Original Contribution

Endothelial Nitric Oxide Synthase (NOS3) Genetic Variants, Maternal Smoking, Vitamin Use, and Risk of Human Orofacial Clefts

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Orofacial clefts have been associated with maternal cigarette smoking and lack of folic acid supplementation (which results in higher plasma homocysteine concentrations). Because endothelial nitric oxide synthase (NOS3) activity influences homocysteine concentration and because smoking compromises NOS3 activity, genetic variation in NOS3 might interact with smoking and folic acid use in clefting risk. The authors genotyped 244 infants with isolated cleft lip with or without cleft palate (CL/P), 99 with isolated cleft palate, and 588 controls from a California population-based case-control study (1987–1989 birth cohort) for two NOS3 polymorphisms: A(-922)G and G894T. Analyses of gene-only effects for each polymorphism revealed a 60% increased risk of CL/P among NOS3 A(-922)G homozygotes (odds ratio (OR) = 1.6, 95% confidence interval (CI): 1.0, 2.6). There was some evidence for higher risk of CL/P with maternal periconceptional smoking in infants with an NOS3 A(-922)G allele (for homozygotes, OR = 2.5, 95% CI: 1.2, 5.6) but not in those with an 894T allele. For CL/P risk, odds ratios were over 4 among mothers who smoked, who did not use vitamins, and whose infants had at least one variant allele for each NOS3 polymorphism (for A(-922)G, OR = 4.6, 95% CI: 2.1, 10.2; for 894T, OR = 4.4, 95% CI: 1.8, 10.7). No similar patterns were observed for risk of cleft palate.

abnormalities; cleft lip; cleft palate; genetics; nitric-oxide synthase; risk factors; smoking; vitamins

Abbreviations: NCBI, National Center for Biotechnology Information; NOS3, endothelial nitric oxide synthase; SNP, single nucleotide polymorphism.

Orofacial clefts are suspected of being etiologically heterogeneous (1–4). A certain number of orofacial clefts occur as part of recognizable patterns of malformation or have genetic etiologies (5–7). Epidemiologic studies indicate that increased risks of clefting may be associated with prenatal exposures, such as exposure to cigarette smoke (8–12), anti-convulsants (13, 14), retinoic acid (15), alcohol (16–18), agricultural pesticides (19), or organic solvents (20, 21), and with lack of folic acid supplementation during pregnancy (8, 22–29).

A promising approach to identifying etiologies of orofacial clefts is exploration of possible gene-environment interactions. Such an approach has been used to explore relations between maternal smoking and gene variants (9, 12, 29–35) and between maternal vitamin use and gene variants (34, 36–42). However, we are not aware of any study that has investigated the potential multiple interactions of maternal smoking, maternal vitamin use, and gene variants, which was the central approach used in the current study.

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Our study focused on endothelial nitric oxide synthase (NOS3), which regulates nitric oxide production and is expressed in human endothelial cells (43) and mouse embryos (44). Brown et al. (45) demonstrated that the NOS3 G894T single nucleotide polymorphism (SNP) was associated with homocysteine concentrations. Some of their study groups comprising persons with the TT genotype showed more than double the homocysteine concentrations of subgroups comprising persons with the GG genotype. These investigators proposed that nitric oxide modulated homocysteine levels via an effect on folate catabolism. It has also been demonstrated that cigarette smoking compromises NOS3 activity (46). Because risk of clefting has been associated with maternal cigarette smoking and lack of folic acid supplementation (which results in higher plasma homocysteine concentrations), we reasoned that genetic variation in NOS3 might interact with these two exposures. Here we investigated a potential association between clefting risks and NOS3 gene variants and whether the association was modified by maternal cigarette smoking and intake of folic acid supplements during the periconceptional period.

MATERIALS AND METHODS

Details on this case-control study have been provided previously (9, 23). Included as cases were infants and fetal deaths (≥20 weeks’ gestation) diagnosed with orofacial clefts within 1 year after delivery among women residing in most California counties. All infants or fetal deaths with delivery occurring between January 1987 and December 1989 (among 552,601 total infants or fetal deaths) were eligible. Case eligibility was determined by one clinical geneticist (E. J. L.) who reviewed detailed diagnostic information from medical records of all hospitals and genetics centers in the surveillance area. Orofacial cleft cases were defined as infants or fetuses born with cleft palate or with cleft lip with or without cleft palate (hereafter called cleft lip/palate) that was confirmed by clinical description, surgical report, or autopsy report. This distinction in phenotype is consistent with embryologic underpinnings (1). Cases were further classified on the basis of the nature of accompanying congenital anomalies. Cases with no other major anomaly or with anomalies considered minor were classified as isolated. Cases with at least one accompanying major anomaly were classified as multiple. Only isolated cases of cleft palate and cleft lip/palate were considered in these analyses. Infants diagnosed with single gene disorders, trisomies, or Turner’s syndrome (45,X) were excluded.

As controls, 972 infants were randomly selected from all infants born alive in the same geographic area and time period as the cases. Control infants had no major congenital anomalies identified before their first birthday, as defined by the California Birth Defects Monitoring Program (47).

Telephone interviews were completed with 489 mothers of isolated orofacial cleft cases (85 percent of those eligible) and 734 control mothers (76 percent). Interviews were completed within an average of 3.7 years from the date of delivery for cases and within 3.8 years for controls. Interviews elicited maternal information on medical and reproductive histories and activities associated with various lifestyles. The interviewer assisted each woman in establishing a 4-month periconceptional period, ranging from 1 month before conception to 3 months after conception, that was referred to throughout the interview to elicit information. Women were asked whether they had used vitamin and mineral supplements during this period and which supplements (types or brands) they had used in each month. We divided women into two categories relative to their use of vitamins containing folic acid: 1) “use” was defined as starting vitamin use anytime during the period ranging from 1 month before conception through the end of the third month after conception and 2) “nonuse” was defined as starting vitamin use after the third month from conception (post-dating the relevant embryologic timing of the studied phenotypes) or absence of use during pregnancy. For assessment of active maternal smoking exposures, women were asked how many cigarettes they had smoked daily during the 4-month periconceptional period (1 month before conception through the first trimester) and in each month during that period.

Our analyses were restricted to 1) cases and controls whose mothers were interviewed and 2) liveborn case and control infants, because the source of DNA was residual newborn screening blood specimens (filter paper). For the 489 infants with isolated cleft lip/palate or isolated cleft palate, 343 (244 with cleft lip/palate and 99 with cleft palate) had DNA available and were genotyped. Among the 652 control infants for whom DNA was available, 588 were genotyped. All interviews and samples were obtained with approval from the State of California Health and Welfare Agency Committee for the Protection of Human Subjects.

In addition to the G894T SNP, we were able to explore two other NOS3 SNPs that were available to us on a panel containing multiple known SNPs. Thus, case and control infants were genotyped for three NOS3 SNPs: A(—922)G (rs1800779), C(—690)T (rs3918226), and G894T (E298D) (rs1799983).

Genotyping was accomplished in a manner similar to that of Cheng et al. (48) using a multilocus sequence-specific hybridization assay developed by Roche Molecular Systems, Inc. (Alameda, California). Briefly, multiplex polymerase chain reaction with a blend of biotinylated primer pairs is used to amplify each polymorphic site. Biotin-tagged amplification products are hybridized to a linear array of immobilized oligonucleotide probes specific for each allele under stringent conditions. Chromogenic reagents are used to visualize the biotin-tagged amplicons that remain hybridized.

After color development, arrays were manually scored and genotypes were interpreted by two observers. When the observers’ interpretations were discrepant, the samples were reassayed. All genotyping was performed blinded to the subjects’ case/control status. Genotypic frequencies for each NOS3 SNP were evaluated for Hardy-Weinberg equilibrium among the controls, both overall and in each of the three racial/ethnic groups studied (non-Hispanic White, Hispanic, or other). Each SNP showed distributions consistent with Hardy-Weinberg expectations. The frequency of homozygosity for the C(—690)T SNP was very low—one infant with cleft lip/palate and three control infants. Thus, we...
did not include this SNP in further analyses. The observed allele frequencies associated with the other two SNPs were consistent with allele frequencies reported in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). That is, for G894T, we observed the frequency of the T allele to be 0.26 among controls as compared to 0.30 in the NCBI database. For the A(922)G SNP, we observed the frequency of the G allele to be 0.31 as compared to 0.24 in the NCBI database. For the A(922)G SNP, we observed the frequency of the T allele to be 0.26 among controls as compared to 0.30 in the NCBI database.

Logistic regression was used to estimate odds ratios and 95 percent confidence intervals for each NOS3 genotype and for each cleft phenotype (cleft lip/palate or cleft palate). Infants with homozygous variant genotypes or heterozygous variant genotypes were compared with infants with homozygous wild-type genotypes for estimation of gene-only effects and gene-smoking effects. To investigate gene-smoking-vitamin combination effects, we used a dominant genetic model; that is, infants whose genotypes were either homozygous variant or heterozygous were compared with infants with homozygous wild-type genotypes. In combination effects, we used a dominant genetic model for gene-smoking effects. To investigate gene-smoking-vitamin combination effects, we used a dominant genetic model; that is, infants whose genotypes were either homozygous variant or heterozygous were compared with infants with homozygous wild-type genotypes for estimation of gene-only effects and gene-smoking effects. To investigate gene-smoking-vitamin combination effects, we used a dominant genetic model; that is, infants whose genotypes were either homozygous variant or heterozygous were compared with infants with homozygous wild-type genotypes for estimation of gene-only effects and gene-smoking effects. To investigate gene-smoking-vitamin combination effects, we used a dominant genetic model; that is, infants whose genotypes were either homozygous variant or heterozygous were compared with infants with homozygous wild-type genotypes for estimation of gene-only effects and gene-smoking effects.

RESULTS

Analyses investigating gene-only effects of each NOS3 SNP revealed a 60 percent increased risk of cleft lip/palate among A(922)G homozygotes (table 2). Risks of this magnitude were not observed for cleft palate (table 2). We also performed analyses comparing persons who were homozygous variant for either NOS3 SNP with persons who were homozygous wild-type for both NOS3 SNPs. These analyses yielded odds ratios of 1.2 (95 percent confidence interval: 0.8, 1.9) for cleft lip/palate and 0.5 (95 percent confidence interval: 0.2, 1.1) for cleft palate.

We investigated potential modification of risk between NOS3 SNPs and maternal cigarette smoking. Results (table 3) showed some evidence for higher risk of cleft lip/palate in infants whose mothers smoked cigarettes periconceptionally and who had the A(922)G SNP but not in those who had the G894T SNP. However, these results did not provide statistical evidence for heterogeneity; that is, p values associated with the interaction model term for gene variant × maternal smoking were 0.3 for A(922)G and 0.8 for G894T. We did not observe such gene-smoking effects on risks of cleft palate (table 4). We extended these analyses by comparing persons who were homozygous variant for either NOS3 SNP with those who were homozygous wild-type for both NOS3 SNPs in combination with maternal smoking. These analyses did not produce results markedly different from those displayed in tables 3 and 4 (data not shown).

Gene-smoking effects on risks of cleft lip/palate and cleft palate were further explored in combination with maternal periconceptional vitamin use. For these analyses, we used a dominant genetic model; that is, infants whose genotypes were either homozygous variant or heterozygous were
combined and compared with infants whose genotypes were wild-type. These combinations were made because of small numbers of available cases or controls for some comparisons and because of the direction of the results revealed in tables 3 and 4. The results (shown in table 5) indicate higher risks (odds ratios > 4) of cleft lip/palate in infants whose mothers smoked cigarettes, whose mothers did not use vitamins periconceptionally, and who had at least one variant allele for either of the two NOS3 SNPs. These results, however, did not provide statistical evidence for heterogeneity; that is, p values associated with the interaction model term for gene variant × maternal smoking × maternal vitamin use were 0.2 for A(−922)G and 0.5 for G894T. No such consistent risk pattern was observed for risk of cleft palate (table 6). The estimated risks displayed in tables 5 and 6 were not substantially different after results were adjusted for the potentially confounding effects of maternal race/ethnic background (data not shown).

We explored whether the A(−922)G and G894T SNPs were in linkage disequilibrium. We observed modest evidence for linkage disequilibrium (D’ = 0.47, p < 10⁻⁴).

Because the similar findings observed for each SNP could have been a function of an overlapping haplotype, we estimated risks associated with each combination of A(−922)G and G894T genotypes, relative to infants with wild-type genotypes for both. The observed results did not provide results substantially different from those observed for the two SNPs analyzed separately (data not shown).

**DISCUSSION**

We hypothesized that a potential association between clefting risks and NOS3 gene variants could be modified by maternal cigarette smoking and vitamin supplement intake during the periconceptional period. Our population-based study of California infants revealed sizable increased risks of cleft lip/palate from the combination between a NOS3 variant SNP and maternal smoking. This risk was further modified by lack of maternal vitamin use. No such pattern, however, was consistently observed for risk of cleft palate.
Several studies have identified an association between maternal smoking during the periconceptional period and delivery of infants with orofacial clefts (8–12). There are several lines of evidence suggesting that folate-homocysteine metabolism is implicated in the risk of orofacial clefts. These include 1) reduced risks of orofacial clefts in infants whose mothers took folic acid vitamin supplements periconceptually (8, 22–29) and 2) higher postnatal plasma homocysteine concentrations in mothers who deliver infants with clefts than in mothers who deliver nonmalformed infants (49). However, the underlying explanations for the associations between smoking and clefting and between folate-homocysteine metabolism and clefting are unknown. Lack of knowledge about these associations, coupled with recent information about \( NOS3 \) being involved in folate-homocysteine metabolism and \( NOS3 \) activity being compromised by smoking, motivated us to explore the combined effects of these exposures and gene variants. Moreover, Plachta et al. (50) recently observed that different levels of endogenous nitric oxide in different time periods influenced the balance between cell cycle progression and programmed cell death in the developing neural plate of chick embryos—cells that contribute to facial development.

Thus, a common pathogenetic theme that can be hypothesized from the observed results and the available literature points toward elevated homocysteine concentrations. Evidence exists that the \( NOS3 \) 894TT genotype is associated with elevated serum homocysteine levels in comparison with the GG genotype (45). Lower folate intake is associated with elevated plasma homocysteine (51), as is smoking (52). Our observation of the largest risks for clefting among infants whose mothers smoked, whose mothers did not take vitamin supplements, and who carried genetic variation in \( NOS3 \) is consistent with this pathogenetic theme. Nevertheless, \( NOS3 \) participates in other biologic pathways, including control of vascular tone (53), and in pregnant rats, inhibition of nitric oxide results in hypertension and fetal growth retardation (54). The functional significance of these other pathways for our observed results is unknown.

Some additional evidence in support of our findings associating \( NOS3 \) variation and risk of birth defects can be found in a recent study by Brown et al. (55). These investigators observed a modestly elevated risk of spina bifida, another neural-crest-cell anomaly, among infants who were heterozygous for the \( NOS3 \) G894T SNP (55).

<table>
<thead>
<tr>
<th>( NOS3 ) polymorphism and genotype</th>
<th>Smoking</th>
<th>Vitamin use</th>
<th>No. of cases</th>
<th>No. of controls</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
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<td></td>
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<td></td>
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<tr>
<td>Wild-type</td>
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<td>56</td>
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<td>18</td>
<td>12</td>
<td>4.6</td>
<td>2.1, 10.2</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>Yes</td>
<td>54</td>
<td>197</td>
<td>1.0‡</td>
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<td>2.5</td>
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<td>No</td>
<td>12</td>
<td>10</td>
<td>4.4</td>
<td>1.8, 10.7</td>
</tr>
</tbody>
</table>

* Cigarette smoking was defined as any smoking during the periconceptional period (from 1 month before conception through 3 months after conception).

† Vitamin use was defined as any use of a vitamin containing folic acid during the periconceptional period (from 1 month before conception through 3 months after conception).

‡ Reference category.

§ “Variant” refers to the dominant model in which homozygotes and heterozygotes for the variant genotype were combined.
To our knowledge, our study is the first to report this association. Therefore, these analyses need to be replicated before a stronger inference can be drawn. Our results should be considered relative to some limitations as well. First, the functional significance of both \textit{NOS3} SNPs studied here has not been fully established. Second, there may be unaccounted genetic diversity that extends beyond the three \textit{NOS3} SNPs included in this study; that is, the observations we made could reflect associations with an unmeasured genetic marker that is in linkage disequilibrium with the studied SNPs. Third, many of the associations identified in this study were statistically imprecise. Moreover, because so many analytic comparisons were made, some might be expected to have revealed associations by chance alone.

This study had several strengths. It was relatively large and was among the first to investigate the effects of “gene-environment-environment” interactions on risk of birth defects. Furthermore, case infants with orofacial clefts were identified using a population-based registry system with systematic review of case eligibility. Control infants were randomly identified from birth files and therefore provided a population-based sample of livebirths from the same study base as the case infants. The analyses adequately accounted for the potentially confounding influence of maternal race/ethnicity. Lastly, this study revealed some important clues for further investigation that may help fill the data gap about the underlying process by which folic acid facilitates a reduced risk of human birth defects.

\textbf{ACKNOWLEDGMENTS}

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\begin{table}
\centering
\caption{Risks of isolated cleft palate associated with single nucleotide polymorphisms in the endothelial nitric oxide synthase (\textit{NOS3}) gene in combination with maternal cigarette smoking* and vitamin use among California infants relative to nonmalformed population-based controls, 1987–1989} \label{tab6}
\begin{tabular}{llllll}
\hline
NOS3 polymorphism and genotype & Smoking & Vitamin use & No. of cases & No. of controls & Odds ratio & 95\% confidence interval \\
\hline
A(−922)G & & & & & & \\
Wild-type & No & Yes & 26 & 169 & 1.0† & \\
Wild-type & No & No & 5 & 36 & 0.9 & 0.3, 2.5 \\
Wild-type & Yes & Yes & 16 & 52 & 2.0 & 1.0, 4.0 \\
Wild-type & Yes & No & 3 & 15 & 1.3 & 0.4, 4.8 \\
Variant§ & No & Yes & 24 & 188 & 0.8 & 0.5, 1.5 \\
Variant§ & No & No & 7 & 46 & 1.0 & 0.4, 2.4 \\
Variant§ & Yes & Yes & 12 & 57 & 1.4 & 0.6, 2.9 \\
Variant§ & Yes & No & 2 & 12 & 1.1 & 0.2, 5.1 \\
G894T & & & & & & \\
Wild-type & No & Yes & 23 & 197 & 1.0† & \\
Wild-type & No & No & 9 & 42 & 1.8 & 0.8, 4.2 \\
Wild-type & Yes & Yes & 17 & 60 & 2.4 & 1.2, 4.8 \\
Wild-type & Yes & No & 2 & 17 & 1.0 & 0.2, 4.6 \\
Variant§ & No & Yes & 27 & 160 & 1.4 & 0.8, 2.6 \\
Variant§ & No & No & 3 & 40 & 0.6 & 0.2, 2.2 \\
Variant§ & Yes & Yes & 11 & 49 & 1.9 & 0.9, 4.2 \\
Variant§ & Yes & No & 3 & 10 & 2.6 & 0.7, 10.0 \\
\hline
\end{tabular}
\end{table}

* Cigarette smoking was defined as any smoking during the periconceptional period (from 1 month before conception through 3 months after conception).
† Vitamin use was defined as any use of a vitamin containing folic acid during the periconceptional period (from 1 month before conception through 3 months after conception).
‡ Reference category.
§ “Variant” refers to the dominant model in which homozygotes and heterozygotes for the variant genotype were combined.
Although this research was partially funded by the Environmental Protection Agency (EPA), it was not subjected to any EPA review and therefore does not necessarily reflect the views of the EPA. No official endorsement should be inferred.

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REFERENCES


