Evidence of Gene–Environment Interaction for Two Genes on Chromosome 4 and Environmental Tobacco Smoke in Controlling the Risk of Nonsyndromic Cleft Palate

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Abstract

Nonsyndromic cleft palate (CP) is one of the most common human birth defects and both genetic and environmental risk factors contribute to its etiology. We conducted a genome-wide association study (GWAS) using 550 CP case-parent trios ascertained in an international consortium. Stratified analysis among trios with different ancestries was performed to test for GxE interactions with common maternal exposures using conditional logistic regression models. While no single nucleotide polymorphism (SNP) achieved genome-wide significance when considered alone, markers in SLC2A9 and the neighboring WDR1 on chromosome 4p16.1 gave suggestive evidence of gene-environment interaction with environmental tobacco smoke (ETS) among 259 Asian trios when the models included a term for GxE interaction. Multiple SNPs in these two genes were associated with increased risk of nonsyndromic CP if the mother was exposed to ETS during the peri-conceptual period (3 months prior to conception through the first trimester). When maternal ETS was considered, fifteen of 135 SNPs mapping to SLC2A9 and 9 of 59 SNPs in WDR1 gave P values approaching genome-wide significance (10^{-6} < P < 10^{-7}) in a test for GxE interaction. SNPs rs3733585 and rs12508991 in SLC2A9 yielded \( P = 2.26 \times 10^{-7} \) in a test for GxE interaction. SNPs rs6820756 and rs7699512 in WDR1 also yielded \( P = 1.79 \times 10^{-7} \) and \( P = 1.98 \times 10^{-7} \) in a df test for GxE interaction. Although further replication studies are critical to confirming these findings, these results illustrate how genetic associations for nonsyndromic CP can be missed if potential GxE interaction is not taken into account, and this study suggest SLC2A9 and WDR1 should be considered as candidate genes for CP.

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Introduction

Nonsyndromic cleft palate (CP) is a common birth defect and has a complex and heterogeneous etiology, involving both genetic and environmental risk factors [1]. The prevalence of CP is about 1/2500 live births, much lower than the 1/1000 live births prevalence for nonsyndromic cleft lip with or without cleft palate (CL/P). About half of all CP cases have another congenital anomaly or represent a recognized malformation syndrome, with the remaining half representing isolated nonsyndromic CP cases [2].

Genetic risk factors play an important role in the etiology of CP. A recent twin study in Denmark showed heritability of CP is as high as 90%, and the proband-wise concordance rate for CP among monozygotic twins was much higher compared to dizygotic twins: 33% vs. 7% [3]. Both family studies and population based studies have identified multiple candidate genes associated with increased risk of CP, including FOXE1, ALX3, MKX, PDGFc, and SUMO1 [4–6]. However, evidence of association between reported candidate genes and CP remains inconsistent. Compared to the candidate gene approach, genome wide association studies (GWAS) have the advantage of providing better coverage of the human genome and are unbiased from a genetic perspective. Although several GWAS have identified strong signals at several chromosomal regions in multiple populations for CL/P [7–11], the variants controlling risk of CP have proven more difficult to find.

A few studies of CP have investigated potential GxE interaction for candidate genes and maternal exposure to cigarette smoking [12–14]. Environmental tobacco smoke (ETS) has also been reported to interact with certain SNPs to influence the risk of nonsyndromic CL/P and CP [15–20]. However, the evidence of GxE interaction has been rather inconclusive [21]. Possible reasons for the difficulty in documenting potential GxE interactions include: limited power due to modest sample size, different study designs and lack of available replication data. Integrating GxE interaction analysis into GWAS design is a powerful strategy for identifying more genetic factors influencing risk of complex disease, which could be overlooked when such interaction is ignored. A recent GWAS using the case-parent trio design found markers in several genes (MLLT3, SMC2, TRB1, ZNF236, and BAILC) showed statistically significant interaction with common maternal exposures, although no single SNP achieved genome-wide significance when such GxE interaction was ignored [22]. Beaty et al. (2011) combined CP trios from 12 different recruitment sites in their analysis, which involved considerably different rates of exposure to certain maternal exposures [22]. While the case-parent trio design has the advantage of being robust to confounding due to population stratification (compared to case-control designs), therefore allowing multi-site studies to amass large sample sizes, this advantage may not hold when considering GxE interaction especially if the exposure rates vary across sites.

In this study, we performed stratified analysis of the CP case-parent trios from the International Cleft Consortium [22] among trios with different ancestries to test for GxE interactions with common maternal exposures, including maternal cigarette smoking, alcohol consumption, ETS and multivitamin supplementation. Here we classified the trios used by Beaty et al. (2011) into groups of Asian and European ancestry and explored the potential GxE interactions.

Subjects and Methods

Case-parent Trios

Research protocols were reviewed and approved by institutional review boards (IRB) at each institution, including IRBs at The Johns Hopkins School of Public Health, University of Iowa, University of Pittsburgh, Utah State University, and all foreign collaborators. The review process for the consortium was approved by Johns Hopkins’ IRB. Written informed consent was obtained from parents. Case-parent trios were drawn from an international consortium which conducted a GWAS using a case-parent trio design to search for genes controlling risk of nonsyndromic, isolated oral clefts [10]. Most cases were ascertained through surgical treatment centers at a surgical or post-surgical visit. Racial/ethnic background of participants was originally based on self-report and most of the 550 CP trios were of European or Asian ancestry, but this was confirmed by genotyping. Table 1 lists characteristics of the CP probands noting gender and recruitment site, stratified by European or Asian ancestry. To minimize potential misclassification of nonsyndromic CP, all probands were examined for other congenital anomalies or major developmental delays by either a clinical geneticist or experienced health care provider to rule out syndromic forms of oral clefts. As expected, there were slightly more female CP cases (56.1%) compared to males. None of the parents of these CP cases were themselves affected.

Table 1. Gender of isolated, nonsyndromic cleft palate (CP) cases in the International Cleft Consortium by recruitment site.

| Site                  | Males | Females | Total |
|-----------------------|-------|---------|-------|
| Singapore             | 20    | 30      | 50    |
| Taiwan                | 29    | 50      | 79    |
| Shandong Prov., China | 16    | 22      | 38    |
| Hubei Prov., China    | 19    | 26      | 45    |
| Sichuan Prov., China  | 18    | 22      | 40    |
| Other*                | 4     | 3       | 7     |
| Subtotal              | 106   | 153     | 259   |

| Site                  | Males | Females | Total |
|-----------------------|-------|---------|-------|
| Denmark               | 8     | 5       | 13    |
| Norway                | 52    | 58      | 110   |
| Iowa                  | 18    | 22      | 40    |
| Maryland              | 14    | 21      | 35    |
| Pittsburg             | 6     | 8       | 14    |
| Utah                  | 29    | 27      | 56    |
| Singapore             | 1     | 3       | 4     |
| Subtotal              | 128(47.1%) | 144(52.9%) | 272   |
| Other ancestries**    | 10(52.0%) | 9(48.0%)   | 19    |
| Total                 | 244(43.9%) | 306(56.1%) | 550   |

*other sites include Maryland, Utah, and Korea.
**other ancestries include African American, Hispanic, Malay and others.
Genotyping
The Center for Inherited Disease Research (CIDR) genotyped DNA samples using Illumina’s 610 Quad platform and 99.1% of SNPs (99.56% of those attempted) were released and then underwent further QC analysis to set up 4 types of QC flags for each SNP: 1) unacceptably high rates (>5%) of missing genotype calls, 2) low minor allele frequency (MAF < 0.01), 3) unacceptably high rates of Mendelian errors (>5%) between parents and child, and 4) significant deviation (p < 10^{-5}) from Hardy Weinberg equilibrium (HWE) among parents within recruitment site or across European and Asian populations separately. This QC process flagged 14.6% of all SNPs (mostly for low MAF), leaving ~490 K SNPs available for analysis.

Exposure Assessment
Maternal exposure information, including cigarette smoking, ETS, multivitamin supplementation, and alcohol consumption was collected through direct interviews of mothers. Only Asian sites collected complete information on ETS. Environmental exposures were defined as being exposed from three months prior to pregnancy through the first trimester. The question measuring ETS status during certain periods asked “did someone smoke in your home, workplace or any other place near you?”. Maternal exposures were assessed as simple yes/no responses. See Table II in Beaty et al. (2011) for details of the exposure rates for all CP trios. The proportion of infants exposed to maternal cigarette smoking and alcohol consumption was very low among Asian mothers (around 4%), so only maternal ETS and multivitamin supplementation could be analyzed in this group. The proportion of exposure to ETS and multivitamin supplementation among Asians were 40.3% and 20.2%, respectively.

Statistical Analysis
MAFs were computed using parents only. Pairwise linkage disequilibrium (LD) was measured as r^2 for all SNPs using the Haploview program, and was used to identify linkage disequilibrium (LD) blocks [23]. In this study, we used a closed form genotypic transmission disequilibrium test (gTDT) developed by Schwender et al. (2012) to test for genetic association of each SNP [24]. To perform this gTDT, a “pseudo-control” dataset was created based on the observed genotype of the case and all alternative possible genotypes given the parental mating type. The gTDT has a number of advantages compared to allelic TDT [25].

While assuming different models of inheritance, the gTDT can be used to estimate the relative risks (RRs) of each genotype and a term for GxE interaction can also be incorporated. Schwender et al. (2012) developed a method with a closed form solution providing parameter estimates for genome-wide markers efficiently [24] and is implemented in the R package Trio (v 1.5.0).

All autosomal markers were examined using the conditional logistic regression model assuming an additive model of inheritance. The log-odds of being the observed case in the i-th trio is modeled as: logit[Pr(case,|)] = β0 + β1G + β2GxE, where G = 0, 1, or 2 stands for the number of risk alleles in the case; “pseudo-control” set (representing a 1:3 matching), and where E = 0 or 1 reflects unexposed or exposed mothers, respectively. A 2 degrees of freedom (df) likelihood ratio test (LRT) for joint effects of G and GxE interaction was first performed, followed by a 1 df LRT for GxE interaction alone. The 2 df test examines the inherent effect of the SNP after taking into account effects of GxE interaction, while the 1 df test focuses exclusively on GxE interaction. We used RR(CP|G and E) = exp(β2G) to represent the estimated RRs of being a case with one copy of the risk allele in the absence of maternal environment exposures, while RR(CP|G and E) = exp(β2G+β2GxE) reflects the RR of being a case carrying one copy of the risk allele in the presence of maternal exposure.

Results
A conventional search for marginal gene (G) effects in the total sample of 550 CP trios, as well as in the stratified analysis of trios of Asian and European ancestry, showed no markers achieved significance at a genome-wide level (P = 10^{-7}, data not shown).

A genome-wide screen for GxE interaction was carried out using Trio (1.5.0), where conditional logistic regression models were used to estimate effects of GxE interaction alone (LRT with 1 df), as well as the combined effects of gene (G) and gene-environment (GxE) interaction (LRT with 2 df). This screening process yielded no significant signals among European trios (see Figure S1 in File S1 for GxE interaction results on maternal smoking, alcohol consumption and multivitamin supplementation among European trios), but revealed several markers with suggestive evidence of GxE interaction (10^{-6} < P < 10^{-4}), among Asian trios clustered on chromosome 4p16, especially in the 1 df test for GxE interaction. Figure 1 presents a conventional Manhattan plot for all autosomal SNPs where –log10(P) from the 1 df/LRT for GxE interaction was plotted (See Figure S2 in File S1 for a Q-Q plot of GxE interaction among Asian trios). Therefore, we mainly present results for GxE interaction among Asian trios here.

To further investigate this evidence, Figure 2 presents a “double Manhattan plot” to summarize joint evidence for G and GxE interactions on chromosome 4p (over the region 8988690 kb–10636912 kb). Table S1 in File S1 showed the physical location and MAFs of SNPs in this region (19 SNPs with MAF < 0.01 were dropped in this region). The bottom half of this plot shows the –log10(P) for the conventional family-based test of SNP effects ignoring exposure (where more significant results fall farther below the mid-line). In the top half of Figure 2, –log10(P) are shown for each autosomal SNP from both the 2 df test of G and GxE interaction together (red dots) and the 1 df test for GxE interaction alone (blue dots). Dashed lines connect P-values from the marginal test ignoring exposures (below the mid-line) to those models considering GxE interaction (above the mid-line). As seen in Figure 2, more than 20 markers gave P values approaching genome-wide significance levels in tests for GxE interaction, including 15 SNPs in SLC2A9 and 9 in WDR1 on chr. 4p16.1 (Figure S3 in File S1 shows LD plots for these two genes).

Although none of 135 SNPs mapping to SLC2A9 approached genome-wide significance level when maternal exposure to ETS was ignored (lower half of Figure 2), a cluster of 61 SNPs identified a region spanning 125 kb yielded P values approaching genome-wide significance levels when interaction with maternal ETS was considered. In this region, fifteen SNPs showed suggestive evidence of GxE interaction in the 1 df test (Table 2). SNPs rs3733585 and rs12508991 suggested GxE interaction in the 1 df test (P = 2.26 × 10^{-6}).

Regression coefficients from the conditional logistic regression model provide an estimate of exposure specific RRs under this additive model. When both G and GxE terms were included in the conditional logistic regression model, RRs were also calculated for both exposed and unexposed heterozygous carriers of the apparent risk allele. Figure 3 shows estimated RR(CP|G no E) and RR(CP|G and E) for 15 SNPs in SLC2A9 along with P-values from the LRT for both the 2 df and 1 df test. Here, the apparent “risk allele” became the target allele (which was the minor allele for rs10022499, rs10016075, rs2240725, rs3733585, rs733175, but...
the major allele for rs4447863, rs998676, rs689717, rs11723970, rs17187075, rs12499857, rs10939650, rs4622999, rs7657096, rs12508991—see Table 2). Estimated RR(CP|G and E) and their 95% CI for a heterozygous child whose mother was exposed to ETS were distinctly higher (open circles) compared to a similar heterozygous child of unexposed mothers (solid circles). For the two most significant SNPs (rs3733585 and rs12508991) being a heterozygous child of an exposed mother was associated with a 2.58-fold increase in risk (RR = 2.58; 95% CI: 1.61–4.14), but not among children of unexposed mothers (RR = 0.60; 95% CI: 0.43–0.83). The 1 df LRT for GxETS interaction in this conditional logistic regression model approached genome-wide significance (P = 2.26 × 10⁻⁷).

WDR1 on chr. 4p16.1 is located next to SLC2A9 and encompasses 59 SNPs. Like SLC2A9, none of these SNPs achieved genome-wide significance levels alone, however, a block of 9 SNPs

Figure 1. CP Asian ETS GxE Manhattan Plot. Manhattan plot with P values from likelihood ratio tests with 1 degree of freedom testing for GxETS interaction among 259 Asian CP trios (492,698 SNPs were left in Asian trios after quality control).
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Figure 2. Gene × Environmental Tobacco Smoke Interaction among Asian CP Group. Double Manhattan plots for SNP effects ignoring maternal exposures (black dots in the lower half) and considering G and GxE interaction for environmental tobacco smoke on selected region on chromosome 4p among 259 Asian trios. Blue dots represent -log₁₀(P) from the 1 df test of GxE interaction alone; red dots represent -log₁₀(P) from the 2 df test of G and GxE interaction. Dashed lines connect SNP showing this level of significance in one test considering GxE interaction with their corresponding P-value when interaction was ignored.
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showed suggestive GxETS interaction in the 1 df test for GxE interaction (Table 3). SNPs rs6820756 and rs7699512 yielded $P = 1.79 \times 10^{-5}$ and $P = 1.98 \times 10^{-5}$ in the 1 df test for GxE interaction, and two adjacent SNPs also approached genome-wide significance (rs6834555, $P = 2.28 \times 10^{-6}$; rs6834555, $P = 2.73 \times 10^{-5}$). Figure 4 shows estimated RR(CP|G no E) and RR(CP|G and E), plus their 95%CI, for these 9 SNPs under an additive model. The risk of having nonsyndromic CP was 1.97–2.75 times higher when the fetus carried the risk allele and the mother was exposed to ETS compared to carriers whose mothers were not exposed.

Examining the imputed genotypes generated by the GENEVA Coordinating Center [26] using 1000 Genomes reference populations after pre-phasing haplotypes using IMPUTE2 [27] yielded additional evidence of GxETS interaction. Analysis of imputed SNPs in the region of these two genes yielded genome-wide significance for several markers (see Figure S4 in File S1).

Similar analysis for potential GxE interaction with maternal multivitamin supplementation in these same Asian CP trios showed no significant GxE interaction. Because the exposure rate for maternal multivitamin supplementation was lower in this sample of Asian CP trios ($20\%$), however, this sample had less statistical power to detect GxE interaction unless the causal allele

### Table 2. Estimated RR(case|G no E) and RR(case|G and E) from conditional logistic regression using cases and 3 pseudo-controls in 259 Asian CP case-parent trios for 15 SNPs in SLC2A9 considering GxE interaction between each SNP and maternal exposure to environmental tobacco smoke.

| SNP       | Physical location | TA (freq) | RR(case|G no E)       | RR(case|G and E)      | LRT 2 df P values | LRT 1 df P values |
|-----------|-------------------|-----------|---------------------|---------------------|--------------------|--------------------|
| rs4447863 | 9548067           | C(0.593)  | 0.64(0.46,0.90)     | 2.07(1.32,3.25)     | 1.61 $\times 10^{-4}$ | 2.94 $\times 10^{-5}$ |
| rs998676  | 9557662           | G(0.587)  | 0.64(0.46,0.90)     | 2.07(1.32,3.25)     | 7.51 $\times 10^{-5}$ | 1.36 $\times 10^{-5}$ |
| rs6849717 | 9567817           | C(0.590)  | 0.66(0.47,0.91)     | 2.26(1.44,3.55)     | 5.07 $\times 10^{-5}$ | 9.01 $\times 10^{-6}$ |
| rs11723970| 9589560           | T(0.588)  | 0.64(0.46,0.90)     | 2.21(1.42,3.46)     | 4.67 $\times 10^{-5}$ | 8.14 $\times 10^{-6}$ |
| rs17187075| 9599426           | C(0.596)  | 0.84(0.46,0.89)     | 2.58(1.63,3.95)     | 8.22 $\times 10^{-6}$ | 1.38 $\times 10^{-6}$ |
| rs12499857| 9604474           | C(0.600)  | 0.62(0.44,0.86)     | 2.46(1.63,3.95)     | 8.57 $\times 10^{-6}$ | 1.36 $\times 10^{-6}$ |
| rs10939650| 9607538           | T(0.500)  | 0.71(0.50,1.01)     | 2.61(1.63,3.88)     | 3.91 $\times 10^{-5}$ | 1.22 $\times 10^{-5}$ |
| rs4622999 | 9612493           | C(0.600)  | 0.61(0.44,0.86)     | 2.35(1.63,3.95)     | 8.08 $\times 10^{-6}$ | 1.28 $\times 10^{-6}$ |
| rs7657096 | 9613098           | A(0.528)  | 0.70(0.49,0.99)     | 2.33(1.49,3.66)     | 7.74 $\times 10^{-5}$ | 2.03 $\times 10^{-5}$ |
| rs10022499| 9615635           | C(0.492)  | 0.71(0.50,1.00)     | 2.37(1.51,3.72)     | 5.87 $\times 10^{-5}$ | 1.60 $\times 10^{-5}$ |
| rs10016075| 9615761           | G(0.492)  | 0.71(0.50,1.00)     | 2.33(1.49,3.66)     | 8.24 $\times 10^{-5}$ | 2.15 $\times 10^{-5}$ |
| rs2240723 | 9630249           | A(0.465)  | 0.67(0.47,0.95)     | 2.32(1.49,3.62)     | 3.93 $\times 10^{-5}$ | 8.81 $\times 10^{-6}$ |
| rs3733585 | 9645437           | C(0.412)  | 0.60(0.43,0.83)     | 2.58(1.61,4.14)     | 1.52 $\times 10^{-6}$ | 2.26 $\times 10^{-7}$ |
| rs12508991| 9650202           | C(0.588)  | 0.60(0.43,0.83)     | 2.58(1.61,4.14)     | 1.52 $\times 10^{-6}$ | 2.26 $\times 10^{-7}$ |
| rs733175  | 9659239           | G(0.488)  | 0.69(0.49,0.98)     | 2.71(1.70,4.33)     | 6.12 $\times 10^{-6}$ | 1.89 $\times 10^{-6}$ |

TA: target allele and its frequency among parents of Asian ancestry.
were highly polymorphic (MAF > 0.15) and the true interaction effects were at least as large as those seen in test of GxETS interaction (RRGE > 2.5).

**Discussion**

While the initial GWAS of 550 CP case-parent trios stratified by European and Asian ancestry did not yield any markers achieving genome-wide significance (i.e., when GxE interaction was ignored), multiple markers in two adjacent genes on chr. 4p16.1 (SLC2A9 and WDR1) showed P-values approaching genome-wide significance when GxETS interaction was incorporated into the analysis of Asian trios. Our results suggested SLC2A9 and/or WDR1 located at position 9 Mb on chromosome 4p16.1 may influence risk of nonsyndromic CP through interaction with maternal exposure to ETS, though independent replication studies are still needed to confirm these findings. Our study did not yield any compelling evidence of GxE interactions approaching genome-wide significance among trios of European ancestry.

Identifying GxE interaction will lead to better understanding of the etiology of common birth defects and potential biological mechanisms, as well as create opportunities for designing effective prevention strategies. Several studies have shown maternal smoking is not only an independent risk factor for CP [28,29], but may interact with genetic variants to influence risk [12–14]. GxSmoking interaction has been suggested for markers in the chr. 4p16 region. A previous case-control study and case-parent trio studies showed evidence of GxSmoking for markers near MSX1 on chr. 4p16 among CP trios or combined CL/P and CP trios [30,31]. This 4p16 region has been suggested to be associated with increased risk of nonsyndromic oral clefts, including CL/P and CP in a previous analysis [32]. Ingersoll et al. (2010) used 381 case-parent trios from four populations including Asian samples from Singapore, Korea, and Taiwan [32]. Their analysis focused on the 2 Mb region around MSX1 and showed SNP effects in STK32B, the EVC–EVC2–CRMP1 region, and the STX18–MSX1 region were significantly associated with risk to CP, especially among Asian trios. A Dutch study showed smoking by both parents may interact with SNPs in MSX1 to increase the risk of nonsyndromic oral clefts [16]. SLC2A9 and WDR1, the most significant genes seen here, are located about 3 Mb downstream of MSX1. ETS has been shown to interact with candidate genes to influence risk of nonsyndromic oral clefts in different populations.

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**Table 3. Estimated RR(case|G no E) and RR(case|G and E) from conditional logistic regression using cases and 3 pseudo-controls in 259 CP case-parent trios for 9 SNPs in WDR1 considering maternal exposure to environmental tobacco smoke.**

| SNP     | Physical location | TA (freq) | RR(case|G no E) | RR(case|G and E) | LRT 2 df p-value | LRT 1 df p-value |
|---------|-------------------|-----------|--------------|---------------|------------------|------------------|
| rs6834555 | 9671424 | G(0.482) | 0.71(0.50,1.00) | 2.75(1.72,4.39) | 5.61 × 10^{-6} | 2.28 × 10^{-6} |
| rs6820756 | 9671947 | A(0.482) | 0.70(0.50,0.99) | 2.75(1.72,4.39) | 4.93 × 10^{-6} | 1.79 × 10^{-6} |
| rs2241469 | 9689560 | A(0.652) | 0.68(0.48,0.97) | 2.36(1.48,3.77) | 8.73 × 10^{-5} | 1.97 × 10^{-5} |
| rs2241482 | 9708912 | G(0.654) | 0.69(0.49,0.98) | 2.37(1.47,3.83) | 1.06 × 10^{-4} | 2.31 × 10^{-5} |
| rs717615 | 9713768 | C(0.527) | 0.63(0.45,0.88) | 2.42(1.50,3.89) | 1.67 × 10^{-5} | 2.73 × 10^{-5} |
| rs4697922 | 9719703 | C(0.657) | 0.68(0.48,0.97) | 2.43(1.50,3.96) | 7.70 × 10^{-5} | 1.58 × 10^{-5} |
| rs7699512 | 9734906 | T(0.519) | 0.56(0.40,0.79) | 2.11(1.34,3.30) | 9.90 × 10^{-6} | 1.98 × 10^{-6} |
| rs10489072 | 9882342 | G(0.499) | 0.65(0.46,0.92) | 1.97(1.27,3.05) | 4.08 × 10^{-4} | 7.81 × 10^{-5} |
| rs6833142 | 9885080 | G(0.494) | 0.66(0.47,0.93) | 1.97(1.27,3.05) | 4.65 × 10^{-4} | 8.98 × 10^{-5} |

TA: target allele and its frequency among parents of Asian ancestry.

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**Figure 4. WDR1: SNP x Environmental Tobacco Smoke.** Estimated RR(CP|G no E) and RR(CP|G and E) from conditional logistic regression model considering SNP effects and their interaction with maternal exposure to ETS on 259 CP case-parent trios of Asian ancestry for nine SNPs in WDR1. P-values from the 2 df and 1 df LRT for GxE interaction are shown along the X axis.

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Previous studies have shown ETS may interact with IRF6, RUNX2, and BMP4 among Chinese CL/P case-parent trios [17,19,33]. Another French study yielded suggestive evidence for interaction between CIP1A1 and ETS among nonsyndromic oral cleft trios [15]. Li et al. (2011) also found maternal ETS interacted with one SNP in microRNA-140 gene to increase the risk of nonsyndromic CP using case-control design in a Chinese population [10].

The WDR1 gene (WD repeat domain 1, also called actin-interacting protein 1) is downstream from SLC2A4 and it is highly conserved in eukaryotes and promotes cofilin-mediated actin filament disassembly [34]. Kato et al. (2008) noted WDR1 has an important role in unidirectional cell migration by promoting cofilin activity. Protein aggregates of actin and cofilin in the brains of twins with dystonia and CL/P were described by Gearing, et al. (2010) [35]. While neither of these studies is proof of any link between WDR1 and nonsyndromic oral clefts, they suggest a possible biological mechanism involving disruption of cell migration during development of the palate. The SLC2A4 gene (solute carrier family 2, member 9) is located on chromosome 4p16.1, and encodes a member of the SLC2A4 facilitative glucose transporter gene family, which is critical for maintaining glucose homeostasis. Multiple association studies across several populations showed consistent findings that this gene is associated with uric acid concentration and risk of gout [36–43], with a higher effect size among females compared to males. In our study, markers in these genes were in high LD. Therefore, the significant findings of GxETS interaction in SLC2A4 may reflect its close physical proximity to WDR1. We also performed the GxETS analysis using imputed genotype data in this chromosomal region among Asian trios, and the imputed genotypes yielded greater significance (including several achieving genome-wide significance) in the region (Figure S4 in File S1). In addition, we tested for parent-of-origin effects among exposed and unexposed trios using the parent-of-origin likelihood ratio test [44], but found no significant signals. Our study suggested genes in this region may play a role in the etiology of CP not only through gene effects but also may through potential GxE interactions, at least among Asian populations. Although it is unclear how either of these genes affects cleft development, our results suggest these two genes (especially WDR1) should be considered as candidate genes for nonsyndromic CP.

We acknowledge the suggestive GxETS interactions on chr. 4p16.1 region seen in the present study require further confirmation in independent samples. However, adequate sample size will be a challenge. Our results argue maternal ETS appears to increase risk of nonsyndromic CP in Asian cases carrying certain genotypes in SLC2A4 and WDR1. Exposure rate of ETS among Asian mothers in this sample was as high as 40%, reflecting the high prevalence of smoking among Asian males (about 60%) [45]. If this observation can be confirmed, such a GxE interaction creates opportunities for an effective intervention to reduce the risk. Our suggestive evidence of interaction between ETS and two genes on Chromosome 4 would be strengthened if we could test for GxSmoking interaction in this same population. However, such analysis would be severely underpowered due to low rates of personal smoking among Asian women. Further analyses will be required to understand how maternal exposure to ETS could interact with genes to affect fetal development. In addition, the analysis testing for the interaction between maternal genes and environmental exposures could also be informative. While the case-parent trio design is robust to population stratification [46,47], and stratification into Asian/European ancestries minimizes potential confounding due to differences in exposure rates, this study illustrates the importance of considering possible GxE interaction in the etiology of CP. Still statistical interaction does not guarantee biological interaction, and the functional gene may be located some distance from the statistical signals for GxE interaction seen here.

Supporting Information

File S1 Supporting information. Figure S1.1: Manhattan plot with P values from likelihood ratio tests with 1 degree of freedom testing for GxSmoking interaction among 272 European CP trios. Figure S1.2 Manhattan plot with P values from likelihood ratio tests with 1 degree of freedom testing for GxAlcohol consumption interaction among 272 European CP trios. Figure S1.3 Manhattan plot with P values from likelihood ratio tests with 1 degree of freedom testing for GxETS interaction among 259 Asian CP trios (492,698 SNPs were left in Asian trios after quality control). The gray shaded region indicates 95% confidence band for order statistics. The numbers on the top axis indicate the respective locations for (ordered) expected –log10 P-values. (e.g., the number 1 (10) indicates the expected value, on the –log10 scale, for the minimum (i.e. the tenth smallest) P-value). Figure S3.1 LD plots for SLC2A4 among 259 Asian CP trios. Black squares represent r² = 1; gray squares represent 0<r²<1; white squares represent r² = 0. Figure S3.2 LD plots for WDR1 among 259 Asian CP trios. Black squares represent r² = 1; gray squares represent 0<r²<1; white squares represent r² = 0. Figure S4: P values from likelihood ratio test with 1 degree of freedom testing for GxETS interaction after including the imputed SNPs among Asian CP trios. Circles represent imputed genotypes using 1000 Genomes as a reference population and squares represent observed SNPs. Table S1. (DOCX)

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Author Contributions

Conceived and designed the experiments: THB JCM MLM RGM. Performed the experiments: TW IR AFS MT SL KYL. Analyzed the data: TW IR AFS MT SL KYL. Contributed reagents/materials/analysis tools: HS IR. Wrote the paper: TW IR AFS MT SL KYL. Conducted field investigation and collected samples: TW IR AFS MT SL KYL.
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