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ORIGINAL INVESTIGATION

Evidence that TGFA influences risk to cleft lip with/without cleft palate through unconventional genetic mechanisms

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Abstract This study examined the association between markers in transforming growth factor alpha (*TGFA*) and isolated, non-syndromic cleft lip with/without palate (CL/P) using a case–parent trio design, considering parent-of-origin effects. We also tested for gene–environmental interaction with common maternal exposures, and for gene–gene interaction using markers in TGFA and another recognized causal gene, IRF6. CL/P case–parent trios from four populations (76 from Maryland, 146 from Taiwan, 35 from Singapore, and 40 from Korea) were genotyped for 17 single nucleotide polymorphisms (SNPs) in *TGFA*. The transmission disequilibrium test was used to test individual SNPs, and the parent-of-origin likelihood ratio test (PO-LRT) was used to assess parent-of-origin effects. We also screened for possible gene–environment interaction using PBAT, and tested for gene–gene interaction using conditional logistic regression models. When all trios were combined, four SNPs showed significant excess maternal transmission, two of which gave significant PO-LRT values [rs3821261: $P = 0.004$ and OR(imprinting) = 4.17; and rs3771475: $P = 0.027$ and OR(imprinting) = 2.44]. Haplotype analysis of these two SNPS also supported excess maternal transmission. We saw intriguing but suggestive evidence of G × E interaction for several SNPs in TGFA when either individual SNPs or haplotypes of adjacent SNPs were considered. Thus, *TGFA* appears to influence risk of CL/P through unconventional means with an apparent parent-of-origin effect (excess maternal transmission) and possible interaction with maternal exposures.

Introduction

Oral clefts are one of the most common birth defects in humans and represent a significant public health problem

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both in terms of medical and economic burdens for affected individuals and their families. Non-syndromic cleft lip with or without palate (CL/P) is considered 'complex' or 'multifactorial' in its etiology, in that both genes and environmental risk factors control risk (Wyszynski et al. 1997; Cobourne 2004). Transforming growth factor alpha (TGFA) gene is a well-studied candidate gene for CL/P, but has shown inconsistent evidence of association with CL/P across a number of studies (Vieira 2006). Some studies have also tested for potential gene–environmental (G × E) interactions between markers in TGFA and common maternal exposures (particularly maternal smoking) (Hwang et al. 1995; Beaty et al. 1997; Shaw et al. 1998; Shaw et al. 1996; Zeiger et al. 2005). Jugessur et al. (2003a, b) raised the possibility of interaction between TGFA and MTHFR, so it would also be important to consider gene–gene interaction (G × G). Here we consider interaction between TGFA and IRF6, another frequently studied candidate gene. IRF6 on chromosome 1 has been identified as responsible for a majority of cases with van der Woude syndrome (VWS), an autosomal dominant malformation syndrome which often includes oral clefts. In addition, several studies have reported strong association between polymorphic markers with isolated non-syndromic CL/P (Zucchero et al. 2004; Park et al. 2007).

It is important to consider parent-of-origin effects when studying birth defects because maternal genotype controls the in utero environment of the developing fetus, and separating maternal genotypic effects from imprinting effects remains an important scientific question (Weinberg and Umbach 2005; Wilkins and Haig 2003). Maternal parent-of-origin effects have been suggested for several genes associated with non-syndromic CL/P (van Rooij et al. 2003; Rubini et al. 2005; Sull et al. 2008). However, to date no study has focused on whether *TGFA* gene may influence risk of CL/P through a parent-of-origin effect.

In a previous paper, we reported an association between markers in *TGFA* and risk of CL/P in three populations (Beaty et al. 2006). Here, we tested for association in 297 CL/P case–parent trios from 4 populations, while specifically considering parent-of-origin effects, as well as testing for gene–environmental interactions between markers in TGFA and three common maternal exposures (maternal smoking, alcohol consumption and vitamin supplementation), plus testing for interaction between markers in the TGFA and IRF6 genes.

Methods

Sample description

As part of an international study of oral clefts, we collected data on case–parent trios recruited through treatment centers in Maryland (MD) (Johns Hopkins and University of Maryland), the Chang Gung Memorial Hospital in Taiwan (TW), KK Women's and Children's Hospital in Singapore (SP), and Yonsei Medical Center in South Korea (KR). Research protocols were reviewed and approved by institutional review boards (IRB) at each institution. Table 1 lists the gender of all CL/P probands. The majority of cases were infants seen during a surgical or postsurgical visit. All parents of probands were unaffected in the Singapore, Taiwan, and Korean trios, but 4 parents among the 76 Maryland trios also had an oral cleft. All probands underwent clinical genetics evaluation (including assessing other congenital anomalies or major developmental delays) and were classified as having an isolated, non-syndromic CL/P. First-trimester maternal exposure information, including cigarette smoking, vitamin supplementation, and alcohol consumption was collected from a face-to-face interview of mothers (although our first group of trios from Taiwan had substantial rates of missing data). For maternal smoking, about 20% of mothers in Maryland reported smoking during the critical period of pregnancy (during the 3 months before conception through the first trimester). Among women in Taiwan and other Asian populations, however, smoking rates were substantially lower (about 5%). The proportion with alcohol consumption and vitamin supplementation during the first-trimester was also higher in Maryland than in the Asian populations (Table 2).

SNP selection, DNA and genotyping

Single nucleotide polymorphisms (SNPs) were selected in a region surrounding *TGFA* on chromosome 2p13, with a goal of identifying one SNP per 5 kb of physical distance. Variants with "SNP scores" (an assessment of design quality of the Illumina assay based on a proprietary algorithm) above 0.6, high validation levels in dbSNP (this included validation levels where the submitter had validated the SNP on multiple platforms), and high heterozygosity levels (particularly in multiple populations), were given higher priority during the selection process. From 24 selected SNPs, 20 were polymorphic in all 4 populations. Two of these SNPs had low genotype call rates and one SNP

Table 1 Gender among 297 non-syndromic cleft lip with or without cleft palate (CL/P) cases from 4 populations

Population	CL/P cases		
	Total (*n*)	Male (*n*)	Female (*n*)
Taiwan	146	95	51
Singapore	35	24	11
Korea	40	22	18
Maryland	76	44	32
Total	297	185	112

Table 2 Distribution of three maternal environmental factors among 297 CL/P case–parent trios from 4 populations

	Maternal smoking			Maternal alcohol			Maternal vitamin supplements		
	Exposed	Unexp	Unknown	Exposed	Unexp	Unknown	Exposed	Unexp	Unknown
TW	5	82	59	1	86	59	27	58	61
SP	3	25	7	2	26	7	5	23	7
KR	0	40	0	0	37	3	6	33	1
MD	16	60	0	12	64	0	61	9	6
Total	24	207	66	15	213	69	99	123	75

deviated significantly from HWE in three of four populations, leaving only 17 SNPs with reasonable heterozygosity (Table 3).

Genomic DNA samples were prepared from peripheral blood by the protein precipitation method described previously (Bellus et al. 1995). DNA concentration was determined using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Inc., Eugene OR) and all DNA samples were stored at −20°C. A 4 µg aliquot of each genomic DNA sample was dispensed into a bar-coded 96-well microtiter plate at a concentration of 100 ng/µl and genotyped for SNP markers using the Illumina Golden-Gate™ chemistry with Sentrix® Array Matrices from the manufacturer (Oliphant et al. 2002) at the SNP Center of the Genetic Resources Core Facility (GRCF), a part of the McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins School of Medicine. Two duplicates and four CEPH controls were included on each plate to evaluate genotyping consistency within and between plates and to insure correct plate orientation. Genotypes were generated on a BeadLab 1000 system (Fan et al. 2003). No Mendelian inconsistencies were found for these 17 SNPs.

Statistical analysis

Within each population, the minor allele frequency (MAF) was computed among parents. Pairwise linkage disequilibrium (LD) was measured as r^2 for all SNPs using the Haploview program (Barrett et al. 2005). LD blocks were identified in each population separately using pairwise LD (Fig. 1). Four blocks of LD were identified, consisting of 9, 2, 3 and 3 SNPs, respectively. Clayton's extension of the Transmission Disequilibrium Test (TDT) incorporated into STATA 8.2 (Spielman et al. 1993; Cordell et al. 2004) was

Table 3 Minor allele frequencies (MAF) among parents of 297 CL/P cases from 4 populations

No.	SNP name	Physical location[a]	Minor allele	Minor allele frequency			
				Taiwan	Singapore	Korea	Maryland
1	rs473698	70587236	G	0.309	0.237	0.333	0.391
2	rs4852595	70591414	T	0.134	0.179	0.184	0.119
3	rs1880039	70598707	C	0.055	0.062	0.06	0.130
4	rs3771516	70606538	C	0.167	0.193	0.158	0.088
5	rs3755384	70616166	T	0.375	0.316	0.395	0.457
6	rs1807968	70619916	G	0.375	0.316	0.395	0.461
7	rs3771503	70623896	T	0.410	0.351	0.428	0.483
8	rs2902345	70628254	T	0.373	0.328	0.393	0.443
9	rs3755378	70632258	A	0.424	0.443	0.462	0.350
10	rs3771494	70637007	C	0.211	0.228	0.184	0.218
11	rs3821261	70640908	G	0.153	0.123	0.112	0.113
12	rs404420	70645181	T	0.184	0.190	0.230	0.255
13	rs455125	70658381	C	0.216	0.216	0.184	0.229
14	rs426081	70662186	C	0.130	0.138	0.026	0.227
15	rs3771475	70680983	G	0.304	0.319	0.303	0.123
16	rs12473408	70688039	C	0.359	0.368	0.367	0.467
17	rs11466191	70691736	C	0.290	0.293	0.253	0.264

[a] Based on NCBI Human Genome build 35.1

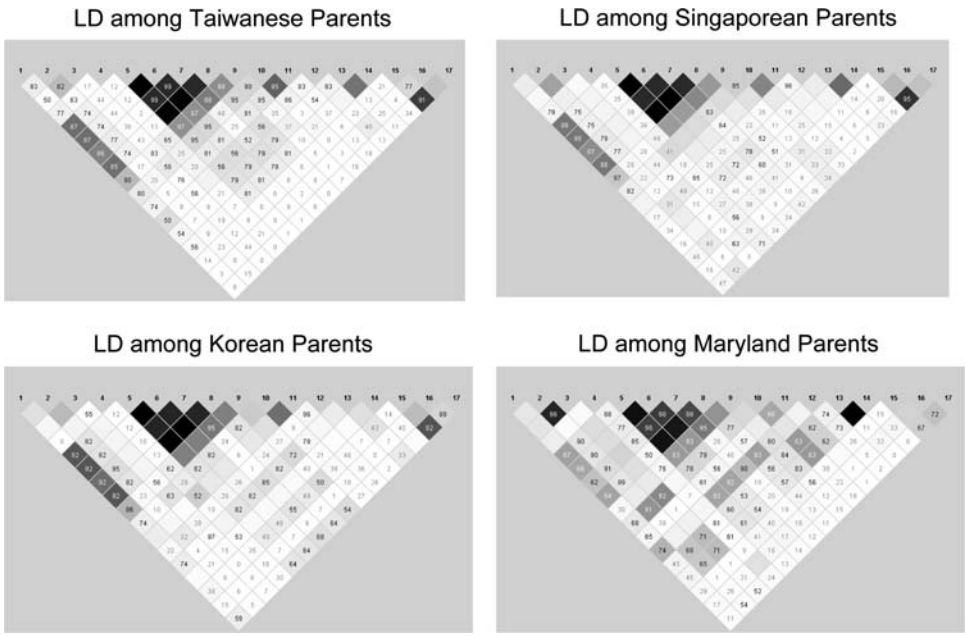

Fig. 1 Linkage disequilibrium as measured by r^2 in *TGFA* among parents of CL/P children from four populations. *White* $r^2 = 0$. *Shades of gray* $0 < r^2 < 1$. *Black* $r^2 = 1$

used on individual SNPs to test for evidence of linkage and LD in the combined sample of 297 CL/P trios.

Parent-of-origin analyses were conducted on the combined sample. We used the likelihood-based approach proposed by Weinberg (1999) to test for parent-of-origin effects while considering genotypes of both mother (2 parameters for maternal genetic effects) and child (2 parameters for child genetic effects). This log-linear model considers the three mating types in which the mother and father carry different numbers of variant alleles, with further stratification by the number of alleles inherited by the child. Like Weinberg, we will call this latter method the "parent-of-origin likelihood ratio test" (PO-LRT). This model considers maternally mediated in utero effects (maternal genotypic effects on the phenotype of the fetus) which could otherwise confound assessment of parent-of-origin effects, along with a separate term for imprinting (Weinberg 1999). Here imprinting reflects a differential transmission of alleles to the affected child from mothers versus fathers. This PO-LRT was executed using the LEM software (van Den Oord and Vermunt 2000).

The FAMHAP package was used to estimate haplotype frequencies and to test for excess transmission of multi-SNP haplotypes (Becker et al. 2006). The FAMHAP package calculates maximum likelihood estimates (MLEs) of haplotype frequencies (for up to 20 SNPs) in nuclear families via the expectation–maximization algorithm and is robust in handling missing SNPs (Becker and Knapp 2004). This program provides a haplotype-based test for nuclear family data. The test statistic is based on Monte-Carlo simulations where the set of transmitted and non-transmitted genotypes/haplotypes is randomly permuted for 10,000 replicates (Zhao et al. 2000; Knapp and Becker 2003). In this analysis, the chi-square statistic for marker combinations is replaced with the maximum chi-square over all haplotypes (maximum TDT statistic). The program gives an empiric *P* value, corrected for the multiple haplotypes being considered. Haplotype analysis was also stratified for maternal and paternal transmission for individual SNPs showing a parent-of-origin effect.

We also screened for possible gene–environment interaction between the 17 SNPs in TGFA and three common maternal exposures (maternal smoking, alcohol consumption and vitamin supplementation). In this analysis we used the strategy proposed by Vansteelandt et al. (2007) where family-based association tests are first evaluated for individual SNPs while allowing for potential gene–environment (G × E) interaction in a 2 degree of freedom (*df*) test, followed by a separate 1 *df* test for G × E interaction alone. This approach is implemented in the PBAT package (v3.6; http://www.biostat.harvard.edu/~clange/default.htm) and computes a combined score test with 2 *df* for main effects of genotype (G) and G × E interaction, and then using a score test with 1 *df* for G × E interaction alone.

We tested for possible interactions between alleles at two genes (TGFA and IRF6). We focused on SNP rs3771494 in TGFA and SNP rs2235373 in IRF6 because these SNPs gave the most significant results in the present and previous analysis (Park et al. 2007). Conditional logistic regression models were used for the gene–gene interaction test.

Results

Among these 17 SNPs, there was some variation in allele frequencies among parents from MD and the three Asian

populations (Table 3). The three Asian populations (TW, SP, and KR) had very low minor allele frequencies (MAF) for SNP 3 (rs1880039) and SNP 14 (rs426081) compared to the MD trios. Pairwise LD measures across the entire gene were calculated within each population, and Fig. 1 shows similar patterns across all populations.

When individual markers were screened in the combined dataset from all four populations using the TDT without considering parent-of-origin, the odds ratio of transmission for the minor allele, OR(transmission), was significant for SNP rs3771494 (OR = 1.59, $P = 0.004$), SNP rs3821261 (OR = 1.56, $P = 0.022$), and SNP rs426081 (OR = 1.51, $P = 0.030$) (Table 4).

Parent-of-origin effects were investigated for all SNPs in the combined dataset (Table 4). The PO-LRTs were only significant for SNP 11 (rs3821261) [$P = 0.004$, OR(imprinting) = 4.17] and SNP 15 (rs3771475) [$P = 0.027$, OR(imprinting) = 2.44]. These significant PO-LRTs gave estimated risk ratios for an imprinting effect ranging between 2.44 and 4.17 for SNP 11 and 15, suggesting excess maternal transmission compared to paternal transmission in this region.

Table 4 TDT analysis of 17 SNPs in TGFA among 297 CL/P case–parent trios ignoring parent-of-origin

No.	SNP name	TDT					PO-LRT[a]	
		T	NT	χ^2	*P* value	OR[b]	OR[c]	*P* value
1	rs473698	127	115	0.60	0.440	1.10	1.52	0.257
2	rs4852595	61	59	0.33	0.855	1.03	0.70	0.389
3	rs1880039	31	21	1.92	0.166	1.48	0.40	0.138
4	rs3771516	60	60	0.00	1.000	1.00	1.98	0.64
5	rs3755384	139	120	1.39	0.238	1.16	1.31	0.438
6	rs1807968	117	140	2.06	0.151	0.84	1.41	0.329
7	rs3771503	126	127	0.01	0.949	0.99	1.46	0.291
8	rs2902345	134	119	0.89	0.346	1.13	1.30	0.448
9	rs3755378	117	91	3.25	0.071	1.29	1.08	0.838
10	*rs3771494*	*100*	*63*	*8.40*	***0.004***	*1.59*	*1.64*	*0.205*
11	*rs3821261*	*67*	*43*	*5.24*	***0.022***	*1.56*	***4.17***	***0.004***
12	rs404420	93	95	0.02	0.884	0.98	0.77	0.498
13	rs455125	93	72	2.67	0.102	1.29	0.52	0.105
14	rs426081	68	45	4.68	**0.030**	1.51	0.68	0.391
15	*rs3771475*	*95*	*81*	*1.11*	*0.291*	*1.17*	***2.44***	***0.027***
16	*rs12473408*	*125*	*104*	*1.93*	*0.165*	*1.20*	*1.79*	*0.121*
17	*rs11466191*	*100*	*86*	*1.05*	*0.305*	*1.16*	*1.97*	*0.098*

Values given in italics indicate inferred blocks of strong linkage disequilibrium (LD)

Bold values represent results significant at the $P < 0.05$ level

T Transmitted, *NT* not transmitted, *OR* odds ratio, *TDT* transmission disequilibrium test

[a] Parent-of-origin likelihood ratio test (PO-LRT) for a separate imprinting term

[b] OR(transmission): Odds Ratio of transmission of minor allele

[c] OR: Odds Ratio for imprinting effect (i.e. differential transmission from mothers vs. from fathers)

Haplotypes of rs3821261 and rs3771475 were analyzed using the FAMHAP program in each population. Taiwan, Singapore, and Korean parents had very similar haplotype frequencies, so all Asian trios were combined. In the combined Asian data, haploytpes T-G and G-G showed strong evidence of excess transmission to CL/P children (maximum TDT = 5.652, $P = 0.054$ for transmission ignoring parent-of-origin). As seen in Table 5 (maximum TDT = 7.686, $P = 0.007$), this is largely attributed to excess maternal transmission among these Asian trios, although the Maryland trios showed similar patterns. No haplotypes showed significant deviation from expected Mendelian transmission when transmitted from fathers.

Figure 2 shows results of screening for gene–environment interaction between these 17 SNPs in TGFA and three common maternal exposures (maternal smoking, alcohol consumption and vitamin supplementation). Here $-\log_{10}(P$ values) are plotted over physical distance for both the 2 *df* test of G and G × E effects considered jointly (triangles) and the 1*df* test for G × E effects alone (squares). A nominal 5% significance level corresponds to $-\log_{10}(P$ value) = 1.3, a 1% significance corresponds to 2.0, etc. in this plot. The positions of the three SNPs showing marginal evidence of linkage in the presence of LD (rs3771494, rs3821261 and rs426081) when maternal exposures were ignored are noted below the *X*-axis, and one of these SNPs (rs3821261) showed nominal statistical significance in the score test for G × E (with 1 *df*) for all three maternal exposures when considered individually or in 2- and 3-SNP haplotypes. Haplotypes including rs3771494 and rs3821261 also showed statistically significant evidence of G × E interaction in the 1 *df* test for maternal vitamin supplementation. SNP rs455125 showed significant evidence in the 1 *df* test for G × E for alcohol consumption. SNP rs426081 gave nominally significant evidence of both G effects and G × E interaction in the 2 *df* test, and for G × E alone in the 1 *df* test for maternal alcohol consumption when considered alone, and for all maternal exposoures when considered as haplotypes of adjacent SNPs. SNP rs11466191 also gave significant evidence of possible interaction with maternal vitamin supplementation and alcohol consumption in the 2 *df* test for G and G × E ($P = 0.04$ and $P = 0.03$ for the 2 *df* test and $P = 0.004$ for the 1 *df* test with vitamin use). Since the marginal effect of this SNP alone (ignoring exposures) was not at all significant, this analysis illustrates the importance of considering possible G × E interaction in the etiology of CL/P.

The patients who received a C allele at rs3771494 in TGFA and a G allele at rs2235373 in IRF6 showed a 5.66-fold increased risk of being a case compared to those who

Table 5 Testing for excess transmission of haplotypes of SNPs rs3821261 (T/G) and rs3771475 (A/G) in TGFA in 297 CL/P case–parent trios considering maternal and paternal transmission separately (implemented with the FAMHAP program)

Population	Haplotype	Frequency	Paternal			Maternal		
			T	NT	Maximum TDT (*P* value)	T	NT	Maximum TDT (*P* value)
Asian (221 trios)	TA	0.612	53.3	41.6	2.005 (0.357)	30.1	55.8	**7.686 (0.007)**
	TG	0.257	27.7	36.4		36.9	30.2	
	GA	0.078	11.2	18.9		16.4	7.7	
	GG	0.053	10.8	6.1		16.6	6.3	
Maryland (76 trios)	TA	0.790	7.8	9.2	1.000 (1.000)	6.5	13.8	2.652 (0.149)
	TG	0.114	2.5	4.5		7.2	4.5	
	GA	0.089	5.7	3.3		7.0	2.7	
	GG	0.007	1.0	0.0		0.3	0.0	

Bold values represent results significant at the $P < 0.05$ level

received the T allele and A allele at these two respective SNPs, and the estimated OR for this combined effect excluded the null hypothesis (Table 6). However, the LRT formally comparing a logistic model with the G × G interaction terms to the baseline model argued this interaction was not statistically significant ($P = 0.2105$).

Discussion

Our study of case–parent trios from different populations (comprising a total of 297 CL/P case–parent trios) showed significant evidence of linkage and disequilibrium for three individual SNPs (rs3771494, rs3821261, and rs426081) in the *TGFA* gene when parent-of-origin was ignored. In screening for parent-of-origin effects, we found suggestive evidence of excess maternal transmission for at least two SNPs: rs3821261 and rs3771475. Analysis of haplotypes of these two SNPs also showed significant deviation from expected for maternal transmission, but not paternal transmission. Several SNPs in the *TGFA* gene also showed possible gene–environment interactions with maternal smoking and alcohol consumption, plus maternal vitamin supplementation. Lastly, we found intriguing patterns of transmission that raise the possibility of interaction between *TGFA* and *IRF6*.

Transforming growth factor alpha (*TGFA*) is a well-characterized mammalian growth factor (Tricoli et al. 1986), first identified as a candidate based on its expression patterns in the developing head and face. *TGFA* is expressed at the medial edge epithelium of fusing palatal shelves (Miettinen et al. 1999). Since the first report of an association between *TGFA* and oral clefts (Ardinger et al. 1989), several other case–control (Christensen and Mitchell 1996) and family-based studies (Maestri et al. 1997; Jugessur et al. 2003a) have found significant evidence of being associated with risk of oral clefts. However, other studies showed no association with risk to CL/P (Vieira 2006). The failure to detect association between oral clefts (in particularly CL/P) and TGFA could be due to both bias and genuine population diversity (Ioannidis et al. 2001). Additionally, previous case–parent trio studies did not consider parent-of-origin effects in testing for linkage in the presence of LD. Our study screened for parent-of-origin effects of individual SNPs and haplotypes and found evidence of excess maternal transmission.

Maternal genotypic effects for non-syndromic CL/P have also been reported for several other candidate genes (*MTHFR*, *CBS*, and *RUNX2*), but these have yet to be confirmed (Van Rooij et al. 2003; Rubini et al. 2005; Sull et al. 2008). Our results for several SNPs in *TGFA* (both analyzed alone and as haplotypes) showed evidence of excess maternal transmission, which could reflect an imprinting effect.

There are several examples of potential gene–environment (G × E) interactions in studies of oral clefts (Vieira 2006), but they have proven difficult to confirm across all studies. *TGFA* is the single most widely studied candidate gene for oral clefts, although several other candidate genes have been examined. Hwang et al. (1995) found evidence of gene–environment interaction between the Taq1 marker in TGFA and maternal smoking for cases of CP, but not CL/P cases. Infants carrying the C2 allele were more likely to have CP if the mother smoked (OR = 5.5, 95% confidence interval = 2.1–4.6). Similar patterns of interaction between TGFA and maternal smoking were found by Shaw et al. (1996), but Zeiger et al. (2005) found substantial heterogeneity among five published CP case–control studies in a meta-analysis. Shaw et al. (1998) found evidence for an interaction between this same *TGFA* marker and nutrient intake; where risk was highest for isolated CL/P (OR = 3.0, 95% CI = 1.4–6.6) among infants with the C2 allele whose mothers did not use vitamin supplements.

In tests for gene–environment interaction presented here, SNPs showing marginal significant evidence of gene effects

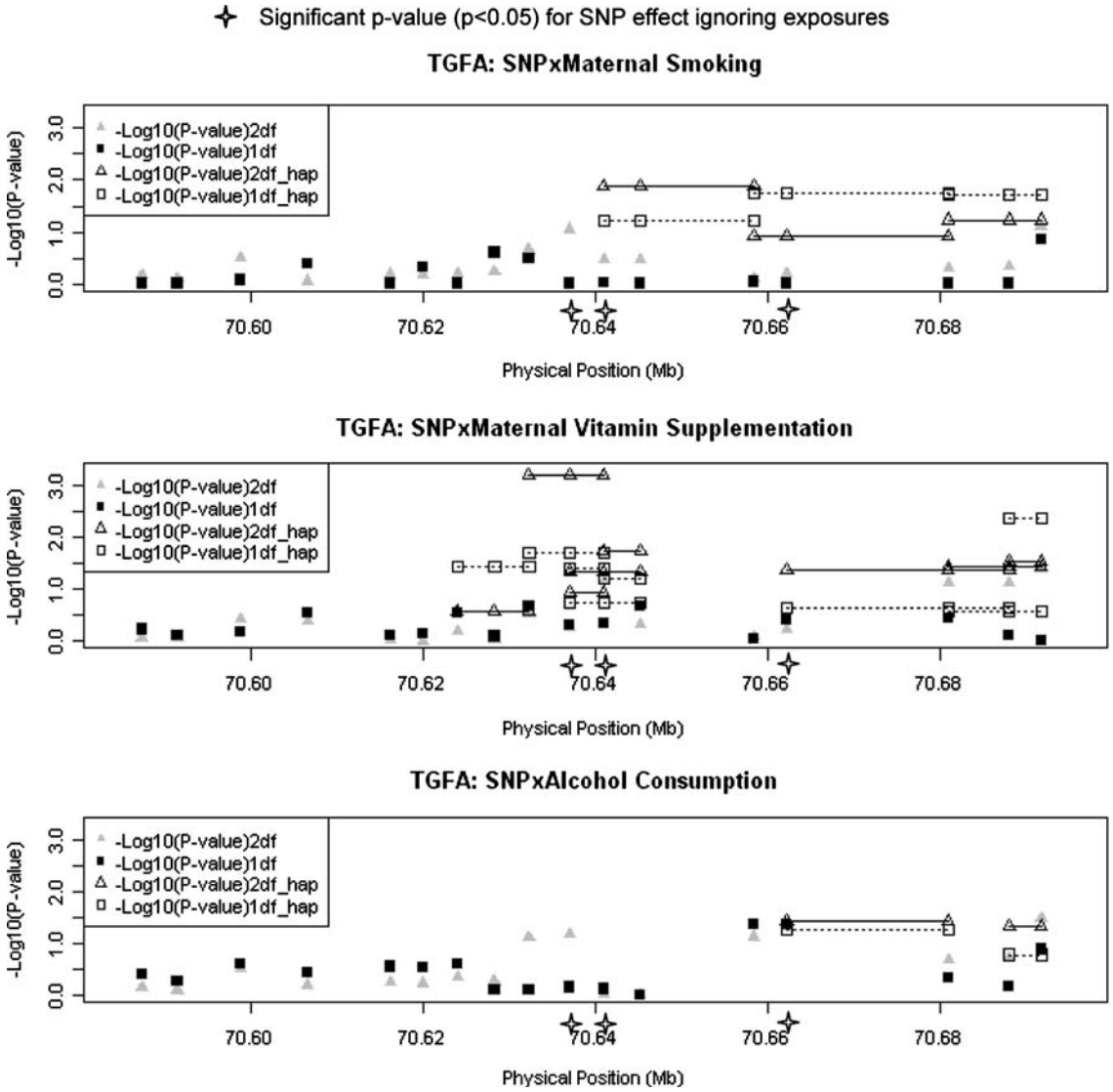

Fig. 2 Testing for main effects (G) of individual SNPs and gene–environment interaction (G × E) for three common maternal exposures in 297 CL/P case–parent trios from 4 populations. *Triangles* represent the 2 *df* test of G and G × E interaction, *squares* represent the 1 *df* test of G × E only. Haplotypes of 2- and 3-SNPs are connected by *dashed lines* (only nominally significant haplotypes are shown here)

Table 6 Odds ratio (OR) for effects of rs3771494 in TGFA and rs2235373 in IRF6 on CL/P main and combined effect

TGFA (rs3771494)	Main effect	IRF6 (rs2235373)	
		Allele 1 (A) OR (95% CI)	Allele 2 (G) OR (95% CI)
		1.0 (reference)	2.43 (1.79–3.31) ($P < 0.0001$)
	OR (95% CI)[a]	Combined effect	
Allele 1 (T)	1.0 (reference)	1.0 (reference)	2.26 (1.61–3.18) ($P < 0.0001$)
Allele 2 (C)	1.64 (1.18–2.27) ($P = 0.003$)	1.53 (0.85–2.74) ($P = 0.17$)	5.66 (3.28–9.76) ($P < 0.0001$)

[a] CI: confidence interval, * P for interaction = 0.2105

ignoring exposures also gave intriguing evidence of possible interaction with common maternal exposures, when considered individually or as haplotypes. We note the exposure prevalence was low for alcohol and smoking, especially in the Asian populations and there were problems with missing data in this study. Low exposure rates

and small sample sizes will become an issue for a valid evaluation of G × E interaction. Therefore, results of these tests for G × E interaction must be considered tentative. The case–parent trio design allows tests for genetic (G) and (G × E) interaction without being grossly affected by heterogeneity in genetic background (a.k.a. population stratification or confounding), but differences in exposure rates remain a source of heterogeneity. Here we found intriguing but tentative evidence of G × E interaction using PBAT in the total sample. Further analysis showed some differences between the MD and Asian trios. For example, rs3771494 showed evidence of a SNP effect ignoring all exposures in the combined sample (where 138 trios were informative for this SNP), and the 1 *df* score test for G × Maternal smoking was significant ($P = 0.022$). Among the 77 MD trios (minor allele frequency, MAF = 0.218 among parents), only 30 trios were informative, but the apparent SNP effect remained significant. Among Asian trios (MAF = 0.208) there were 108 informative trios and the SNP effect was only marginally significant (P value = 0.10), but the 1 *df* score test for G × Maternal vitamin use also approached signficiance (P value = 0.075) despite the lower exposure frequency. Obviously, further confirmation will be needed before firm conclusions can be drawn regarding G × E interaction, but clearly considering G × E interactions may become important in understanding the etiology of CL/P.

For non-syndormic oral clefts, gene–gene interactions have been suggested for TGFA (Vieira 2006). *Interferon Regulatory factor 6* (*IRF6*) is a well-known candidate gene for non-syndromic CL/P, as well as being the primary causal gene for van der Woude syndrome, an autosomal dominant malformation syndrome which can include an oral cleft in carriers. Our previous study also reported several SNPs in *IRF6*, including rs2235373, were associated with CL/P (Park et al. 2007). Recently, IRF6-TGFA gene–gene interactions were observed in a case–parent trios study in human tooth agenesis (Vieira et al. 2007). In the present study, we tested for possible gene–gene interaction between these two genes (IRF6 and TGFA) for CL/P. Patterns consistent with G × G interaction were observed, but were not statistically significant.

Even though this candidate gene study involved a modest number of SNPs in the TGFA gene, addressing the issue of multiple comparisons is necessary before an overall statement about the significance of our findings can be made. Here we relied on a hypothesis-driven approach for 17 SNPs and conducted tests for genetic main effect, haplotype-based test statistics, imprinting, gene–environment interaction with three exposures. SNPs in strong LD typically have highly correlated P values, adjusting significance levels via Bonferroni correction is overly conservative. Therefore, following the strategy in Sull et al. (2008), we adjusted empirical P values for the number of LD blocks, rather than the number of SNPs. In the present study, we have four LD blocks in the gene. In the second block with two SNPs (seen in Table 4), we found evidence against the null hypothesis (the empirical P value of 0.013 would still be marginally significant after correcting for the number of LD blocks). We also used haplotype-based test statistics based on permutation analysis of case–parent trio data. Salyakina et al. (2005) argue permutation tests are generally preferred over adjustments of asymptotic P values based on the estimated correlation structure among multiple markers or on conventional Bonferroni adjustment (which can be too conservative) (Nyholt 2004).

The case–parent trio design offers the advantage of testing directly for maternal versus paternal effects, and allows separating these from effects of the fetal genotype versus parental origin in a robust manner (Cordell et al. 2004; Starr et al. 2005; Sinsheimer et al. 2003). Another advantage of this design is it minimizes issues of confounding that plague traditional case–control designs. This feature permitted pooling trios from diverse populations into a combined test of allelic effects on the OR(transmission) to the affected child, while testing for parent-of-origin effects. The present study suggests maternal transmission effects for markers in *TGFA* and risk of non-syndromic CL/P, and we have found suggestive evidence of gene–environment interaction between markers in TGFA and maternal smoking, alcohol consumption and vitamin use, although further work is still needed to confirm its ultimate impact on risk.

Web resources

HAPLOVIEW: http://www.broad.mit.edu/mpg/haploview/index.php/.

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim/.

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