Assessing the impact of nicotine dependence genes on the risk of facial clefts: An example of the use of national registry and biobank data

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ABSTRACT

Background: Maternal smoking during pregnancy has consistently been associated with risk of facial clefts in offspring, although these studies cannot establish causation. The association between maternal smoking and clefting risk may be caused by genes that influence nicotine dependence and other risk behaviors. Gamma-aminobutyric acid B receptor 2 (*GABBR2*), dopa decarboxylase (*DDC*), and cholinergic receptor nicotinic alpha 4 (*CHRNA4*) are three examples of genes that have previously shown strong associations with nicotine dependence.

Methods: We used a population-based sample of 377 case-parent triads of cleft lip with or without cleft palate (CL/P) and 762 control-parent triads from Norway (1996-2001) to investigate whether variants in *GABBR2, DDC* and *CHRNA4* are associated with maternal first-trimester smoking and with clefting risk. We used HAPLIN (Gjessing et al. 2006), a statistical software tailored for family-based association tests, to perform haplotype-based analyses of 12 SNPs in these genes (rs10985765, rs1435252, rs3780422, rs2779562, and rs3750344 in *GABBR2*; rs2060762, rs3757472, rs1451371, rs3735273, and rs921451 in *DDC*; rs4522666 and rs1044393 in *CHRNA4*).

Results: When analyzed one at a time, there was little evidence of association between any of the 12 SNPs and maternal first-trimester smoking. In haplotype analyses, however, one copy of the maternal G-G-c-G-c haplotype in *DDC* (SNP order as above) was linked with smoking prevalence (odds ratio=1.5; 5% confidence interval: 1.0-2.1). This same haplotype also increased the risk of isolated CL/P in offspring by 1.5-fold with one copy and 2.4-fold with two copies (P_{trend}=0.06). No statistically significant associations were detected with *GABBR2* and *CHRNA4*.

Conclusions: Despite strong associations previously reported between nicotine dependence and variants in *GABBR2*, *DDC* and *CHRNA4*, these genes were poor predictors of maternal first-trimester smoking in our data. The direct association of the *DDC* haplotype with CL/P suggests that this haplotype may either have direct effects on clefts or it may influence clefting risks through other yet unexplored risk behavior(s).

INTRODUCTION

Maternal smoking during the first trimester of pregnancy has consistently been linked to an increased risk of facial clefts in offspring (1-3). Several mechanisms have been proposed to explain the detrimental effects of cigarette smoking on pregnancy outcomes. A reduced capacity of the mother or the fetus to detoxify teratogenic compounds from tobacco smoke is one plausible mechanism, and a number of genes involved in detoxification pathways have already been examined for their potential roles in facial clefting (4,5). Another mechanism is through hypoxia, whereby carbon monoxide from tobacco smoke binds to embryonic and maternal hemoglobin, reducing the amount of oxygen available to the embryo. Both human (6-10) and animal studies (11-13) support a role for hypoxia in clefting of the lip and palate. A third hypothesis is that the association between maternal first-trimester smoking and clefting risk is the result of genes that influence nicotine dependence and other risk behaviors.

Genetic variants in *GABBR2*, *DDC*, and *CHRNA4* have previously shown strong associations with nicotine dependence (14-16). In family-based haplotype analysis of *GABBR2*, there was a highly significant (P = 0.0003) inverse association of the C-A-C-A haplotype (composed of SNPs rs1435252-rs3780422-rs2779562-rs3750344) with the heaviness-of-smoking index (HSI) of nicotine dependence in a European-American sample (14). Another haplotype composed of the same four SNPs showed a significant association with the following three adjusted nicotine-dependence measures: smoking quantity, HSI, and Fagerström test for nicotine dependence score (FTND). In another study by the same group (16), a high-risk haplotype of *DDC* (composed of SNPs rs921451-rs3735273-rs1451371-

rs3757472) showed a strong association (P = 0.005) with smoking quantity and nicotine dependence in a European-American sample. As to *CHRNA4*, analysis of six SNPs in a European-American sample showed that rs2273504 was associated with smoking quantity and rs1044396 was associated with nicotine dependence (15).

Following these initial reports, several other studies have found similar associations between these genes and nicotine dependence [with *GABBR2* in (17,18), *DDC* in (19,20), and *CHRNA4* in (21-25)], with a few exceptions [for example with *CHRNA4* in (26)]. Most of the reported associations are not with the same SNPs or haplotypes identified in the original reports (14-16), preventing a formal cross-study comparison.

An association between genetic variants in *GABBR2*, *DDC*, and *CHRNA4* and smoking is biologically plausible given the functions of these genes. GABA is a key neurotransmitter in the mammalian brain, and both human and animal studies implicate GABA receptors in drug dependence (18,27,28). *DDC* is involved in the synthesis of two other key neurotransmitters in the brain: dopamine and serotonin. Nicotine stimulates local energy metabolism and triggers dopamine release in the shell compartment of the *nucleus accumbens*—the key structure of the brain responsible for reward, motivation and dependence (29). *CHRNA4* influences nicotine dependence through neuronal nicotinic acetylcholine receptors, and activation of alpha-4 acetylcholine receptors is sufficient for nicotine-induced reward, tolerance and sensitization (30). *CHRNA4* encodes an integral membrane receptor subunit which interacts with nicotinic acetylcholine receptors β-2 or β-4 to form a functional receptor. The α-4 and β-2 subunits comprise the majority of the high-affinity binding sites in the brain, and nicotine exerts its effects by binding to these receptors (31). Absence of such high-affinity binding sites in *Chrna4*$^{-/-}$ mice results in greatly subdued nicotine dependence (32).

To investigate whether the above genes and SNPs are associated with maternal first-trimester smoking and with clefting risk, we used a population-based study of facial clefts from Norway (1996-2001), in which detailed information on maternal first-trimester smoking and genotypes on 377 case-parent triads of cleft lip with or without cleft palate (CL/P) and 762 control-parent triads were available for analysis.

METHODS

Study population

Data for this study derive from a nationwide case-control study of babies born with facial clefts in Norway in the period 1996-2001. Mothers of babies with clefts were invited to participate in the study through the two surgical clinics that treat all clefts in Norway. The participation rate was 88% in the case group. Overall, 377 cases with CL/P and 196 cases with cleft palate (CP) were recruited (N = 573). During the same years, controls were randomly selected from all live births recorded in the Norwegian Medical Birth Registry. Of 1006 eligible control-mothers, 76% (N = 763) agreed to participate. Further details on study design and participants have been provided elsewhere (33,34).

Questionnaires and biological specimens

After the consent form was returned (approximately one month after the baby's birth), mothers received a self-administered questionnaire for the assessment of a spectrum of environmental exposures, demographic characteristics, reproductive history and maternal health. To assess active maternal smoking, women were asked if they had smoked during the first trimester of pregnancy, and if yes, to state the average number of cigarettes smoked per day. Details are provided in (35) and the questionnaire is available in its entirety at http://www.niehs.nih.gov/research/atniehs/labs/epi/studies/ncl/question.cfm.

The median time from the infant's delivery to the mother's completion of the questionnaire was 14 weeks for cases and 15 weeks for controls (36). After returning the questionnaire, control-mothers were asked to provide cheek swabs from themselves, the proband, and the father and siblings of the proband. Mothers who agreed to provide swabs were mailed a kit containing sterile cotton-tipped sticks and alcohol-containing plastic tubes. As control-fathers were asked to provide buccal swab samples starting in November 1998, a subset of the control families initially recruited consists of mother-child dyads only (N = 347), which leaves 416 control-parent triads for the current analysis. Case-parents and babies donated blood at the treatment centers.

Approval for this study was obtained from the Norwegian Data Inspectorate, the Regional Committee on Research Ethics for Western Norway, and the respective institutional review boards of the US National Institute of Environmental Health Sciences (NIEHS) and the University of Iowa. Clinicopathological information and biologic specimens for DNA extraction were obtained with the informed consent of the mothers and fathers, and all aspects of this research are in compliance with the tenets of the *Declaration of Helsinki* for human research.

Genotyping

Genotyping was carried out by the TaqMan® assay and endpoint reactions were analyzed using the proprietary SDS software from Applied Biosystems (Foster City, California). Various characteristics of the 12 SNPs in *GABBR2*, *DDC* and *CHRNA4* are summarized in Table 1. These SNPs were selected on the basis of their statistical associations with nicotine dependence in the original reports (14-16), a preference for being located within coding or regulatory regions of the gene, and for having a minor allele frequency above 5%.

Pairwise linkage disequilibrium (LD) plot

Pairwise LD plots were created using the HAPLOVIEW software (37), version 4.1. This software calcu-

Table 1. Characteristics of *GABBR2*, *DDC* and *CHRNA4* SNPs in 416 population-based control-parent triads from Norway (1996-2001).

Gene	Chromosome	SNP Name[a]	Type and genetic location[b]	Alleles[c]	Amino acid change	HWE *P* Value	Maf	Overall likelihood ratio *P* Value[d]
GABBR2	9q22.1-q22.3	rs10985765	Non-synonymous (Exon 18)	c,T	Ala[A] to Thr[T]	0.45	19.6	0.26
		rs1435252	Intron 13	t,C	–	0.77	30.4	0.63
		rs3780422	Intron 11	a,G	–	0.64	44.0	0.46
		rs2779562	Intron 3	c,T	–	0.62	44.0	0.60
		rs3750344	Synonymous (Exon 2)	g,A	Ala[A] to Ala[A]	0.58	18.9	0.85
DDC	7p11	rs2060762	Intron 14	a,G	–	0.60	15.4	0.69
		rs3757472	Intron 11	t,G	–	0.18	29.8	0.29
		rs1451371	Intron 9	c,T	–	0.71	44.8	0.85
		rs3735273	Intron 5	a,G	–	0.13	22.9	0.05
		rs921451	Intron 1	c,T	–	0.24	40.9	0.57
CHRNA4	20q13.2-q13.3	rs4522666	3' of *CHRNA4*	g,A	–	0.35	35.6	0.53
		rs1044393	Synonymous (Exon 5)	t,C	Asp[D] to Asp[D]	0.21	11.4	0.63

[a] SNPs in bold are the same as those in the original reports (14-16).
[b] According to dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP).
[c] Uppercase denotes the major allele in the Norwegian control-parent triads.
[d] This *P* value represents an overall test for deviation from Mendelian transmission among the 416 control-parent triads.

Abbreviations: *GABBR2*, gamma-aminobutyric acid B receptor 2; *DDC*, dopa decarboxylase; *CHRNA4*, cholinergic receptor nicotinic alpha 4; SNP, single-nucleotide polymorphism; HWE, Hardy-Weinberg equilibrium; Maf, minor allele frequency.

lates allele frequencies for each SNP, tests for deviations from Hardy-Weinberg equilibrium (HWE), and screens for Mendelian inconsistencies within the offspring-parent triads. Genotype data for the 416 control-parent triads were fed into HAPLOVIEW and the marker positions entered as specified (detailed instructions can be found at http://www.broadinstitute.org/haploview). In the LD plots, the *D'* statistic is used to quantify LD. This is the ratio of *D* to its maximum possible absolute value given the allele frequencies (38). The interpretation is that *D'* = 1 if two SNPs have not been separated by recombination or recurrent mutation during the history of the sample; they are thus perfectly correlated and predictive of each other. In contrast, *D'* = 0 if two SNPs are in complete linkage *equilibrium* such that the frequency of a given haplotype is the simple product of the individual allele frequencies at the two loci.

Statistical analyses

Data analysis was divided into three stages. In the first, we performed single-marker analyses to look for association between each of the 12 SNPs and maternal first-trimester smoking. A standard chi-squared test (2 degrees of freedom) was used to compare the prevalence of maternal first-trimester smoking by genotype at each SNP (Table 2). In addition, we used a one-way ANOVA test to compare the average number of cigarettes smoked per day during the first trimester by maternal genotype. These comparisons were first done on control-mothers, since these represent a randomly selected sample from the total population of pregnant women, and then repeated in the pooled sample of all mothers to verify whether the results differed by the addition of case-mothers.

In the second stage of analysis, we focused on haplotypes of *GABBR2*, *DDC* and *CHRNA4* and used the HAPLIN statistical software package[(39); available at http://www.uib.no/smis/gjessing/genetics/software/haplin] to reconstruct haplotypes from complete mother-father-offspring triads. Whenever haplotypes could not be uniquely identified, HAPLIN used the expectation-maximization (EM) algorithm to determine the probability distribution of haplotypes within each offspring-parent triad. The predicted haplotypes were then used to test for association with maternal first-trimester smoking. In all analyses, the most frequent haplotype was used as reference. We used logistic regression to study the prevalence of smoking and Gaussian linear regression to study the number of cigarettes smoked in the smoking group (Tables 3-5). Both sets of analyses used probability weights for the predicted probabilities of different maternal haplotypes when a unique haplotype could not be determined from a given offspring-parent triad.

In the final stage of analysis, we performed logistic regression to look for associations between maternal haplotypes of each gene and isolated CL/P (Table 6). As before, the haplotypes were analyzed using probability weights. All statistical analyses were done using the STATA software version 9 (Stata Corporation, College Station, Texas) and the *R* statistical package (40).

RESULTS

SNP selection and linkage disequilibrium (LD)

To enable cross-study comparisons, we sought to evaluate the same SNPs in *GABBR2*, *DDC* and *CHRNA4* that produced the strongest associations with nicotine dependence in the original reports (14-16). For *GABBR2* and *DDC*, we were able to genotype the same combination of SNPs that gave rise to the high-risk haplotypes in their European-American samples; namely rs1435252-rs3780422-rs2779562-rs3750344 for *GABBR2* and rs3757472-rs1451371-rs3735273-rs921451 for *DDC* (highlighted in bold in Table 1). In contrast, both *CHRNA4* SNPs in Li *et al.* (2005) failed assay-design and were replaced with rs4522666 and

Table 2. Proportion of smoking mothers and average number of cigarettes smoked per day stratified by maternal genotype at each of the SNPs in *GABBR2*, *DDC* and *CHRNA4*.

			Control-mothers only (N = 763)[a]				Combined case- and control-mothers (N = 1336)[b]			
Gene	SNP	Genotype (%)	% smokers[c]	P-diff.[d]	Mean no. of cigarettes[e]	P-diff.[f]	% smokers[c]	P-diff.[d]	Mean no. of cigarettes[e]	P-diff.[f]
GABBR2	rs10985765	CC (3)	42		6.4		36		6.0	
		CT (28)	34	0.48	6.3	0.96	41	0.15	6.5	0.93
		TT (69)	31		6.1		35		6.4	
	rs1435252	TT (8)	31		5.1		31		6.1	
		TC (42)	34	0.56	4.9	0.63	38	0.31	6.9	0.27
		CC (50)	30		6.1		35		6.1	
	rs3780422	AA (20)	31		6.2		37		7.1	
		AG (50)	32	0.99	6.5	0.27	35	0.75	6.5	0.10
		GG (30)	32		5.3		38		5.7	
	rs2779562	CC (24)	32		6.8		34		6.9	
		CT (49)	31	0.85	5.8	0.43	36	0.38	5.8	0.09
		TT (27)	33		5.9		39		6.8	
	rs3750344	GG (3)	35		2.4		32		8.6	
		GA (32)	31	0.89	5.4	0.79	35	0.63	6.3	0.29
		AA (65)	32		4.9		37		6.4	
DDC	rs2060762	AA (3)	30		6.7		23		7.3	
		AG (28)	30	0.96	7.0	0.40	37	0.19	7.3	0.06
		GG (69)	31		5.9		36		6.1	
	rs3757472	TT (9)	33		5.8		34		6.7	
		TG (40)	31	0.94	7.2	0.08	37	0.90	6.9	0.20
		GG (51)	31		5.6		36		6.0	
	rs1451371	CC (21)	32		5.5		39		6.1	
		CT (50)	31	1.0	6.6	0.42	35	0.52	6.6	0.72
		TT (29)	32		6.0		36		6.3	
	rs3735273	AA (7)	35		5.2		34		6.8	
		AG (34)	29	0.62	6.9	0.36	35	0.65	6.6	0.70
		GG (59)	32		6.1		37		6.3	
	rs921451	CC (17)	31		7.4		35		7.3	
		CT (46)	32	0.97	5.8	0.27	37	0.81	6.1	0.23
		TT (37)	31		6.2		35		6.3	
CHRNA4	rs4522666	GG (12)	34		6.4		33		5.8	
		GA (48)	31	0.86	5.4	0.08	37	0.47	6.3	0.56
		AA (40)	31		7.1		35		6.6	
	rs1044393	TT (1)	42		8.8		40		8.2	
		TC (23)	32	0.74	5.8	0.42	35	0.45	6.4	0.70
		CC (76)	32		6.2		36		6.4	

[a] Control-mothers were randomly selected among all women who gave birth in Norway in the period 1996-2001.
[b] Pooled sample of control-mothers and mothers of babies born with facial cleft in Norway (1996-2001).
[c] Smoking was defined as reporting having smoked one cigarette per day or more in the first trimester of pregnancy.
[d] Chi-squared test (2 degrees of freedom) for difference in the proportion of smoking mothers.
[e] Average number of cigarettes smoked among smokers.
[f] One-way ANOVA test for difference in mean number of cigarettes smoked.

Abbreviations: *GABBR2*, gamma-aminobutyric acid B receptor 2; *DDC*, dopa decarboxylase; *CHRNA4*, cholinergic receptor nicotinic alpha 4; SNP, single-nucleotide polymorphism.

rs1044393. rs4522666 was selected for its location in the 3' end of the gene, for the detection of association signals from putative regulatory elements in that region; rs1044393 in exon 5 was chosen to tag useful LD with other alleles in the immediate intragenic regions of *CHRNA4*.

Since analyses based on offspring-parent triads can be vulnerable to deviations from Mendelian transmission, we searched for evidence of such deviations among the 416 control-parent triads. With the possible exception of rs3735273 in *DDC* (P = 0.053), none of the SNPs showed any evidence of deviation from Mendelian transmission. Furthermore, no deviations from HWE were noted among the genotyped SNPs (Table 1).

The LD plots in Figure 1 show a low degree of LD between the *GABBR2* SNPs; the highest *D'* value was 0.53. The lack of LD is even more pronounced between the two *CHRNA4* SNPs (*D'*=0.07). They are thus more useful in the association tests because they do not contribute redundant genetic information. In contrast, the *DDC* SNPs are more strongly correlated, with LD between rs1451371 and rs921451 very close to that reported earlier in a European-American sample (19).

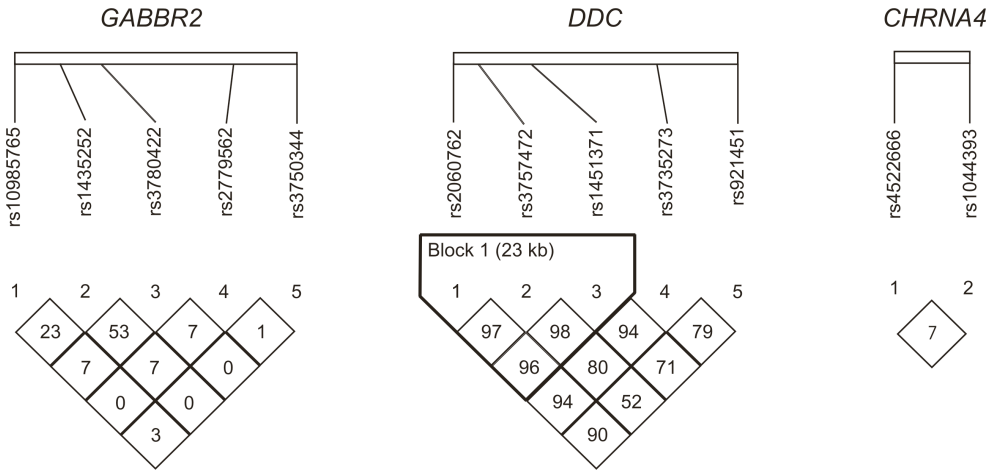

Figure 1. ***Linkage disequilibrium (LD) between SNPs in GABBR2, DDC and CHRNA4.*** The pairwise LD plot was created by the HAPLOVIEW software (see text for details) using the population-based control-parent triads for generating *D'* values. The *D'* value for a given pair of SNPs is indicated by the number (expressed as a percentage) inside the corresponding diamond.

Table 3. Maternal smoking by *GABBR2* haplotypes among 1022[a] case- and control-mothers.

			OR of smoking[c]			No. of cigarettes smoked		
Haplotype[b]	Haplotype frequency (%)	One or two copies	OR	95% CI	*P* value[e]	Coefficient[d]	95% CI	*P* value[e]
C-a-c	14	One	0.9	0.6-1.2	0.58	0.6	−0.7-1.9	0.32
		Two	1.3	0.5-3.4		3.0	−1.3-7.2	
t-a-c	12	One	0.8	0.6-1.1	0.41	1.1	−0.4-2.5	0.36
		Two	0.8	0.3-2.3		0.3	−2.9-3.5	
C-G-c	23	One	0.8	0.6-1.1	0.33	−0.3	−1.6-0.9	0.39
		Two	0.8	0.4-1.4		−1.5	−3.6-0.7	
C-a-T	11	One	1.0	0.7-1.4	0.64	0.3	−1.1-1.8	0.76
		Two	0.5	0.2-1.9		0.9	−1.7-3.6	
t-a-T	12	One	1.1	0.8-1.5	0.03	0.5	−0.9-1.9	0.33
		Two	0.1	0.0-0.6		−1.6	−4.5-1.2	
C-G-T	28	One						
		Two	Ref.	–	–	Ref.	–	–

[a] Haplotypes could be predicted for a total of 1022 mothers from the offspring-parent triads using HAPLIN.
[b] Haplotypes of rs1435252-rs3780422-rs2779562.
[c] Odds ratio of maternal smoking during the first trimester of pregnancy estimated by frequency-weighted logistic regression, using the probability distribution of possible haplotypes for each mother as predicted by HAPLIN from the offspring-parent triads.
[d] Regression analysis of number of cigarettes smoked per day during the first trimester of pregnancy by frequency-weighted linear regression, using the probability distribution of possible haplotypes for each mother as predicted by HAPLIN from the offspring-parent triads.
[e] *P* values for effect of one or two copies of the haplotype.

Abbreviations: *GABBR2*, gamma-aminobutyric acid B receptor 2; OR, odds ratio; CI, confidence interval; ref, reference haplotype.

The *D'* value between rs1451371 and rs921451 is 0.71 in our control-parent triads and the two SNPs do not form part of a common haplotype block. Note, however, that the two SNPs are 70 kilobases apart, which may partly explain the erosion in LD.

GABBR2, DDC, CHRNA4 and maternal first-trimester smoking

In the first stage of analysis, we tested for association between each SNP in *GABBR2, DDC,* and *CHRNA4* and maternal first-trimester smoking. Thirty-two percent of control-mothers and 42% of case-mothers in our sample reported smoking during the first trimester. Among control-mothers, none of the SNPs in *GABBR2*, *DDC* or *CHRNA4* on their own was associated with the prevalence of first-trimester smoking. These results were unaffected by the addition of 573 case-mothers (Table 2). Similarly, there was no statistically significant difference in the mean number of cigarettes smoked in the first trimester of pregnancy across strata of maternal genotype, again when assessing one SNP at a time.

In the next stage of analysis, we focused on haplotypes in *GABBR2*, *DDC* and *CHRNA4* (Table 3-5) as opposed to the single-marker analyses above. Mothers homozygous for the t-a-T haplotype in *GABBR2* had a lower prevalence of smoking compared with mothers carrying the reference haplotype C-G-T (OR=0.1; 95%

Table 4. Maternal smoking by *DDC* haplotypes among 1177[a] case- and control-mothers.

Haplotype[b]	Haplotype frequency (%)	One or two copies	OR of smoking[c] OR	95% CI	*P* value[e]	No. of cigarettes smoked Coefficient	95% CI	*P* value[e]
a-t-T-a-c	15	One	1.1	0.9-1.5	0.48	0.5	−0.8-1.7	0.74
		Two	0.8	0.4-1.6		0.1	−3.3-3.5	
G-t-T-a-c	4	One	0.7	0.5-1.1	<0.001	0.0	−1.4-1.4	0.90
		Two	29.2	5.8-147.5		0.8	−2.7-4.3	
G-G-c-G-c	7	One	1.5	1.0-2.1	0.13	−0.9	−2.4-0.6	<0.001
		Two	1.0	0.1-8.5		2.9	1.6-4.2	
G-G-T-G-c	12	One	1.0	0.8-1.4	0.89	−0.1	−1.4-1.1	0.94
		Two	1.2	0.5-2.8		−0.4	−2.9-2.0	
G-G-T-a-T	4	One	1.1	0.6-2.1	0.90	−2.5	−4.4-−0.7	0.03
		Two	1.0	0.2-5.5		−1.7	−8.6-5.2	
G-G-c-G-T	36	One	Ref.	–	–	Ref.	–	–
		Two						
G-G-T-G-T	13	One	1.1	0.8-1.5	0.41	−0.8	−2.0-0.4	0.38
		Two	0.5	0.2-1.7		0.5	−5.4-6.4	
G-t-T-G-T	7	One	1.2	0.8-1.7	0.59	−0.2	−1.5-1.1	0.06
		Two	1.2	0.2-6.2		−2.8	−5.0-−0.5	

[a] Haplotypes could be predicted for 1177 mothers from the offspring-parent triads using HAPLIN.
[b] Haplotypes of rs2060762-rs3757472-rs1451371-rs3735273-rs921451.
[c] Odds ratio of maternal smoking during the first trimester of pregnancy estimated by frequency-weighted logistic regression, using the probability distribution of possible haplotypes for each mother as predicted by HAPLIN from the offspring-parent triads.
[d] Regression analysis of number of cigarettes smoked per day during first trimester of pregnancy by frequency-weighted linear regression, using the probability distribution of possible haplotypes for each mother as predicted by HAPLIN from the offspring-parent triads.
[e] *P*-values for effect of one or two copies of the haplotype.

Abbreviations: *DDC*, dopa decarboxylase; OR, odds ratio; CI, confidence interval; ref, reference haplotype.

Table 5. Maternal smoking by *CHRNA4* haplotypes among 1309[a] case- and control-mothers.

Haplotype[b]	Haplotype frequency (%)	One or two copies	OR of smoking[c] OR	95% CI	*P* value[e]	No. of cigarettes smoked Coefficient[d]	95% CI	*P* value[e]
A-C	53	One	Ref.	–	–	Ref.	–	–
		Two						
g-C	31	One	1.2	1.0-1.5	0.14	−0.5	−1.5-0.4	0.53
		Two	0.9	0.6-1.3		−0.1	−1.6-1.5	
A-t	8	One	1.2	0.9-1.6	0.02	0.5	−0.8-1.9	0.01
		Two	7.9	1.6-38.4		−2.4	−4.2-−0.6	
g-t	7	One	1.0	0.7-1.5	0.83	−0.1	−1.6-1.3	<0.001
		Two	0.5	0.1-4.8		−4.7	−6.5-−2.9	

[a] Haplotypes could be predicted for 1309 mothers from offspring-parent triads using HAPLIN.
[b] Haplotypes of rs4522666-rs1044393.
[c] Odds ratio of maternal smoking during the first trimester of pregnancy estimated by frequency-weighted logistic regression, using the probability distribution of possible haplotypes for each mother as predicted by HAPLIN from the offspring-parent triads.
[d] Regression analysis of number of cigarettes smoked per day during first trimester of pregnancy by frequency-weighted linear regression, using the probability distribution of possible haplotypes for each mother as predicted by HAPLIN from the offspring-parent triads.
[e] *P* values for effect of one or two copies of the haplotype.

Abbreviations: *CHRNA4*, cholinergic receptor nicotinic alpha 4; OR, odds ratio; CI, confidence interval; ref, reference haplotype.

CI: 0.0-0.6; Table 3). These mothers also smoked on average 1.6 fewer cigarettes per day compared with other mothers, although this difference was not statistically significant (95% CI: −4.5-1.2).

Haplotype G-G-c-G-c in *DDC* was associated with a higher odds ratio of smoking among heterozygous mothers (OR=1.5; 95% CI: 1.0-2.1), but with no increase in the number of cigarettes smoked (Table 4). In contrast, homozygotes for G-G-c-G-c smoked on average 2.9 more cigarettes per day compared with other mothers (95% CI: 1.6-4.2), but with no evidence of an association with smoking prevalence. Other associations were less consistent (Table 5).

Table 6. Odds ratio[a] of isolated cleft lip with or without cleft palate (CL/P) by maternal haplotypes and maternal smoking (165 cases vs. 615 controls).

			Without adjustment for smoking		With adjustment for smoking[c]	
	Haplotype[b]	One or two copies	OR**	95% CI	OR**	95% CI
GABBR2	t-a-T	One	1.0	0.7-1.6	1.0	0.7-1.6
		Two	0.3	0.0-2.2	0.4	0.1-2.6
Smoking					1.8***	1.2-2.5
DDC	G-G-c-G-c	One	1.5	1.0-2.5	1.5	0.9-2.4
		Two	2.4	0.2-28.0	2.3	0.3-20.5
Smoking					1.6***	1.2-2.5
CHRNA4	g-C	One	1.2	0.9-1.6	1.2	0.9-1.6
		Two	1.2	0.7-1.9	1.2	0.7-2.0
	A-t	One	1.1	0.8-1.7	1.1	0.8-1.6
		Two	0.2	0.0-1.3	0.2	0.0-1.1
Smoking					1.6***	1.2-2.2

[a] Odds ratio of maternal smoking during the first trimester of pregnancy estimated by frequency-weighted logistic regression, using the probability distribution of possible haplotypes for each mother as predicted by HAPLIN from the offspring-parent triads.

[b] Haplotypes of rs1435252-rs3780422-rs2779562 for *GABBR2*; rs2060762-rs3757472-rs1451371-rs3735273-rs921451 for *DDC*; and of rs4522666-rs1044393 for *CHRNA4*.

[c] With maternal smoking during the first trimester of pregnancy included as an adjustment variable in the analysis.

** Using the most frequent haplotype for each gene as reference.

*** Effect of smoking *vs.* no-smoking.

Abbreviations: *GABBR2*, gamma-aminobutyric acid B receptor 2; *DDC*, dopa decarboxylase; *CHRNA4*, cholinergic receptor nicotinic alpha 4; OR, odds ratio; CI, confidence interval; ref, reference haplotype.

GABBR2, DDC, CHRNA4 and isolated CL/P

In the final stage of analysis, we assessed whether haplotypes of the three nicotine-dependence genes were associated with risk of facial clefts and found an increased risk of isolated CL/P among mothers who were heterozygous for the G-G-c-G-c haplotype of *DDC* (OR=1.5; 95% CI: 1.0-2.5; Table 6). Interestingly, the odds ratio was unaffected by adjustment for smoking (OR=1.5; 95% CI: 0.9-2.4).

DISCUSSION

The specific SNPs evaluated in our analyses had non-significant effects on maternal first-trimester smoking when assessed one at a time, despite prior evidence showing their strong associations with nicotine dependence. The lack of a robust association with smoking for these variants when assessed individually may be due to our limited sample size, although this study represents one of the largest collections of cleft triads to date. It may also be related to the fact that we are studying smoking *during pregnancy*, which may be less related to nicotine dependence than smoking behaviors during other periods. Many women either quit smoking during pregnancy or reduce their smoking intensity, both of which significantly reduce the variation in smoking. Therefore, the general utility of a gene in predicting smoking behavior may be more apparent during other periods. Furthermore, given the potential genetic and allelic heterogeneity inherent to complex behaviors such as smoking, and the correlation of SNPs within the same gene, analyses focusing on one SNP at a time may not show their full effects on smoking especially if those SNPs are confounders for each other. Additional studies might be useful to evaluate the simultaneous effects of these SNPs and potential SNP interdependencies.

In addition to the points noted above, the lack of replication in our data compared with the literature has several possible interpretations. It may be due to differences in the specific measures and quality of the smoking data, or due to variations in LD patterns. In Ma *et al.* (2005), nicotine dependence was assessed by smoking quantity (the number of cigarettes smoked per day), heaviness-of-smoking index (HSI), and Fagerström test for nicotine dependence score (FTND). The smoking measure closest to the one in our study is smoking quantity, where mothers reported the average number of cigarettes smoked per day (or per month, if less than one per day) during the first trimester of pregnancy. In Ma *et al.* (2005), rs921451 was significantly associated with smoking quantity and HSI under various genetic models in their European-American sample, and the maternal haplotype G-T-G-T was positively associated with all three adjusted smoking measures.

In haplotype analyses, G-G-c-G-c in *DDC* was associated with an increased prevalence of smoking. Compared with the reference G-G-c-G-T haplotype, the only difference is the allele of the last SNP (rs921451 T>c). This is noteworthy because, among all the *DDC* SNPs tested by Ma *et al.* (2005), rs921451 was the only one that was significantly associated with nicotine dependence in their European-American sample. Unfortunately, this haplotype was too uncommon (7%) in our sample to be particularly useful. Four of the *DDC* SNPs in our study (rs3757472, rs1451371, rs3735273 and rs921451) were the same as those in Ma *et al.* (2005), and although the maternal G-T-G-T haplotype (SNP order as above) was significantly

associated with nicotine dependence in their paper, we found no such evidence in our sample.

It is likely that different risk haplotypes influence the prevalence of smoking in different study populations. Although our use of haplotypes may have improved the power for detecting an association, inadequate SNP coverage may still be a concern in this study, particularly for *CHRNA4* where only two SNPs were successfully genotyped. Our analyses are reliant on LD to tag the real causative variant(s). Depending on the population LD structure, more than one haplotype may proxy the same causative variant(s). Moreover, the same haplotype may appear risk-conferring in one population but protective in another—a phenomenon known as genetic "flip-flop" in the literature (41,42). The reference category in the haplotype analyses, defined here on the basis of only a few genotyped SNPs, may effectively aggregate over a set of corresponding haplotypes. This set may differ across different study populations and the odds ratios would thus be computed relative to different aggregations of reference haplotypes, producing conflicting results.

In our assessments of whether variants in *GABBR2*, *DDC*, and *CHRNA4* are associated with risk of facial clefts, the maternal G-G-c-G-c haplotype in *DDC*, found to be associated with increased smoking prevalence in the first stage of analysis, also appeared to increase the risk of isolated CL/P among heterozygous mothers. Adjustment for maternal first-trimester smoking had no effect on the risk estimates, ruling out the possibility that the association was mediated through maternal smoking. The direct association of the *DDC* haplotype with CL/P suggests that this haplotype may influence clefting risks through other health behaviors.

Our study of *GABBR2*, *DDC*, and *CHRNA4* is based on data from a nationwide study of facial clefts in Norway (1996-2001), providing one of the largest collections of case and control offspring-parent triads to date, as well as extensive information on maternal smoking collected in the first months after the baby's birth. Since the Norwegian healthcare system covers all expenses related to the treatment of clefts at the two surgical departments appointed to treat all cleft patients in the country, we had virtually 100% case ascertainment. Although participation was slightly lower in the control group (76%) compared to the case group (88%), these controls had the advantage of being randomly drawn from the entire population of births. However, as with other studies where exposure data are collected through self-reporting, there is always the risk of recall bias. Retrospective reporting of smoking indeed appeared to be more complete for first-trimester smoking than prospective reporting in a previous study of the same Norwegian study population (35). Although the first trimester is the most relevant period for smoking exposure in studies of birth defects and facial clefts in particular, smoking in this period might not adequately reflect genetically-determined nicotine dependence. Another issue relates to population stratification, which may affect all three variables simultaneously and, therefore, cannot be corrected for in the analyses presented herein. However, the impact of population structure is likely to be small given the relatively small sample size and genetically homogeneous nature of our study population.

Cigarette smoking is one of the most commonly measured exposures in perinatal epidemiology. Therefore, further efforts are needed to identify genes for this complex exposure that are better at targeting the first trimester of pregnancy. Recent insights into lung cancer susceptibility have provided several important candidates, with confirmed associations with the cholinergic receptor, nicotinic, alpha 5 gene (*CHRNA5* on chr 15q24) in multiple, independent studies (43-47). Additional susceptibility loci have been identified through recent genome-wide association studies, including the genes for acetylcholine receptors *CHRNB3* and *CHRNA6* on chr 8p11, egl nine homolog 2 (*EGLN2*) on chr 9q13, brain-derived neurotrophic factor (*BDNF*) on chr 11p13, *CHRNA3* on chr 15q24, and cytochrome P450 genes *CYP2A6* and *CYP2B6* on chr 19q13 (47-49). However, as emphasized by our study, these new genes will need to be tested for their usefulness in specifically targeting maternal first-trimester smoking.

In summary, the SNPs selected in the three nicotine dependence genes, *GABBR2*, *DDC* and *CHRNA4*, did not show a convincing association with maternal first-trimester smoking in our data, except for weak associations with a few haplotypes. Further efforts are needed to evaluate the utility of these genes and those recently proposed (e.g. *CHRNA5*) in studies of smoking during pregnancy.

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