al., 1998). PCR amplification and fragment analysis was used to genotype *MSX1* p(CA) using an ABI 7300 sequencer (Applied Biosystems) (Hwang et al., 1998). To assess precision of genotyping, 5% of samples, randomly selected, were replicated. These gene variants are further described in Table 1.

Putative gene-environment and gene-gene joint effects were chosen primarily based on previous reports in the literature. We tested for joint effects of folic acid supplementation and folate metabolism genes *MTHFR* and *MTHFD1*, as well as for joint effects of smoking and each of the genes investigated. Based on reports suggesting sex-dependent effects of *MSX1*, we investigated possible differences in the association with *MSX1* by sex (Blanco et al., 1998; Blanco et al., 2001). We also investigated putative gene-gene joint effects: *TGFA-MTHFR* and *TGFA-MSX1* (Jugessur et al., 2003a; Jugessur et al., 2003b).

Statistical Analyses

We performed case-parent triad analyses using a log-linear regression model that incorporates an expectation-maximization algorithm to allow inclusion of triads for which one or both of the parent’s genotypes were missing (Wilcox et al., 1998; Weinberg, 1999). The model was implemented using the GENECMT command in Stata 9 (http://www.biostat-resources.com). We estimated rela- ?3 tive risks (RRs) and 95% confidence intervals (CIs) for the independent effects of the mother’s and child’s genotypes. For all analyses, we used the homozygous wildtype genotype as the reference group and compared heterozygous and homozygous variant individuals to the reference group. For gene-environment and gene-gene joint effects, we stratified on the factor of interest and estimated the RRs and 95% CIs separately within each stratum. We excluded from all analyses triads for which the child’s genotype was missing, and we excluded from the gene-environment joint effects any triads with missing data on the environmental factor of interest. We also excluded any triad in which the child’s genotype was not one that could have been inherited from the parents according to Mendelian inheritance.

Results

Overall, 1020 families participated in the study, including 728 families of infants with nsCL±P and 292 families of infants with nsCP. We found that nsCL±P was more common among male infants (65%) and nsCP was more common among female infants

Table 2 Associations Between Mother's and Child's Genotypes and Oral Clefts[a]

		Mother's Genotype, RR (95% CI)[b]		*Child's Genotype, RR (95% CI)[b]*	
	N	*One Variant Allele*	*Two Variant Alleles*	*One Variant Allele*	*Two Variant Alleles*
MTHFR:c.677C>T					
CL/P	728	0.90 (0.72,1.12)	0.79 (0.56, 1.11)	0.88 (0.73, 1.08)	0.70 (0.49, 0.99)
CP	292	0.90 (0.62, 1.32)	0.90 (0.52, 1.57)	0.97 (0.71, 1.33)	0.53 (0.31, 0.92)
MTHFD1:c.1958G>A					
CL/P	665	1.03 (0.81, 1.31)	0.95 (0.68, 1.32)	0.90 (0.73, 1.11)	0.83 (0.61, 1.12)
CP	242	0.84 (0.55, 1.26)	0.74 (0.44, 1.22)	0.99 (0.69, 1.42)	0.93 (0.55, 1.57)
TGFA TaqI[c]					
CL/P	499	0.85 (0.61, 1.18)	0.64 (0.18, 2.25)	0.93 (0.67, 1.27)	0.72 (0.22, 2.32)
CP	181	0.72 (0.421, 1.23)	-	0.96 (0.57, 1.62)	0.86 (0.09, 8.20)
SATB2:c.IVS4+35G>C					
CL/P	536	1.20 (0.92, 1.57)	1.01 (0.61, 1.69)	1.20 (0.92, 1.57)	1.01 (0.61, 1.69)
CP	209	0.70 (0.44, 1.12)	0.53 (0.22, 1.24)	1.06 (0.70, 1.60)	1.18 (0.58, 2.37)
MSX1 p(CA)					
CL/P	530	1.47 (1.04, 2.08)	1.01 (0.70, 1.45)	1.08 (0.81, 1.44)	0.94 (0.67, 1.33)
CP	180	1.32 (0.75, 2.32)	1.32 (0.68, 2.53)	1.03 (0.65, 1.64)	1.12 (0.64, 1.97)

[a] N = number of triads; RR = relative risk; CI = confidence interval; CL/P = cleft lip with or without cleft palate; CP = cleft palate.
[b] Reference group is no variant allele (homozygous wildtype).
[c] Model did not converge for CP.

(57%). Each of the 11 study sites enrolled between 11 and 358 triads and genotyped three to five variants. One study site did not collect information on periconceptional folic acid supplementation or smoking and so triads from this site were excluded from the gene-environment analysis. Among sites collecting information on lifestyle factors, the prevalence of reported folic acid supplementation ranged from 36% to 78% (overall prevalence in study sample: 62%), and the prevalence of reported maternal smoking ranged from 10% to 46% (overall prevalence: 22%).

Genetic Effects

Few associations were observed between specific genotypes and CL±P or CP (Table 2). We found inverse associations between child's *MTHFR* genotype and both outcomes, with T allele apparently acting as dominant for CL±P risk and recessive for CP risk. Moreover, we found a positive association with the maternal heterozygous *MSX1* genotype and CL±P (RR, 1.47; 95% CI, 1.04–2.08). Considering multiple testing, after Bonferroni correction none of these associations reached a significant threshold.

Gene-Environment Joint Effects

The RRs were slightly larger for mothers carrying one or more variant *MTHFR* alleles and not taking folic acid supplements compared with mothers taking supplements (Table 3). The models for joint effects between *TGFA* and smoking did not converge, and, considering

Table 3 Associations Between Mother's and Child's *MTHFR* and *MTHFD1* Genotypes and Orofacial Clefts, Stratified by Periconceptional Folic Acid Supplementation[a]

		Mother's Genotype, RR (95% CI)[b]		*Child's Genotype, RR (95% CI)[b]*	
	N	*One Variant Allele*	*Two Variant Alleles*	*One Variant Allele*	*Two Variant Alleles*
MTHFR:c.677C>T					
CL/P					
Folic acid	442	0.82 (0.63, 1.08)	0.60 (0.45, 0.96)	0.95 (0.74, 1.22)	0.72 (0.46, 1.22)
No folic acid	193	1.13 (0.66, 2.13)	1.31 (0.67, 2.55)	0.79 (0.53, 1.17)	0.74 (0.39, 1.41)
CP					
Folic acid	176	0.86 (0.53, 1.41)	0.80 (0.38, 1.66)	0.90 (0.60, 1.34)	0.52 (0.27, 1.02)
No folic acid	86	1.13 (0.55, 2.33)	1.18 (0.49, 2.87)	1.39 (0.74, 2.61)	0.67 (0.23, 1.95)
MTHFD1:c.1958G>A					
CL/P					
Folic acid	389	1.12 (0.81, 1.55)	1.14 (0.74, 1.74)	0.85 (0.65, 1.12)	0.74 (0.50, 1.11)
No folic acid	179	0.78 (0.49, 1.25)	0.52 (0.26, 1.02)	1.16 (0.77, 1.75)	1.15 (0.65, 2.04)
CP					
Folic acid	141	0.71 (0.41, 1.23)	0.38 (0.18, 0.80)	1.02 (0.64, 1.63)	1.03 (0.52, 2.03)
No folic acid	72	1.14 (0.54, 2.40)	1.55 (0.66, 3.67)	0.83 (0.42, 1.65)	0.65 (0.24, 1.77)

[a] N = number of triads; RR = relative risk; CI = confidence interval; CL/P = cleft lip with or without cleft palate; CP = cleft palate.
[b] Reference group is no variant allele (homozygous wildtype).

Table 4 Associations Between Mother's and Child's Genotypes and Oral Clefts, Stratified by Maternal Smoking During Pregnancy[a]

		Mother's Genotype, RR (95% CI)[b]		*Child's Genotype, RR (95% CI)[b]*	
	N	*One Variant Allele*	*Two Variant Alleles*	*One Variant Allele*	*Two Variant Alleles*
MTHFR:c.677C>T					
CL/P					
Smoking	156	0.85 (0.53, 1.36)	1.48 (0.60, 3.68)	0.96 (0.63, 1.46)	0.78 (0.37, 1.65)
No smoking	484	0.90 (0.69, 1.18)	0.66 (0.43, 0.99)	0.88 (0.69, 1.13)	0.69 (0.45, 1.05)
CP					
Smoking	65	0.43 (0.17, 1.10)	0.36 (0.11, 1.14)	0.86 (0.44, 1.67)	0.32 (0.10, 0.98)
No smoking	204	1.16 (0.74, 1.81)	1.19 (0.60, 2.34)	1.05 (0.72, 1.53)	0.69 (0.36, 1.31)
MTHFD1:c.1958G>A					
CL/P					
Smoking	135	0.89 (0.53, 1.49)	0.83 (0.41, 1.70)	0.97 (0.61, 1.54)	1.09 (0.55, 2.15)
No smoking	443	1.05 (0.77, 1.42)	0.91 (0.61, 1.36)	0.95 (0.74, 1.24)	0.88 (0.61,1.27)
CP					
Smoking	46	0.68 (0.26, 1.85)	0.47 (0.12, 1.82)	0.78 (0.35, 1.77)	0.32 (0.09, 1.20)
No smoking	176	0.88 (0.55, 1.43)	0.80 (0.45, 1.41)	0.99 (0.65, 1.53)	1.14 (0.62, 2.10)
SATB2:cIVS4+35G>C					
CL/P					
Smoking	123	1.45 (0.84, 2.49)	0.95 (0.36, 2.50)	1.53 (0.92, 2.53)	1.02 (0.39, 2.70)
No smoking	407	1.11 (0.82, 1.52)	1.08 (0.58, 2.00)	1.11 (0.84, 1.47)	1.19 (0.79, 1.81)
CP					
Smoking	43	0.79 (0.30, 2.11)	1.32 (0.15, 11.78)	0.93 (0.36, 2.41)	2.05 (0.24, 17.55)
No smoking	161	0.70 (0.41, 1.19)	0.48 (0.18, 1.28)	1.09 (0.68, 1.73)	1.19 (0.56, 2.55)
MSX1 p(CA)					
CL/P					
Smoking	125	1.01 (0.50, 2.05)	1.03 (0.48, 2.20)	1.56 (0.83, 2.93)	1.96 (0.94, 4.10)
No smoking	359	1.35 (0.91, 2.01)	1.38 (0.90, 2.10)	0.90 (0.65, 1.25)	0.97 (0.65, 1.45)
CP					
Smoking	43	1.11 (0.35, 3.55)	1.02 (0.26, 4.06)	1.15 (0.42, 3.12)	1.64 (0.49, 5.47)
No smoking	131	1.39 (0.72, 2.66)	1.37 (0.65, 2.89)	1.05 (0.61, 1.82)	1.08 (0.55, 2.10)

[a] N = number of triads; RR = relative risk; CI = confidence interval; CL/P = cleft lip with or without cleft palate; CP = cleft palate.
[b] Reference group is no variant allele (homozygous wildtype).

Bonferroni correction, there were no obvious joint effects between any other genes and smoking (Table 4).

Sex-dependent differences in effect were observed for *MSX1* p(CA) (Table 5). Among male infants, no association was observed between *MSX1* genotype and CL±P. Among female infants, increased risks of CL±P were observed when mothers carried one or more variant alleles (one variant allele: RR, 2.20; 95% CI, 1.18–4.10; two variant alleles: RR, 2.21; 95% CI, 1.13–4.34). Similar trends were observed for CP.

Gene-Gene Joint Effects

Potential gene-gene joint effects were observed for child's *MTHFR*.c:677C>T and *TGFA* TaqI genotypes (Table 6). The *MTHFR* C/T heterozygotes had an almost twofold increased risk of nsCL±P among *TGFA* TaqI deletion carriers (RR, 1.89; 95% CI, 1.01–3.55), whereas they had a reduced risk among *TGFA* wildtype homozygotes (RR, 0.72; 95% CI, 0.55–0.96). By contrast, for nsCP, *MTHFR* C/T heterozygotes had a

Table 5 Associations Between Mother's and Child's *MSX1 p(CA)* Genotype and Oral Clefts, Stratified by Sex of the Affected Child[a]

		Mother's Genotype, RR (95% CI)[b]		*Child's Genotype, RR (95% CI)[b]*	
	N	*One Variant Allele*	*Two Variant Alleles*	*One Variant Allele*	*Two Variant Alleles*
CL/P					
Male	323	0.93 (0.64, 1.43)	0.98 (0.63, 1.54)	1.01 (0.70, 1.45)	1.00 (0.64, 1.54)
Female	165	2.20 (1.18, 4.10)	2.21 (1.13, 4.34)	1.04 (0.63, 1.71)	1.62 (0.90, 2.93)
CP					
Male	66	0.95 (0.39, 2.33)	1.01 (0.37, 2.79)	0.87 (0.40, 1.87)	0.65 (0.25, 1.69)
Female	111	1.66 (0.79, 3.48)	1.60 (0.67, 3.79)	1.07 (0.59, 1.94)	1.44 (0.70, 2.95)

[a] N = number of triads; RR = relative risk; CI = confidence interval; CL/P = cleft lip with or without cleft palate; CP = cleft palate.
[b] Reference group is no variant allele (homozygous wildtype).

Table 6 Joint Effects of Maternal-Maternal and Child-Child Gene-Gene Interactions Between *MTHFR* and *MSX1* With *TGFA* Genotype on Risk of Oral Clefts[a]

		Mother's Genotype, RR (95% CI)[b,c]		*Child's Genotype, RR (95% CI)*[b,d]	
	N	*One Variant Allele*	*Two Variant Alleles*	*One Variant Allele*	*Two Variant Alleles*
MTHFR:c.677C>T					
CL/P					
TGFA TaqI wildtype	381	0.84 (0.62, 1.14)	0.74 (0.46, 1.20)	0.72 (0.55, 0.96)	0.67 (0.42, 1.08)
TGFA TaqI variant	77	0.82 (0.44, 1.53)	0.89 (0.29, 2.70)	1.89 (1.01, 3.55)	0.67 (0.20, 2.24)
CP[e]					
TGFA TaqI wildtype	135	0.84 (0.62, 1.52)	1.31 (0.64, 2.68)	1.31 (0.81, 2.11)	0.65 (0.29, 1.44)
TGFA TaqI variant	30	0.88 (1.41, 1.87)	2.00 (0.18, 22.06)	0.17 (0.05, 0.61)	0.08 (0.05, 0.49)
MSX1 p(CA)					
CL/P					
TGFA TaqI wildtype	391	1.36 (0.93, 2.00)	1.29 (0.85, 1.94)	1.53 (1.05, 2.22)	1.62 (1.06, 2.48)
TGFA TaqI variant	79	0.99 (0.41, 2.39)	0.83 (0.33, 2.08)	0.87 (0.44, 1.73)	1.38 (0.60, 3.19)
CP					
TGFA TaqI wildtype	150	1.49 (0.80, 2.69)	1.43 (0.71, 2.86)	1.15 (0.69, 1.94)	1.23 (0.65, 2.31)
TGFA TaqI variant	28	0.67 (0.11, 3.99)	0.61 (0.07, 5.28)	0.70 (0.21, 2.37)	1.01 (0.24, 4.24)

Abbreviations: CI, confidence interval; N, number of triads; RR, relative risk.
[a] N = number of triads; RR = relative risk; CI = confidence interval; CL/P = cleft lip with or without cleft palate; CP = cleft palate.
[b] Reference group is no variant allele (homozygous wildtype).
[c] Mother's *MTHFR* or *MSX1* genotype and mother's *TGFA* genotype.
[d] Child's *MTHFR* or *MSX1* genotype and child's *TGFA* genotype.
[e] Models for maternal interactions did not converge.

6-fold reduced risk of nsCP among *TGFA* TaqI deletion carriers (RR, 0.17; 95% CI, 0.05–0.61). Among these carriers, *MTHFR* T/T homozygotes had an even more pronounced reduced risk for nsCP (RR, 0.08; 95% CI, 0.05–0.49), suggesting a dominant effect of the T allele that seems subordinated to the epistatic effect of *TGFA* TaqI deletion.

As regards *MSX1* p(CA) and *TGFA* TaqI variants, no gene-gene joint effects were observed for child's genotypes (Table 6).

No gene-gene joint effects were observed for maternal genotypes.

Discussion

In this study, we investigated genes, gene-environment interactions, and gene-gene interactions previously reported in association with nsCL±P and nsCP. Our results were consistent with much of the literature in not finding associations between these genes and interactions and oral clefts.

We found an inverse association with child's *MTHFR* T/T genotype. A meta-analysis of 15 studies of nsCL/P showed no overall association with this genotype, but based on five studies only, an inverse association with nsCP (Pan et al., 2012). For nsCL/P and nsCP combined, there was a positive association in Asian populations, based on four studies, but not in other ethnicities. A more recent meta-analysis of eight studies of nsCL/P in Asian populations showed a positive association (Zhao et al., 2014), which is also apparent in recent small studies in Asia (Aşlar et al., 2013; Ebadifar et al., 2015a; Ebadifar et al., 2015b).

We also identified a sex-dependent association between *MSX1* and clefts, with evidence that maternal genotype acts as risk factor for female offspring but not for male offspring. However, we did not replicate the *MSX1*-male joint effect referred to child's genotype previously reported (Blanco et al., 1998; Blanco et al., 2001).

In a previous study including 88 nsCP trios, a *MSX1-TGFA* joint effect was found for the child's genotypes (Jugessur et al., 2003b), with *TGFA* TaqI deletion carriers having an almost tenfold increased risk of nsCP among *MSX1* *4/*4 homozygotes but no effect on children carrying other *MSX1* genotypes; however, no joint effect with the child's genotypes was found in the present study that included 181 nsCP trios.

A joint effect of the child's *MTHFR* and *TGFA* genotypes was observed for nsCP in the present study, with the *MTHFR*-nsCP association showing a strong inverse association in the presence of *TGFA* TaqI deletion. A previous study investigating this joint effect reported that the *TGFA*-nsCP association was stronger (RR above the null) when the child had *MTHFR* variant alleles than when the child had the *MTHFR* wildtype genotype, but this was true only in homozygotes for the *TGFA* TaqI deletion (Jugessur et al., 2003a). In the present study, however, the effect of homozygosity for the *TGFA* TaqI deletion could not be analyzed due to the very low frequency of this genotype among CP cases.

EUROCRAN is one of the largest case-parent triad studies of oral clefts. The study is unique in that it includes centers from countries in southern Europe (Italy and Spain), countries in eastern Europe soon after they joined the European Union (Bulgaria,

Estonia, Hungary, Slovakia, and Slovenia), and countries in northwest Europe (Denmark, the Netherlands, and the United Kingdom). This has increased the diversity in prevalence and intensity of a variety of exposures, including tobacco smoking in women of reproductive age, dietary patterns, and use of supplements in the periconceptional period and pregnancy (Mackenbach and McKee, 2013) compared with other studies. While this exposure variability would be expected to increase the power to detect gene-environment joint effects compared with studies done in a less diverse setting, it raises the potential problem of population stratification. However, the case-parent trio design is less vulnerable to bias arising from this source than other designs (Weinberg and Shi, 2009). With regard to possible joint effects between periconceptional use of supplements containing folic acid and *MTHFR* and *MTHFD*, it is noteworthy that Europe does not have programs to fortify grains with folic acid, in contrast to Canada, the United States, and parts of South America and Australia (Berry et al., 2010). In studies done in those settings since the implementation of fortification, it is possible that the very high levels of exposure as reflected by red cell folate levels would have washed out the effect of germline genetic variation in folate metabolism, or it might even have produced counterintuitive effects (Colapinto, 2013). We acknowledge that, as in other studies, misclassification of exposure is an important issue and has a profound effect on the statistical power to adequately assess potential gene-environment joint effects (Burton et al., 2009). Because the study was carried out in the same framework as randomized controlled trials of alternative surgical managements of unilateral nsCL±P, protocols that we were able to put in place for when families were approached and information collected were tighter than usual clinical practice and thus may have reduced misclassification compared with other studies. Although in isolation this study provides few informative results on these interactions, it might be of future use for literature-based and individual participant meta-analyses. It also reinforces the need for large collaborations with pooling of samples to generate sufficient power in genotyping studies.

Replication of previous study results is important to further understand the roles of chance, bias, or population heterogeneity in study results. Replication of earlier findings has often been difficult in genetic association studies, with subsequent studies tending to find weaker associations than the original (Ioannidis et al., 2001). In the present analysis, the variants and most interactions we investigated had previously been reported as associated with either nsCL±P or nsCP in one or more previous reports. Many of these variants had also been found not to be associated with either outcome in other studies. Determining at what point it can be said that no association exists and further studies are unnecessary will be a challenge if a causative variant only works in concert with an environmental exposure or another gene (Greene et al., 2009; Shi and Weinberg, 2011). Investigations of gene-environment and gene-gene interactions as we have done in this study are a start, but the large combination of possible gene-environment and gene-gene interactions makes this a daunting task. Genome-wide gene-environment interaction studies, as have recently been completed for nsCP, can assist in identifying variants that otherwise would not have been identified in oral cleft etiology based on marginal genetic effects alone (Beaty et al., 2011).

The results of this study do not support a role for the majority of these candidate genes in oral cleft etiology in European populations, although the possibility remains that they interact with other environmental exposures or genes to increase risk of nsCL±P or nsCP.

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