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ORIGINAL INVESTIGATION

The *MSX1* allele 4 homozygous child exposed to smoking at periconception is most sensitive in developing nonsyndromic orofacial clefts

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Abstract Nonsyndromic orofacial clefts (OFC) are common birth defects caused by certain genes interacting with environmental factors. Mutations and association studies indicate that the homeobox gene *MSX1* plays a role in human clefting. In a Dutch case-control triad study (mother, father, and child), we investigated interactions between *MSX1* and the parents' periconceptional lifestyle in relation to the risk of OFC in their offspring. We studied 181 case- and 132 control mothers, 155 case- and 121 control fathers, and 176 case- and 146 control children, in which there were 107 case triads and 66 control triads. Univariable and multivariable logistic regression analyses were applied, and odds ratios (OR), 95% confidence intervals (CI) were calculated. Allele 4 of the CA marker in the *MSX1* gene, consisting of nine CA repeats, was the most common allele found in both the case and control triads. Significant interactions were observed between allele 4 homozygosity of the child with maternal smoking (OR 2.7, 95% CI 1.1–6.6) and with smoking by both parents (OR 4.9, 95% CI 1.4–18.0). Allele 4 homozygosity in the mother and smoking showed a risk estimate of OR 3.2 (95% CI 1.1–9.0). If allele 4 homozygous mothers did not take daily folic acid supplements in the recommended periconceptional period, this also increased the risk of OFC for their offspring (OR 2.8, 95% CI 1.1–6.7). Our findings show that, in the Dutch population, periconceptional smoking by both parents interacts with a specific allelic variant of *MSX1* to significantly increase OFC risk for their offspring. Possible underlying mechanisms are discussed.

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Introduction

Orofacial clefts (OFC) are common birth defects and include nonsyndromic cleft lip (CL) and/or cleft palate (CP). They have a birth prevalence varying from 1 in 1,500 to 1 in 2,500 in Caucasian populations (Schutte and Murray 1999). These figures also depend on ethnic background, geographic origin, lifestyle factors and socioeconomic status (Jugessur and Murray 2005). OFC are most often classified into cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). The etiology of OFC is complex, meaning both genetic and environmental factors are involved (Jugessur and Murray 2005; Krapels et al. 2006a;

Gritli-Linde 2007; Schliekelman and Slatkin 2002). Gene expression studies and a transgenic knock-out model with cleft phenotype have suggested that *Msx1* is involved in the etiology of clefting (Satokata and Maas 1994; Davidson 1995).

Msx1 is a highly conserved homeobox gene that plays several key roles in epithelial–mesenchymal tissue interactions during craniofacial development. It regulates cellular proliferation, differentiation and cell death, which is important for balanced cell growth and morphogenesis (Bendall and Abate-Shen 2000). Extensive studies in knockout mice have also demonstrated that *Msx1-Bmp* signaling, regulating expression of *Shh*, is essential for palate development (Zhang et al. 2002) and the identification of a *MSX1* stop mutation in a Dutch family with a combination of tooth agenesis and OFC confirmed *MSX1* as a candidate gene for clefting in humans (van den Boogaard et al. 2000). Sequencing- and association studies have indicated a role for *MSX1* in the etiology of nonsyndromic orofacial clefting (Lidral et al. 1998; Beaty et al. 2001; Blanco et al. 2004; Fallin et al. 2003; Jezewski et al. 2003; Jugessur et al. 2003; Vieira et al. 2003; Moreno et al. 2004; Vieira et al. 2005; Modesto et al. 2006; Tongkobpetch et al. 2006; Park et al. 2007), although results published by others question this role (Mitchell et al. 2001; Koillinen et al. 2003; Etheredge et al. 2005).

There is increasing evidence that the environment can substantially modulate genetic effects and various lifestyle factors, such as smoking, a folate-deficient diet, alcohol intake and the use of medication, have been associated with OFC (Jugessur and Murray 2005; Krapels et al. 2006a, b). It is important to realize that the mother determines the intrauterine environment in which the fetus develops. Our aim was to investigate any association between *MSX1*-CA markers in the mother, father, and child with periconceptional smoking, alcohol, and medication use by both parents and maternal folic acid supplementation in a Dutch population.

Materials and methods

Study population

The study population and design were described previously (van Rooij et al. 2002). Briefly, between 1998 and 2000, a case-control triad study was conducted by nine of the largest cleft palate teams in the Netherlands (Amsterdam VU, Arnhem, Groningen, Leeuwarden, Nijmegen, Rotterdam, Tilburg, Utrecht, and Zwolle). We recruited children with a nonsyndromic OFC, both parents, and healthy controls. In each hospital team, the OFC was diagnosed by a clinician according to a standard registration form developed by the Dutch Association for Cleft Palate and Craniofacial Anomalies (Luijsterberg and Vermeij-Keers 1999). The standardized registration was performed when the index child was approximately 15 months old. Most associated malformations and developmental delays are identified in the first year of life, which is important in selecting cases and controls. The unrelated control children did not have major congenital malformations and were enrolled by friends, acquaintances or neighbors of case parents and through well-baby clinics in and around Nijmegen. (Dutch well-baby clinics provide standard check-ups for all young children's growth and development.) All participants were Dutch Caucasians. DNA was obtained via blood samples or buccal swabs. We defined the periconceptional period for mothers as 3 months before conception to 3 months afterward and for the fathers as 3 months before conception to 2 weeks afterward. The periconceptional period for recommended folic acid use is defined as 4 weeks before conception to 8 weeks afterward.

Both case and control parents filled in a questionnaire at home on demographics and on their periconceptional and first-trimester smoking, alcohol consumption, medication use and maternal folic acid supplementation. The mothers were asked to fill in questionnaires for the period covering 3 months before conception to 3 months afterward, while the fathers reported on the period of 3 months before conception to 2 weeks afterward. The questions on medication use asked about the type of medication, dosage, and frequency of intake, and the period in which medication was taken.

Maternal folic acid supplementation was defined as any taken during the periconceptional period, with a daily intake of at least 400 μg of folic acid, either in a multivitamin supplement or as a single tablet of folic acid from 4 weeks before conception to 8 weeks afterward, as recommended by the Dutch government for all women who want to become pregnant (Health Council and Food and Nutrition Council 1993). There were also questions on the mother's periconceptional use of multivitamins with or without folic acid.

Both parents were also asked about their family history and if they reported any family member with an OFC, the family history was defined as positive.

We selected case and control children and their parents for whom DNA was available and the *MSX1* CA repeats could be successfully analyzed. This resulted in 181 case mothers and 132 control mothers, 155 case fathers and 121 control fathers, and 176 cases and 146 control children. Due to the poor quality of the DNA isolated from a number of buccal swabs, CA repeat data were only available for 107 complete case triads and for 66 complete control triads to be included in our TDT analysis.

Determination of serum and red blood cell folate

A venous blood sample was taken from the mothers to measure their concentrations of serum and red blood cell (RBC) folate. These were measured using a microbiologic assay. Sample collection and laboratory determination were described previously (van Rooij et al. 2003).

Genotyping

The analyses were based on an intronic polymorphic CA repeat in the *MSX1* gene. The CA repeat alleles were determined by polymerase chain reaction (PCR) and fragment analysis. Primers and conditions for PCR were as described previously, with minor modifications (Hwang et al. 1998). After amplifying the DNA, PCR products were run on an ABI 3100 sequencer (Applied Biosystems) and analyzed using genescan and genotyper software (Applied Biosystems). Sequencing analysis was performed on representative samples to determine the exact repeat numbers for different alleles (data not shown). Four different alleles could be identified and were called as in previous studies: allele 1, 12 CA repeats; allele 2, 11 CA repeats; allele 3, 10 CA repeats; allele 4, 9 CA repeats (Jugessur et al. 2003).

Statistical analysis

Sample characteristics were compared between cases and controls using *t* test or Chi-square tests. The frequencies of the four *MSX1* CA repeat alleles were compared between cases and controls, and odds ratios (OR) were derived with 95% confidence intervals (CI), for which allele 1 served as the reference. To test for genetic association in nuclear families, we used the software package UNPHASEDv2.4, which implements a likelihood-based approach allowing for missing parental data (Dudbridge 2008).

The genotypes for the MSX1 CA repeat were allotted to three genotype categories defined in previous studies: CA4 homozygotes (4/4), CA4 heterozygotes (4/x), and CA4 non-carriers (x/x) (Beaty et al. 2002; Fallin et al. 2003; Jugessur et al. 2003). Genotypic odds ratios with 95% CI were derived. We investigated gene–environment interactions by further stratifying environment factors such as parental smoking, alcohol consumption, medication use, and maternal folic acid supplementation.

Because CA4 is the most common allele, the groups of non-exposed CA4 non-carriers were too small to serve as a reference. We therefore combined CA4 heterozygotes and CA4 non-carriers (4/x, x/x) into one category for all the stratified and non-stratified analyses. The limited sample size meant stratified analyses of the different OFC phenotypes were not feasible.

Pooling different types of clefting is consistent with the finding that *MSX1* is involved in both CL/P and CPO (van den Boogaard et al. 2000). Furthermore, a recent study identified occult lip defects with high-resolution ultrasound of the upper lip in a subset of CP cases, showing how difficult it is to classify orofacial clefting in different types (Weinberg et al. 2008).

The data for folic acid values were compared between the groups using Wilcoxon's rank sum test.

Results

Characteristics

The characteristics of the case and control groups are given in Table 1. Case children were almost twice as likely to be boys compared to controls (OR 1.8, 95% CI 1.1–2.8), consistent with the large number of CL/P cases and known male predominance (Jugessur et al. 2003). There were no significant differences in the characteristics of parents and children between the cases and controls, except that a positive family history was reported significantly more frequently by case parents compared to controls (both $P < 0.01$).

In the periconceptional period, case mothers used significantly more medication ($P < 0.01$) and case fathers smoked more than controls ($P = 0.05$). However, maternal medications were rather diverse and included analgesics, decongestive nose-sprays, antibiotics, antimycotics, antihistamic drugs, ovulation-inducing drugs, antidepressive drugs and thyroid drugs. No anti-epileptic drugs, nor vitamin A or its congeners, were reported.

There was no significant difference in the use of folic acid between case and control mothers. In total, 62.8% of case mothers and 71.8% of control mothers took some folic acid in the periconceptional period. However, when maternal folic acid supplementation was defined as daily use from 4 weeks before conception to 8 weeks afterward, case mothers took significantly less folic acid supplementation. Of those who took supplements, all but one mother used 400 µg folic acid; this case mother took 5 mg folic acid daily during the recommended period without clear indication.

Serum and red blood cell folate levels

The median serum and red blood cell (RBC) folate concentrations were within the normal range (>4.8 and >340 nmol/L, respectively) (van Rooij et al. 2003) (Table 2). The median level of serum folate was lower in the case mothers than in control mothers, 13.2 and 15.2 nmol/L. The median level of serum folate was lower in mothers who smoked during the periconceptional period than in non-smoking mothers, 12.7 and 14.0 nmol/L. In mothers who smoked

Table 1 Characteristics of the case and control triads

	Cases	%	Missing	Controls	%	Missing	OR (95% CI)	*P* value
Child	*n* = 176			*n* = 146				
Age at the study moment in months, mean (SD)	15.2	4.2	2	14.7	6.7	6		0.43
Boys, *n* (%)	114	64.8		74	50.7		1.8 (1.1–2.8)	0.01
Boys in CL/P cases, *n* (%)	102	68.0		74	50.7		2.1 (1.3–3.4)	<0.01
Boys in CP cases, *n* (%)	12	46.2		74	50.7		0.8 (0.4–1.9)	0.65
Type of OFC								
CL, *n* (%)	58	33.0						
CP, *n* (%)	26	14.8						
CLP, *n* (%)	92	52.3						
Mother	*n* = 181			*n* = 132				0.54
Age, years, mean (SD)[a]	30.6	3.9	1	30.9	3.8	3		
Family history for OFC positive, *n* (%)	38	21.1	1	11	8.4	1		<0.01
Periconception, yes, *n* (%)								
Smoking[b]	52	28.9	1	27	20.6	1	1.6 (0.9–2.7)	0.10
Alcohol[c]	68	37.8	1	60	45.8	1	0.7 (0.5–1.1)	0.16
Medication[d]	68	37.8	1	26	19.9	1	2.5 (1.5–4.1)	<0.01
Any folic acid use[e]	113	62.8	1	94	71.8	1	0.7 (0.4–1.1)	0.10
Folic acid use/multivitamin use[f]	54	44.6	60	56	60.2	39	0.5 (0.3–0.9)	0.02
Father	*n* = 155			*n* = 121		1		
Age, years, mean (SD)	33.1	4.1	3	33.8	4.8			0.21
Family history for OFC positive, *n* (%)	33	21.9	4	10	8.5	3		<0.01
Periconception, yes, *n* (%)								
Smoking[b]	73	47.7	2	43	35.8	1	1.6 (1.0–2.7)	0.05
Alcohol[c]	137	89.5	2	105	87.5	1	1.2 (0.6–2.6)	0.60
Medication[d]	26	17.0	2	12	10.0	1	1.8 (0.9–3.8)	0.10

SD standard deviation, *CL/P* cleft lip with or without cleft palate, *CL* cleft lip, *CP* cleft palate, *CLP* cleft lip with cleft palate; *OFC* orofacial clefting; *OR* (*95% CI*) = odds ratio, 95% confidence interval; *n* = number for whom *MSX1* CA repeat alleles were successfully analyzed; *P* value tested by Chi-square

[a] At delivery of the child

[b] Any smoking/cigarettes/cigar/pipe

[c] Any alcohol use

[d] Any medication other than oral contraceptives or iron

[e] Any use of supplements containing folic acid

[f] Daily use of supplements containing folic acid with a minimum of 400 μg folic acid from 4 weeks before conception to 8 weeks afterward, one case mother used 5 mg per day. Incidental users and women who started folic acid supplements later than 4 weeks before conception were excluded

and who had the 4/4 genotype the median level of serum folate was lower, 11.3 nmol/L, than in non-smoking 4/4 mothers, 13.0 nmol/L. In addition, the median level of serum folate was lower in case mothers who smoked and who had the 4/4 genotype than in those smoking case mothers not carrying the 4/4 genotype (category 4/x, x/x), 11.3 and 14.5 nmol/L.

The median RBC folate concentrations also revealed no significant differences. The median RBC folate level in case mothers was 654.5 nmol/L, while it was 634.8 nmol/L in control mothers. The median RBC folate in mothers who smoked during the periconceptional period was 641.8 nmol/L, and in non-smoking mothers it was 637.8 nmol/L. The RBC folate concentration was lower in mothers who smoked and had the 4/4 genotype than in non-smoking 4/4 mothers, 527.6 and 594.0 nmol/L, respectively. Furthermore, the median level of RBC folate was lower in case mothers who smoked and had the 4/4 genotype than in case mothers who smoked but did not have the 4/4 genotype (category 4/x, x/x), 554.9 and 681.9 nmol/L, respectively.

CA repeat alleles and *MSX1* genotypes

As in previous studies, four alleles were identified (Jugessur et al. 2003). The distributions of the CA repeat alleles and genotypes were not significantly different among case

Table 2 Plasma and red blood cell folate concentrations in relation to smoking and *MSX1* genotype

	Case mothers	Control mothers	*P* value*
	n = 101	*n* = 80	
	Median (p5–p95)	Median (p5–95)	
Plasma folate (nmol/L)	13.2 (5.7–38.6)	15.2 (6.8–58.3)	0.40
	n = 103	*n* = 82	
Red blood cell folate (nmol/L)	654.5 (325.6–1226.5)	634.8 (348.3–1418.6)	0.61
	Smoking mothers	Non-smoking mothers	*P* value*
	n = 44	*n* = 136	
	Median (p5–p95)	Median (p5–p95)	
Plasma folate (nmol/L)	12.7 (5.2–38.6)	14.0 (6.6–55.9)	0.30
	n = 44	*n* = 139	
Red blood cell folate (nmol/L)	641.8 (266.8–1182.2)	637.8 (342.8–1401.9)	0.26
	Smoking 4/4 mothers	Non-smoking 4/4 mothers	*P* value*
	n = 12	*n* = 46	
	Median (p5–p95)	Median (p5–p95)	
Plasma folate (nmol/L)	11.3 (5.1–26.5)	13.0 (5.9–48.2)	0.19
	n = 12	*n* = 46	
Red blood cell folate (nmol/L)	527.6 (165.7–1024.9)	594.0 (363.5–1446.1)	0.16
	Smoking 4/4 case mothers	Smoking 4/x, x/x case mothers	*P* value*
	n = 10	*n* = 20	
	Median (p5–p95)	Median (p5–p95)	
Plasma folate (nmol/L)	11.3 (5.1–26.5)	14.5 (5.1–38.7)	0.19
	n = 10	*n* = 20	
Red blood cell folate (nmol/L)	554.9 (165.7–1024.9)	681.9 (246.7–1204.4)	0.59

* Wilcoxon's rank sum test

and control children or among their parents. Allele 4 (nine repeats of the CA marker in the *MSX1* allele) was the most common allele in case children (58.0%), mothers (57.7%), and fathers (53.9%), and in control children (56.1%), mothers (53.4%), and fathers (55.8%), regardless of OFC status (Fig. 1; Table 3).

For the TDT analysis, we analyzed 107 informative case triads. Case fathers transmitted allele 1 on three occasions but did not transmit allele 1 on 14 occasions ($P = 0.02$).

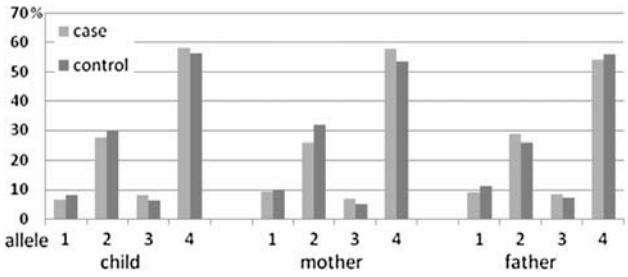

Fig. 1 Allele frequencies (%) in case and control child, mother, and father

Genotype 1/4 was seen less frequently in cases than in controls (OR 0.47, 95% CI 0.2–1.1). However, the frequency of allele 1 was low and the numbers were too small for further analysis.

Interactions between lifestyle factors and *MSX1* genotypes

Gene–environment interaction analysis for parental and child genotype and parental smoking, alcohol use, medication use and maternal folic acid supplement use was performed and is shown in Tables 4 and 5.

Maternal smoking influenced the risk for OFC if either the child or mother was homozygous for allele 4 (OR 2.7, 95% CI 1.1–6.6 and OR 3.2, 95% CI 1.1–9.0, respectively) (Table 4). If both parents smoked, the odds ratio was increased to 4.9 (95% CI 1.4–18.0) (Table 4). The possible effects of paternal smoking are presented in Table 4. Children homozygous for allele 4 of fathers who smoked showed a more than twofold increased OFC risk (OR 2.2, 95% CI 1.0–4.7). When we excluded the children with two

Table 3 Case-control genotype distribution of the *MSX1* CA repeat in children, mothers and fathers in association with risk of orofacial clefting

Genotype	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*n* = 176		*n* = 146		
4/4	56	31.8	45	30.8	1.1 (0.7–1.7)
4/x, x/x[a]	120	68.2	101	69.2	1.0 (Reference)
Mother	*n* = 181		*n* = 132		
4/4	61	33.7	33	25.0	1.5 (0.9–2.5)
4/x, x/x[a]	120	66.3	99	75.0	1.0 (Reference)
Father	*n* = 155		*n* = 121		
4/4	44	28.4	37	30.6	0.9 (0.5–1.5)
4/x, x/x[a]	111	71.6	84	69.4	1.0 (Reference)

[a] Combined genotype category, x = allele 1, 2, 3

smoking parents from this analysis the odds ratio was 1.2 (95% CI 0.5–3.2).

An association was found between the mother not taking folic acid supplements and increased OFC risk (Table 5a, b). Not taking folic acid daily in the recommended period increased OFC risk for children homozygous for allele 4 (4/4) (OR 2.0, 95% CI 0.9–4.5) as well as for children not carrying the 4/4 genotype (category 4/x, x/x) (OR 1.4, 95% CI 0.7–2.7) (Table 5a). In addition, not taking folic acid supplements daily during the recommended period increased the risk of having a child with OFC in both mothers homozygous for allele 4 (4/4) (OR 2.8, 95% CI 1.1–6.7) and in mothers without the 4/4 genotype (category 4/x, x/x) (OR 1.8, 95% CI 0.96–3.5) (Table 5a). Only a relatively small number of mothers (54 case mothers and 56 control mothers) took the advised 400 μg folic acid daily in the recom-

Table 4 Interaction between parental smoking during periconceptional period and the *MSX1* CA repeat genotype on the risk of orofacial clefting

Genotype	Smoking	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*Mother*	*n* = 175		*n* = 145		
4/4	Yes	23	13.1	7	4.8	2.7 (1.1–6.6)
4/x, x/x[a]	Yes	30	17.1	28	19.3	0.9 (0.5–1.6)
4/4	No	33	18.9	38	26.2	0.7 (0.4–1.2)
4/x, x/x[a]	No	89	50.9	72	49.7	1.0 (Reference)
Child	*Father*	*n* = 172		*n* = 143		
4/4	Yes	28	16.3	12	8.4	2.2 (1.0–4.7)
4/x, x/x[a]	Yes	55	32.0	39	27.3	1.3 (0.8–2.3)
4/4	No	26	15.1	33	23.1	0.7 (0.4–1.4)
4/x, x/x[a]	No	63	36.6	59	41.3	1.0 (Reference)
Child	*Father*[b]	*n* = 119		*n* = 108		
4/4	Yes	12	10.1	9	8.3	1.2 (0.5–3.2)
4/x, x/x[a]	Yes	32	26.9	18	16.7	1.7 (0.8–3.3)
4/4	No	19	16.0	29	26.9	0.6 (0.3–1.2)
4/x, x/x[a]	No	56	47.0	52	48.1	1.0 (Reference)
Child	*Mother + father*[c]	*n* = 114		*n* = 105		
4/4	Yes	16	14.0	3	2.9	4.9 (1.4–18.0)
4/x, x/x[a]	Yes	23	20.2	21	20.0	1.0 (0.5–2.1)
4/4	No	19	16.7	29	27.6	0.6 (0.3–1.2)
4/x, x/x[a]	No	56	49.1	52	49.5	1.0 (Reference)
Mother		*n* = 180		*n* = 131		
4/4	Yes	18	10.0	5	3.8	3.2 (1.1–9.0)
4/x, x/x[a]	Yes	34	18.9	22	16.8	1.4 (0.7–2.5)
4/4	No	42	23.3	28	21.4	1.3 (0.8–2.3)
4/x, x/x[a]	No	86	47.8	76	58.0	1.0 (Reference)
Father		*n* = 153		*n* = 120		
4/4	Yes	22	14.4	12	10.0	1.6 (0.7–3.6)
4/x, x/x[a]	Yes	51	33.3	31	25.8	1.5 (0.8–2.6)
4/4	No	21	13.7	25	20.8	0.7 (0.4–1.5)
4/x, x/x[a]	No	59	38.6	52	43.3	1.0 (Reference)

OR 95% CI = odds ratios 95% confidence interval

[a] Combined genotype category x = allele 1, 2, 3

[b] Independent effect of smoking by the father, children of two smoking parents were excluded

[c] Mother + father; *yes* both parents smoking, *no* both parents not smoking

Table 5 Folic acid use, *MSX1* CA repeat genotype and risk of orofacial clefting

a. Interaction between maternal folic acid use in the recommended periconceptional period and *MSX1* CA repeat genotype on the risk of orofacial clefting

Genotype	Folic acid[b]	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*Mother*	*n* = 117		*n* = 106		
4/4	No	23	19.7	13	12.3	2.0 (0.9–4.5)
4/x, x/x[a]	No	44	37.6	35	33.0	1.4 (0.7–2.7)
4/4	Yes	15	12.8	19	17.9	0.9 (0.4–2.0)
4/x, x/x[a]	Yes	35	29.9	39	36.8	1.0 (Reference)
Mother		*n* = 121		*n* = 93		
4/4	No	22	18.2	9	9.7	2.8 (1.1–6.7)
4/x, x/x[a]	No	45	37.2	28	30.1	1.8 (0.96–3.5)
4/4	Yes	16	13.2	13	14.0	1.4 (0.6–3.3)
4/x, x/x[a]	Yes	38	31.4	43	46.2	1.0(Reference)

b. Interaction between some maternal folic acid use during pregnancy and *MSX1* CA repeat genotype on the risk of orofacial clefting

Genotype	Folic acid[c]	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*Mother*	*n* = 175		*n* = 145		
4/4	No	23	13.1	13	9.0	1.5 (0.7–3.3)
4/x, x/[xa]	No	44	25.1	35	24.1	1.1 (0.6–1.9)
4/4	Yes	33	18.9	32	22.1	0.9 (0.5–1.6)
4/x, x/x[a]	Yes	75	42.9	65	44.8	1.0 (Reference)
Mother		*n* = 180		*n* = 131		
4/4	No	22	12.2	9	6.9	2.3 (0.98–5.3)
4/x, x/x[a]	No	45	25.0	28	21.4	1.5 (0.9–2.7)
4/4	Yes	38	21.1	24	18.3	1.5 (0.8–2.7)
4/x, x/x[a]	Yes	75	41.7	70	53.4	1.0 (Reference)

OR odds ratios, *95% CI* 95% confidence interval

[a] Combined genotype category x = allele 1, 2, 3

[b] Daily use of supplements containing folic acid (400–500 µg) from 4 weeks before conception of the child until 8 weeks after; incidental users and women who started folic acid supplements later than 4 weeks before conception were excluded

[c] Any folic acid use during pregnancy, also incidental users and women who started folic acid supplements later than 4 weeks before conception were included

mended period. The analyses were therefore repeated for mothers taking any folic acid supplements at all (Table 5b).

During pregnancy, case mothers more often took medication (OR 2.5, 95% CI 1.5–4.1) (Table 1). The OFC risk was higher in mothers or their children homozygous for allele 4 if the mother used medication (OR 8.3, 95% CI 2.4–28.7 and OR 1.9, 95% CI 0.9–4.0, respectively), albeit not significantly in the children. If fathers used medication and they or their children were homozygous for allele 4, the OFC risk was also increased, albeit not significantly (OR 3.3, 95% CI 0.9–12.0 and OR 1.2, 95% CI 0.5–3.1, respectively). We found no significant interactions between the 4/4 genotype in mothers, fathers, or children, parental alcohol use and OFC risk (data not shown).

Discussion

This study provides further evidence for an association between the homeobox gene *MSX1* and maternal smoking during the first trimester of pregnancy and offspring with nonsyndromic OFC. In addition, the daily use of folic acid supplement from 4 weeks before conception to 8 weeks afterward reduces OFC risk independent of *MSX1* genotype. The analyses are based on an intronic polymorphic CA repeat in the *MSX1* gene, which identifies four alleles. Consistent with previous observations, allele 4 (nine repeats of the CA marker in the *MSX1* allele) was the most common allele (Jugessur et al. 2003). Our analysis suggests that allele 4 homozygous (4/4) children exposed to periconceptional smoking by both parents, but particularly by the mother, are more susceptible to OFC. The maternal 4/4 genotype and smoking showed a threefold higher OFC risk.

Several hypotheses might explain the mechanisms by which interaction between smoking and *MSX1* influences OFC risk. Studies in mice suggest that maternal cigarette smoke alters the expression of a large number of genes in the fetus. Especially genes involved in response to oxidative stress, DNA and protein repair, signal transduction and cell cycle regulation (CDK4 and CDK6 inhibitors) were upregulated (Izzotti et al. 2003). Interestingly, *Msx1* permits cell cycle progression and proliferation of tissue by inhibiting the expression of CDK inhibitors, resulting in E2F mediated expression of cell cycle genes (Han et al. 2003).

Treatment of mouse hepatoma cells with a component of tobacco smoke [2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)]

resulted in the co-repression of E2F-mediated expression of genes necessary for cell cycle progression (Huang and Elferink 2005).

Another interesting hypothesis is that AhR suppresses *Tgfb3* expression and Tgfb3 has an indirect effect on IRF6. Various studies have shown that both genes play a significant role in the development of the palate and lip (Murray and Schutte 2004). The finding of two IRF binding sites in the promotor and intron of *MSX1* indicates both genes might be part of a common pathway (Kondo et al. 2002). This would suggest that there is a relation between Ahr and *MSX1*, and thus indirectly between smoking and *MSX1*. The effect of the interaction between smoking and *MSX1* might also be a result of gene–gene interaction with genes involved in detoxification pathways. Results of a preliminary analysis suggested gene–gene interaction between *MSX1* with *CYP1B1*, *GSTM1*, *HIF1A*, and *SULT1A1 (*Shi et al. 2007).

Another aspect of smoking by the mother is its possible influence on folate levels. It has been suggested that exposure to tobacco smoke decreases serum folate level and that these lower levels are not completely due to differences in folate intake from diet or supplements (Mannino et al. 2003; Honein et al. 2007). In our study the current level of serum folate was also lower in the mothers who smoked during the periconceptional period than in the non-smoking mothers. This was in contrast to the RBC folate levels. The smoking mothers with the 4/4 genotype had a lower serum and RBC folate level than non-smoking 4/4 mothers. In addition, the level of serum and RBC folate was lower in the case mothers who smoked and had the 4/4 genotype than in the case mothers who smoked without this genotype. However, the numbers are too small to draw any firm conclusions.

Interestingly, knockout studies in mice demonstrated inactivation of the folate-binding protein Folbp1, decreased folate levels and clefting (Tang and Finnell 2003). The *Shh* expression was decreased in the facial primordial in the *Folbp1* mutants, accompanied by an increase of *Bmp4* expression. It is striking that *Msx1* plays a significant role in the expression of *Bmp4*, and that *Msx1*, *Bmp4*, *Shh* and *Bmp2* constitute a pathway essential in the palatogenesis in mice (Zhang et al. 2002). *Pax3* was also found to be upregulated in the nasal processes of the *Folbp1* mutant (Tang and Finnell 2003). In vivo studies showed that complex formation between MSX1 and PAX3 may prevent premature activation of myogenic genes (*MyoD*) in muscle precursor cells, which is important for the maintenance of their proliferative capacity and outgrowth (Bendall and Abate-Shen 2000). We speculate that low levels of folate might influence the function of specific *MSX1* variants, thereby affecting the *MSX1*, *BMP4*, *SHH* and *BMP2* pathway in the embryo. The downregulation of *SHH* affects the expression of *PAX3*, resulting in abnormal outgrowth of tissue. Further studies are needed to evaluate these possible mechanisms and the specific role of *MSX1* in these models in relation to OFC.

An interesting aspect of folic acid is that it functions as a one-carbon donor for DNA methylation, which is important in regulating gene expression. A recent study indicated expression of *Msx1* in embryonic stem cells was regulated by a unique histone modification, which consisted of activating and repressive methylation within their promotor loci (Gan et al. 2007). We cannot rule out that low folate affects this mechanism as well, and it might explain potential associations between smoking, folic acid, *MSX1* and OFC risk.

In this study, only a relatively small number of mothers took the daily 400 μg folic acid in the recommended period. Association between supplemental folic acid use, *MSX1* and OFC risk was observed. Homozygosity for allele 4 in the child or mother combined with the mother not taking folic acid supplements tended to increase OFC risk. However, any interaction between periconceptional folic acid intake and a specific *MSX1* genotype on OFC risk will have a relatively small effect.

Our case fathers smoked more often than control fathers, and paternal smoking may also contribute to OFC risk. Paternal smoking increases maternal exposure to tobacco smoke by passive smoking, thus indirectly influencing the environment of the fetus. Homozygosity for allele 4 in children and paternal smoking showed a twofold higher OFC risk. However, to exclude any possible effect from the mother smoking, we performed an additional analysis which excluded children with two smoking parents. The suggested interaction between paternal smoking, homozygosity for allele 4 in the child and OFC risk could not be confirmed. But again, our sample size was very small.

In all our analyses we used the intronic CA repeat marker. Recent studies (Knight 2004; Spielman et al. 2007) have revealed that polymorphic variants can also be responsible for individual differences in expression level, and specific genetic variation among populations contributes appreciably to differences in gene expression phenotypes. These variations of gene expression and specific gene expression phenotypes could account for a large proportion of susceptibility to complex genetic disorders (Spielman et al. 2007). Given this observation, the CA repeat may also play a role in the etiology of clefting. The *Msx1* gene encodes an antisense RNA, which regulates gene expression, and the antisense strand, in the 3′UTR to the middle of the intron, includes the CA repeat (Blin-Wakkach et al. 2001). We hypothesize that differences in the CA repeat numbers of the opposite alleles may alter the binding between the sense and antisense RNA molecules of both alleles and could thereby affect regulation of gene expression. This could be an explanation for the possibly protective effect of the 1/4 genotype and our finding that this genotype was more prevalent in controls than in cases. The difference

in the repeat numbers of the opposite alleles was most profound in the 1/4 genotype, which may influence regulation of gene expression by antisense RNA. Further studies are needed to evaluate this possible mechanism.

We performed our study using a case-control study design, in which recall bias of periconceptional exposure must be considered. To minimize this issue, we used a standardized study moment, when the child was around 15 months old for both the case and control groups. This moment is in the same season as the periconceptional period and just after the first birthday, which may facilitate parents' recall. However, several studies have indicated that the role of recall bias in case-control studies focusing on congenital malformations can be considered fairly small (Infante-Rivard and Jacques 2000) and our data on lifestyle exposures of the reproductive age group also correspond with those reported in the Dutch population (Statistics Netherlands 2003).

In conclusion, we demonstrate a complex role for the *MSX1* gene, maternal smoking, and folic acid intake in the etiology of OFC and show some interesting gene–environment interactions that influence the risk of OFC. Further studies are needed to resolve the details of the pathogenic mechanisms and to determine the genetic and environmental risk factors playing a decisive role in the occurrence of OFC. Ultimately, this work may be helpful in providing better tailored advice to individuals in preconceptional counseling.

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Conflict of interest statement None.

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