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Studies in the United States have indicated that maternal first trimester smoking and infant transforming growth factor alpha (TGFA) locus mutations are associated with non-syndromic cleft lip and/or palate (CLP) and that a synergistic effect of these two risk factors occurs. Based on a Danish case-control study of CLP, the authors studied the effects of smoking and TGFA alleles in an ethnically homogeneous setting. Interview information was obtained for mothers of 302 CLP cases (96% of eligible) and for 567 mothers of nonmalformed children (94% of eligible). Multivariate logistic regression analyses revealed that smoking was associated with a moderately increased risk of cleft lip ± cleft palate (CL(P)) (odds ratio = 1.40, 95% confidence interval 0.99–2.00). No association between smoking and isolated cleft palate (CP) was observed. TGFA genotype was not associated with either CL(P) or CP, and no synergistic effect with smoking was observed. The "rare" TGFA allele occurred in 25% of both cases and controls compared with an average of 14% in other white control groups. Furthermore, the frequency of CLP in Scandinavia is among the highest in the world. Hence, it is possible that the previously reported association between TGFA and CLP to some degree can be attributable to confounding by ethnicity. Am J Epidemiol 1999; 149:248–55.

case-control studies; cleft lip; cleft palate; confounding factors (epidemiology); smoking; transforming growth factor alpha

The etiology of non-syndromic cleft lip and/or palate (CLP), which is among the most common birth defects, is still largely an enigma. Twin and other family studies have indicated that genetic factors play a major role in the etiology of CLP (1–5). Associations have been found between CLP and the rare TaqI allele at the transforming growth factor alpha (TGFA) locus (6, 7) and between cleft lip ± cleft palate (CL(P)) and mutations at the retinoic acid receptor locus (8). Recent studies of CL(P) have also supported a role for genes on 4q, 6p, and 19q. However, the associations are generally weak and not seen in all populations (9).

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Abbreviations: CI, confidence interval; CLP, cleft lip and/or palate (i.e., all clefts); CL(P), cleft lip ± cleft palate (i.e., excluding CP); CP, isolated cleft palate; OR, odds ratio; PKU, phenylketonuria; TGFA, transforming growth factor alpha.

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Because CLP is a rare condition, no common environmental exposure could in isolate be a sufficient cause of CLP. However, common environmental exposures could play a role in the CLP etiology if they act conditionally on other component causes, such as infrequent genetic factors. The first reports on such interactions in CLP etiology have been published within the last few years. Hwang et al. (20) found for isolated cleft palate (CP) a statistically significant interaction between maternal smoking and infant TGFA genotype. However, as noted by the authors, the study had several limitations. A similar interaction was found for CL(P) in a Californian study reported by Shaw et al. (21). The large ethnic heterogeneity in this study leaves, however, room for confounding (22). Despite the limitations in these gene-environment interaction studies, they provided a possible breakthrough in the etiologic research of CLP.

In a Danish nationwide 3-year case-control study of newly diagnosed non-syndromic CLP, we evaluated the hypotheses that maternal first trimester smoking and infant TGFA mutations are associated with CLP and that a smoking-TGFA interaction occurs. The study had advantages of a high participation rate, ascertainment from an ethnically homogeneous population, and exposure information collected within a few weeks after birth. Furthermore, the self-reported smoking information could be validated through an independent data source, which mainly relied on smoking information collected before the pregnancy outcome was known.

We found a considerably higher frequency of the rare TGFA allele among Danes compared with previous figures for whites in the United States, Great Britain, Australia, and France. In addition, because the frequency of CLP in Scandinavia is among the highest in the world (23), we examined to what degree the association between TGFA and CLP could be attributable to a confounding effect of ethnicity.

MATERIALS AND METHODS

The study was based on a nationwide 3-year case-control study in the Danish population of 5.2 million people. First trimester maternal exposure information was obtained by interviews and from birth records. The latter included prenatally recorded information on maternal smoking during pregnancy. Furthermore, infant genotype information was obtained from stored newborn screening blood spots.

Participants

Case mothers. The case mothers comprised all women in Denmark who gave birth to a CLP child without major associated malformations or syndromes in the period December 1, 1991 to August 31, 1994. Only women who had given birth to a liveborn child, and who were hospitalized in connection with the birth, were eligible. Furthermore, a woman was eligible only if she and the child’s father spoke Danish fluently.

Control mothers. The chosen controls comprised the two mothers of the two preceding births in the hospital where the case mother had given birth. Hence, the two controls for each case were matched on time and place of birth. If a woman gave birth to a child with major malformation(s) or had a stillborn child, she was not eligible and the mother of the preceding birth in the hospital was chosen instead. The same applied if the mother or father of the child did not speak Danish fluently. If a control mother did not wish to participate, another control was not selected instead.

Infants with major malformations other than CLP were not included in the study. Major malformations included anomalies such as neural tube defects, monogenic traits (e.g., Van der Woude), syndromes (e.g., trisomy), and sequences (e.g., Pierre Robin), while anomalies such as clubfoot and syndactylyia of the second and third toes were considered minor defects. “Minimal defects,” such as naevis and descendant testes, were not considered as associated malformations. CLP cases initially suspected to have major associated malformations or syndromes were excluded from the study if the anomalies were confirmed later through the follow-up in the centralized treatment system. A mother was only included in the study if her child was alive at the time planned for the interview.

The Interviews

The treatment of CLP in Denmark has been centralized for half a century. More than 25 years ago, a service was established which provides that mothers who give birth to CLP children shall receive a visit by a specially trained nurse within the first day(s) after birth. The nurse gives advice and help regarding both practical and psychological problems. Usually, after approximately 2 weeks, the same nurse pays the mother a follow-up visit. In the early 1990s, 12 “CLP nurses” maintained this service throughout Denmark. All 12 nurses agreed to act as interviewers in this study in connection with their usual work with CLP families. The interviewers were instructed and trained by one of us (KC) during fall 1991, including a 3-month pilot study comprising a total of 57 interviews. All 53 hospitals, in which births took place in Denmark at that time, agreed to participate in the study. In the study period, only 1 percent of births in Denmark were home confinements.
The questionnaire included questions about previous pregnancies, birth control, diseases, medications, alcohol, smoking, occupation, job exposures, vitamin consumption, diet, socioeconomic status, and family history of congenital malformations. The focus was on maternal exposures in the first trimester. In order to determine this period with greatest possible accuracy, the information about last menstruation’s first day and a calendar were used to identify the dates which delimited the first trimester. Because some women change their life-styles on recognition of pregnancy, information concerning alcohol and smoking was divided into a time period before and a time period after recognition of the pregnancy. This, together with the time for recognition of the pregnancy, provided the basis for a more accurate estimation of the exposures in the first trimester. The time for the recognition of pregnancy was missing for 18 women. For these 18 women, the mean value (39 days) in the sample was used.

The case mothers were interviewed at the follow-up visit, which usually took place 2 weeks after birth. The control mothers were usually interviewed in connection with the nurse’s first visit at the hospital to the case mother. If the control mothers had been discharged from the hospital, they were interviewed in their homes.

Birth record information

Danish birth records from the study period indicate whether the mother was a smoker or nonsmoker at the time of the first visit to the midwife at 12–16 gestational weeks. At most hospitals, this part of the birth record was based on notes made during the first half of the pregnancy. In some hospitals, the information was obtained by asking the women shortly before the delivery.

Blood samples

A biologic bank, consisting of nearly all newborn screening cards (blood spots on filter paper) used for phenylketonuria (PKU) and hypothyroidism screening in Denmark since 1981, has been established at the State Serum Institute in Copenhagen. The newborn screening laboratory receives about 60,000 samples per year. These samples are taken 4–6 days after birth and the laboratory receives them one day later (24). Through personal identification numbers unique for all Danes, it was possible to trace the PKU cards of the individuals who participated in the case-control study. In April 1995, the Danish Medical Research Ethical Committee gave permission to include PKU cards in the case-control study (project no. 95/32 MC). The addresses of all participants were traced and during May 1995 each participant was asked for informed consent to use part of the PKU card for genotyping.

Genotyping

Genotyping for TGFA polymorphism was done using previously reported procedures and PCR-based assays. TGFA genotyping identified the two-allele TaqI RFLP using a modified PCR-based assay (25). All genotyping was carried out in 10 μliter volumes with the TGFA variant analyzed on SSCP (single-stranded conformational polymorphism) gels. DNA samples were prepared from blood spots and DNA extracted using published protocols and purification steps (26). Genotypes were read and interpreted with no knowledge of the case/control status of samples and were repeated when inadequate or uninterpretable results occurred on the first assay. All samples were read by two individuals independently and, when discrepancies resulted, they were re-assayed or resolved by reinterpretation.

Statistical analysis

The data were analyzed using logistic regression including maternal smoking, infant TGFA genotype, and other covariates considered potentially important for CLP, i.e., alcohol and vitamin consumption (19). In the regression models, all independent variables were categorized: smoking consumption (number of cigarettes per day: 0, 1–9, 10–19, ≥20), TGFA-genotype (1.1 (common) or 1.2/2.2 (rare)), alcohol consumption (yes/no), vitamin use (yes/no), time (season and year), and place of birth. It is well established that cleft lip ± cleft palate (CL(P)) and isolated cleft palate (CP) have at least partially different etiology (27). Consequently, analyses were stratified according to cleft type. Controls were matched on time and place of birth, and therefore conditional logistic regression analyses were used initially. Unconditional logistic regression analyses adjusted for time and place of birth (the matching variables) gave, as expected, similar results. The unconditional results are presented here.

We simulated the influence of confounding by ethnicity by assuming that a given population consisted of two subpopulations: subpopulation A, in which 25 percent of the newborns had the rare TGFA allele and in which the overall frequency of nonsyndromic CLP was 1.9/1,000 (comparable with Danish data (4, 5, 28)) and subpopulation B, in which we let the frequency of the rare TGFA allele and the frequency of nonsyndromic CLP vary within the range of previously reported figures (7, 29).

RESULTS

Descriptive characteristics of mothers and infants are shown in table 1. Of the eligible cases and controls, 96 and 94 percent, respectively, participated in the inter-
Table 1 illustrates that women tend to report changes in their life-style after recognition of pregnancy: cigarette consumption decreased one third, and alcohol consumption decreased 60 percent among the interviewed mothers. Table 1 shows that CL(P) case mothers smoked more than controls during the first trimester, and that the frequency of "2" allele was significantly higher in case mothers than in controls. View. Genotyping was obtained on more cases than controls (85 vs. 81 percent of the interview participants, respectively). There was no difference in maternal age between cases and controls. On average, controls were interviewed 5 days sooner after birth than cases (9 vs. 14 days).

*The 604 controls correspond to two controls for each of the 302 participating cases.
†SD, standard deviation; TGFA, transforming growth factor alpha.
‡ Missing values occurred among the first trimester exposures. The variable with most missing values was mean number of beverages, for which no information could be obtained for 17 individuals = 2.0% of the interviewed participants. A question about folic acid was added to the questionnaire when the study had been running for nearly a year and consequently only 618 participants were asked this question (214 cases and 404 controls).
trimester. Furthermore, a tendency of lower alcohol and vitamin consumption was seen among case mothers compared with control mothers. Anti-epileptic drug use was reported for three case mothers and no control mothers.

The TGFA genotype distribution was nearly identical in CL(P) cases, CP cases, and controls and it was in Hardy-Weinberg equilibrium. Some 25 percent of the children had the "rare" TGFA allele. Our simulation studies revealed that most confounding occurs when subpopulation A comprises approximately 20–50 percent of the total population and, as expected, when the differences in TGFA genotype frequencies and CLP occurrence are large. Only in the most extreme scenarios (e.g., population B set to have an 8 percent frequency of the rare TGFA allele and a 0.7/1,000 frequency of nonsyndromic CLP), could confounding account for an odds ratio of approximately 1.4, while in a number of more realistic settings, confounding could account for an odds ratio of 1.1–1.2.

Among the 302 CLP cases, 26 had one affected first-degree relative and one had two affected first-degree relatives. Hence, the recurrence among the 837 first-degree relatives was 3.2 percent, which corresponds to previous findings in Denmark based on register linkage (4, 5). Seven (26 percent) of the 27 cases with affected first-degree relatives had the rare TGFA allele. Additionally, among the most severe cases (49 with bilateral CL(P)), we failed to observe a significantly increased occurrence of the rare TGFA allele (29 percent).

For a total of 817 individuals (94 percent of the interview participants), smoking information was available from interview as well as from birth record. For 758 individuals (93 percent), these two sources agreed on smoking status (table 2). In the interview, 5 percent of both cases and controls reported first trimester smoking, while the birth record stated nonsmoker at first visit to the midwife. More cases than controls (2.8 percent vs. 1.7 percent; p > 0.25) reported that they were nonsmokers after pregnancy was recognized; while the birth record indicated that they were smokers at the first visit to the midwife.

Table 3 shows that first trimester maternal smokers had a higher risk of CL(P). When smoking information from the interview was used, an odds ratio (OR) of 1.35 was found (95 percent confidence interval (CI) 0.99–1.86), while using smoking information from the birth record yielded a stronger risk estimate (OR = 1.56, 95 percent CI 1.12–2.17). When the interview smoking information was adjusted for alcohol and vitamin consumption, infant TGFA genotype, and place and time of birth, this had little impact on the estimate (OR = 1.40, 95 percent CI 0.99–2.00). Excluding the 27 cases with affected relatives slightly reduced all estimates of the risk associated with smoking.

A statistically nonsignificant dose-response pattern was seen both for the adjusted and the nonadjusted results (except in the small high exposure group of ≥20 cigarettes/day). No association between smoking and isolated cleft palate (CP) was observed. Table 4 shows that the rare TGFA allele reduced the risk estimate for smoking for CL(P), although not statistically significantly.

**DISCUSSION**

This study revealed no evidence for an association between the rare TGFA allele and CLP. The TGFA genotype distribution was practically identical among CL(P) cases, CP cases, and nonmalformed children. The discrepancy between this study and the previous reports of associations between TGFA and both CL(P) and CP could be due to different causal mechanisms in different settings. Another possibility is that the present study had less potential for confounding by ethnic differences between cases and controls. Genetic association studies can be very difficult to interpret if conducted in an ethnically heterogeneous setting (22). Denmark is ethnically very homogeneous compared with most countries and restricting our study to parents who speak Danish fluently further reduced the risk of ethnic confounding.

An important finding in the present study is that large differences in the occurrence of the rare TGFA allele occur among Caucasians. In our Danish population...
TABLE 3. Odds ratios (OR) and 95% confidence intervals (CI) by the logistic regression model for the association of smoking with oral cleft occurrence, Denmark, 1991–1994*

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Cleft lip ± cleft palate</th>
<th>Isolated cleft palate</th>
<th>No. of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td><strong>Interview</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking unadjusted (cigarettes per day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>107</td>
<td>1.00</td>
<td>0.70–1.68</td>
</tr>
<tr>
<td>1–9</td>
<td>36</td>
<td>1.08</td>
<td>0.77–2.04</td>
</tr>
<tr>
<td>10–19</td>
<td>63</td>
<td>1.56</td>
<td>1.06–2.94</td>
</tr>
<tr>
<td>≥20</td>
<td>12</td>
<td>1.46</td>
<td>0.70–3.02</td>
</tr>
<tr>
<td>Smoking adjusted† (cigarettes per day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>91</td>
<td>1.00</td>
<td>0.73–2.36</td>
</tr>
<tr>
<td>1–9</td>
<td>32</td>
<td>1.25</td>
<td>1.06–2.94</td>
</tr>
<tr>
<td>10–19</td>
<td>53</td>
<td>1.62</td>
<td>0.70–3.02</td>
</tr>
<tr>
<td>≥20</td>
<td>7</td>
<td>0.93</td>
<td>0.70–1.68</td>
</tr>
<tr>
<td><strong>Birth record</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking unadjusted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>107</td>
<td>1.00</td>
<td>0.70–1.68</td>
</tr>
<tr>
<td>Yes</td>
<td>111</td>
<td>1.35</td>
<td>0.99–1.86</td>
</tr>
<tr>
<td>Smoking adjusted†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>91</td>
<td>1.00</td>
<td>0.70–2.00</td>
</tr>
<tr>
<td>Yes</td>
<td>92</td>
<td>1.40</td>
<td>0.99–2.00</td>
</tr>
</tbody>
</table>

* Maternal epilepsy cases are excluded. The numbers do not total the overall number of subjects because of missing information.
† Adjusted for maternal alcohol and vitamin consumption, infant TGFA-genotype, and place and time of birth.

TABLE 4. Odds ratios (OR) and 95% confidence intervals (CI) for infant transforming growth factor alpha (TGFA) genotype, maternal smoking, and oral clefts, Denmark, 1991–1994

<table>
<thead>
<tr>
<th>Smoking status*</th>
<th>TGFA genotype</th>
<th>No. of controls</th>
<th>No. of cases</th>
<th>Crude OR</th>
<th>Adjusted† OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cleft lip ± cleft palate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>TGFA 1</td>
<td>193</td>
<td>67</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGFA 2†</td>
<td>66</td>
<td>27</td>
<td>1.18</td>
<td>1.20</td>
<td>0.70–2.08</td>
</tr>
<tr>
<td>Smoker</td>
<td>TGFA 1</td>
<td>139</td>
<td>75</td>
<td>1.55</td>
<td>1.64</td>
<td>1.09–2.46</td>
</tr>
<tr>
<td></td>
<td>TGFA 2</td>
<td>46</td>
<td>19</td>
<td>1.19</td>
<td>1.03</td>
<td>0.54–1.94</td>
</tr>
<tr>
<td><strong>Isolated cleft palate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>TGFA 1</td>
<td>193</td>
<td>29</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGFA 2</td>
<td>66</td>
<td>11</td>
<td>1.11</td>
<td>1.19</td>
<td>0.55–2.57</td>
</tr>
<tr>
<td>Smoker</td>
<td>TGFA 1</td>
<td>139</td>
<td>19</td>
<td>0.91</td>
<td>0.98</td>
<td>0.52–1.86</td>
</tr>
<tr>
<td></td>
<td>TGFA 2</td>
<td>46</td>
<td>5</td>
<td>0.72</td>
<td>0.72</td>
<td>0.26–2.00</td>
</tr>
</tbody>
</table>

* Smoking information was obtained through interview.
† Adjusted for maternal alcohol and vitamin consumption as well as place and time of birth. The adjustment reduced the sample size with 5 for the cleft lip ± cleft palate group, 1 for the isolated cleft palate group, and 14 for the control group due to missing information.
‡ Infants with either one or two of the TGFA 2 allele were considered TGFA 2.

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We found that first trimester maternal smoking is a risk factor for CL(P). The intriguing finding of a strong gene-environment interaction between the rare TGFA allele and maternal smoking in the etiology of CLP reported by Hwang et al. (20) and Shaw et al. (21) could not be corroborated in our study or in another recent study from Maryland (30) with 149 white non-syndromic cases and 86 controls. However, the Maryland study had, as noted by the authors, very limited power to detect an interaction as strong as the one reported by Hwang et al. At present, the evidence for a TGFA-smoking interaction in CLP etiology is still rather limited: the Hwang et al. study found the interaction in CP cases, while Shaw et al. found it in CL(P) cases—so it is possible that these findings represent type I errors. Hopefully, additional studies will be able to investigate this possibility.

Our study does not support the notion that the smoking-CL(P) association is due to a tendency for cases to overreport smoking (i.e., recall bias): 1) the association was seen only for CL(P) and not for CP cases; 2) birth record smoking information—which mainly relied on smoking data collected before the outcome of the pregnancy was known—revealed almost similar risk estimates; 3) comparison of smoking information from birth record and interview suggested that the interview smoking information was valid and, if anything, cases tended to underreport smoking.

The study had a very high participation rate among both cases (96 percent) and controls (94 percent). The reason for this high participation rate among controls was probably that the control mothers were in the same ward as the case mother, most often at the same time. Knowing the case mother probably increased the motivation for participation among the control mothers. The study also had drawbacks. The design made it impossible to blind the interviewers toward the case/control status of a participant. However, the interview instrument was highly structured and throughout the 3-year period the interviewers were closely monitored. Smoking information was only available for the mother, but paternal smoking was not found to be associated with CLP in the large Californian study by Shaw et al. (21). The most important limitation in our study was that little information was available for the rarer cleft type (CP) and its possible subtypes (31, 32) due to small CP sample size.

Our study provides additional evidence for the importance of smoking in the etiology of CL(P), but it suggests no role for TGFA gene variants and TGFA-smoking interaction. However, at present only a very limited number of CLP candidate genes and putative environmental risk factors have been studied simulta-}

neously. It seems likely that future progress within the understanding of the etiologic basis for CLP will come from such studies of gene-environment interaction.

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