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Genome-wide analysis of parent-of-origin effects in non-syndromic orofacial clefts

Paras Garg[1,8], Kerstin U Ludwig[2,3,8], Anne C Böhmer[2,3], Michele Rubini[4], Regine Steegers-Theunissen[5,6], Peter A Mossey[7], Elisabeth Mangold[3] and Andrew J Sharp*,[1]

Parent-of-origin (PofO) effects, such as imprinting are a phenomenon where the effect of variants depends on parental origin. Conventional association studies assume that phenotypic effects are independent of parental origin, and are thus severely underpowered to detect such non-Mendelian effects. Risk of orofacial clefts is influenced by genetic and environmental effects, the latter including maternal-specific factors such as perinatal smoking and folate intake. To identify variants showing PofO effects in orofacial clefts we have used a modification of the family-based transmission disequilibrium test to screen for biased transmission from mothers and fathers to affected offspring, biased ratios of maternal *versus* paternal transmission, and biased frequencies of reciprocal classes of heterozygotes among offspring. We applied these methods to analyze published genome-wide single-nucleotide polymorphism (SNP) data from ∼2500 trios mainly of European and Asian ethnicity with non-syndromic orofacial clefts, followed by analysis of 64 candidate SNPs in a replication cohort of ∼1200 trios of European origin. In our combined analysis, we did not identify any SNPs achieving conventional genome-wide significance ($P < 5 \times 10^{-8}$). However, we observed an overall excess of loci showing maternal *versus* paternal transmission bias ($P = 0.013$), and identified two loci that showed nominally significant effects in the same direction in both the discovery and replication cohorts, raising the potential for PofO effects. These include a possible maternal-specific transmission bias associated with rs12543318 at 8q21.3, a locus identified in a recent meta-analysis of non-syndromic cleft (maternal-specific $P = 1.5 \times 10^{-7}$, paternal-specific $P = 0.17$). Overall, we conclude from this analysis that there are subtle hints of PofO effects in orofacial clefting.
European Journal of Human Genetics (2014) **22**, 822–830; doi:10.1038/ejhg.2013.235; published online 30 October 2013

INTRODUCTION

Orofacial clefting (OFC) is one of the most common of all human congenital birth defects with a prevalence rate of 1 in 700 live births worldwide.[1,2] It may either occur in the context of malformation syndromes, or as an isolated anomaly (ie, non-syndromic OFC, NSOFC). Non-syndromic clefts are divided into two categories on the basis of epidemiological and embryological research findings: (i) cleft lip with or without cleft palate (CL/P) and (ii) cleft palate only (CPO).[3,4]

Although genome-wide association studies (GWAS) have identified several genetic loci associated with risk oral clefting,[5–7] there is significant evidence of other biological factors contributing to etiology of OFC.[8] These may include parent-of-origin (PofO) effects such as imprinting,[9] and other effects such as maternal–fetal interactions. In fact, environmental studies have indicated maternal smoking, and potentially alcohol consumption, during pregnancy greatly increase the risk of clefting in offspring, with some evidence of genetic interactions.[2,10,11] Similarly, according to several studies, multi-vitamin supplements with or without folic acid taken during pregnancy have been shown to decrease the risk of oral clefting, with a stronger effect seen in CL/P as compared with CPO.[12–14] If levels of circulating folate are influenced by genetic factors, then it can be hypothesized any maternal genes involved in dictating circulating folate levels could also alter the risk of OFCs in the fetus. Indeed, evidence in support of this idea has recently been published.[15] As such, some genetic effects on disease risk can operate via a mechanism of maternal/fetal interaction. Previous candidate gene studies have suggested evidence for PofO effects in CL/P providing a strong rationale for performing comprehensive analysis to identify unconventional modes of inheritance.[16–19]

However, non-Mendelian inheritance patterns such as imprinting are generally ignored in conventional GWAS, as these tests ignore the differential effects of maternally and paternally inherited alleles on phenotype. Indeed, simple case–control GWAS are unable to address this question. A detailed assessment of the extent of PofO effects across the genome is therefore important for the proper understanding of genome function in relation to disease. Although individual candidate gene studies have suggested possible PofO effects in OFC, a recent GWAS specifically investigated the role of PofO effects in OFCs, but was unable to identify any loci showing genome-wide significant effects.[20]

[1]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, Hess Center for Science and Medicine, New York, NY, USA; [2]Department of Genomics, Life and Brain Center, University of Bonn, Bonn, Germany; [3]Institute of Human Genetics, University of Bonn, Bonn, Germany; [4]Department of Experimental and Diagnostic Medicine, Medical Genetics Unit, University of Ferrara, Ferrara, Italy; [5]Department of Obstetrics and Gynaecology, and Clinical Genetics, Erasmus Medical Center, University Medical Center, Rotterdam, The Netherlands; [6]Department of Epidemiology, Radboud University Nijmegen, Nijmegen, The Netherlands; [7]Orthodontic Unit, Dental Hospital and School, University of Dundee, Dundee, UK
[8]These authors contributed equally to this work.
*Correspondence: Dr AJ Sharp, Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, Hess Center for Science and Medicine, 1470 Madison Avenue, Room S8-116, Box 1498, New York, NY 10029, USA. Tel: +1 212 824 8942; Fax: +1 646 537 8527; E-mail: andrew.sharp@mssm.edu

Previously, we used association methods specifically designed to detect PofO effects, and applied these to successfully detect quantitative trait loci exerting PofO effects on the expression of imprinted genes missed by conventional approaches.[21] These methods take advantage of trio data to first define the parental origin of each allele in the offspring based on rules of Mendelian inheritance, and then perform separate association analyses of the two parental genotypes.

To analyze the role of PofO effects in common complex diseases, here we have employed an extension of the transmission asymmetry test and parental asymmetry test, to detect PofO effects using single-nucleotide polymorphism (SNP) genotyping in trios.[22,23] We applied these methods to re-analyze publicly available genotype data from trios with NSOFC from the database of Genotypes and Phenotypes.[24] Our strategy analyzes associations separately for the maternally and paternally derived alleles, providing considerably increased power to detect PofO effects over conventional GWAS approaches. By first annotating parental origin of each allele in the affected offspring, we can conduct specific tests for association with maternal and paternal alleles independently, together with additional tests to look for differential effects of alleles inherited maternally *versus* paternally. From this initial genome-wide screen, we then selected 64 SNPs showing the strongest putative transmission bias for follow-up in a replication cohort of ~1200 additional trios of European origin. Although we failed to identify any loci with PofO affects achieving genome-wide significance, our results do provide evidence suggesting biases for maternally inherited genetic factors influencing the risk of NSOFC.

METHODS

Genome-wide detection of putative PofO effects

Genome-wide SNP data for 7018 individuals comprising 2339 trios in which each child was affected with any type of NSOFC (CL/P or CPO), were downloaded from dbGaP (Accession number: phs000094.v1.p1).[7] Available genotype data included a total of 1 387 466 SNPs, comprising 601 273 genotyped SNPs and an additional 786 193 SNPs with discrete genotypes imputed by BEAGLE using HapMap Phase II samples as a reference panel. After converting high confidence imputed SNPs at $r^2 \geq 0.9$ to their respective genotypes and filtering non-informative SNPs (see Supplementary Information), we identified the transmitted and non-transmitted alleles in each parent, and the paternally and maternally inherited alleles in each child using rules of Mendelian inheritance. We then performed four different tests to detect putative PofO effects: (i) analysis of transmission bias from heterozygous fathers to affected children (PAT); (ii) analysis of transmission bias from heterozygous mothers to affected children (MAT); (iii) a comparison with the maternal and paternal odds ratios (PofO); and (iv) a comparison of the relative frequency of the two classes of heterozygotes in affected children (HET) (Figure 1). We analyzed this data set using seven different combinations based on disease subtype (NSCL/P and NSCPO[3,4]) and ethnic groups (Europeans and Asians). The number of SNPs and samples used in each analysis are shown in Table 1.

SNPs showing putative parental-specific transmission bias in this discovery cohort were defined as follows: as a primary filter, we first selected those SNPs showing nominal significance ($P_{PofO} < 0.05$) in the PofO test, despite the large number of tests. Then, using a significance threshold of $P < 1 \times 10^{-5}$, SNPs were considered to show a possible PofO bias if they were significant in any of the four tests: PAT, MAT, PofO and HET. A subset of these SNPs was then carried forward for further investigation in a replication cohort (see below).

Using the PLINK -blocks function,[25] we calculated the number of distinct linkage disequilibrium (LD) blocks containing GWAS SNPs showing $P_{PofO} < 0.05$, and either $P_{MAT} < 10^{-4}$ or $P_{PAT} < 10^{-4}$. Enrichment analysis was performed using χ^2-test with d.f. = 1, under the null hypothesis that there should be equal numbers of MAT and PAT LD blocks.

Replication study

We selected 64 SNPs (32 SNPs for NSOFC, 33 SNPs for NSCL/P and 5 SNPs for NSCPO; with six SNPs shared between NSOFC and NSCL/P) for a replication study using 1197 European trios. Seven hundred and forty six trios were part of the EUROCRAN/ITALCLEFT studies (273 from the Netherlands, 124 from Italy, 118 from the UK, 73 from Slovakia, 71 from Hungary, 33 from Bulgaria, 23 from Slovenia, 21 from Estonia and 10 from Spain), and the 451 remaining trios were recruited in Bonn, Germany.[6] Two additional SNPs overlapping *SEMA4D*, a gene with roles in axon guidance,[26] were also included for CLP + CPO replication analysis. At the phenotypic level, the sample was subdivided into 931 trios where the index patient had NSCL/P and 266 with NSCPO (see Supplementary Information).

Genotyping was conducted using Sequenom MALDI-ToF mass spectrometer MassArray system (Sequenom Inc., San Diego, CA, USA). Primers were synthesized at Metabion (Martinsried, Germany). Using Sequenom MassARRAY Assay Design Software 3.4, two multiplex assays comprising all 64 selected SNPs plus the gender-specific variant were designed. Genotype data were analyzed using Sequenom Spectrodesigner Software package. Inter- and intraplate duplicates were included to check for genotype consistencies across DNA plates. Allele peaks were analyzed using Sequenom Typer Analysis software and genotype calls were confirmed by visual inspection of cluster plots. After quality control filtering, our final filtered replication data set comprised 48 SNPs mapping to 38 distinct chromosomal loci (see Supplementary Information). As the patient consent does not allow any unrestricted release of data, even in anonymised form, genotypes were not deposited in a public database. However, genotypes can be provided by the authors upon request.

In the replication sample PAT, MAT, PofO and HET tests for analysis of PofO effects were performed, as described above. Subsequently, combined analyses in which we pooled both the discovery and replication samples were conducted.

RESULTS

Genome-wide analysis of PofO effects

Table 1 summarizes the results for genome-wide analysis of PofO effects in the different phenotypic and ethnic groups analyzed. Based on a low stringency discovery threshold of $P < 1 \times 10^{-5}$ in any of the four tests for PofO effects (PAT, MAT, PofO and HET), among the combined NSCL/P and NSCPO samples, we identified a total of 55 SNPs (representing 13 distinct LD blocks) when the two ethnic groups were analyzed together, 31 SNPs (21 LD blocks) using only European samples, and 9 SNPs (8 LD blocks) using only Asian samples (Supplementary Table 1). Similarly, when performing our analysis based on each disease subtype, we identified an additional 52, 21 and 16 SNPs (falling in 14, 17 and 12 separate LD blocks) associated with NSCL/P based on the analysis of all samples combined (Europeans and Asians), respectively (Supplementary Table 2). In the analysis of NSCPO, we identified 36 SNPs (representing 19 LD blocks) when analyzing all ethnic groups (Supplementary Table 3).

The most significant site, showing a putative association only when transmitted by the mother, was produced by SNP rs3814878 (16p11.2) in the MAT test ($P_{MAT} = 1.69 \times 10^{-7}$). This same SNP showed no significant transmission bias in PAT test ($P_{PAT} = 0.052$) (Figure 2, SNP M11). This region contains an interesting candidate gene for OFC, namely *TBX6* (Supplementary Figure 1).

To investigate whether there was any bias in the transmission ratio of nominally significant associations between the two parental alleles, we calculated the number of independent loci (LD blocks) containing any SNP exceeding a threshold of $P < 1 \times 10^{-4}$ in the MAT and PAT tests for each ethnic group and each category of OFC (Figure 3). In every case the total number of maternal-specific signals equaled or exceeded the number of paternal-specific signals, with a significant excess of maternal-specific signals observed in three categories: All
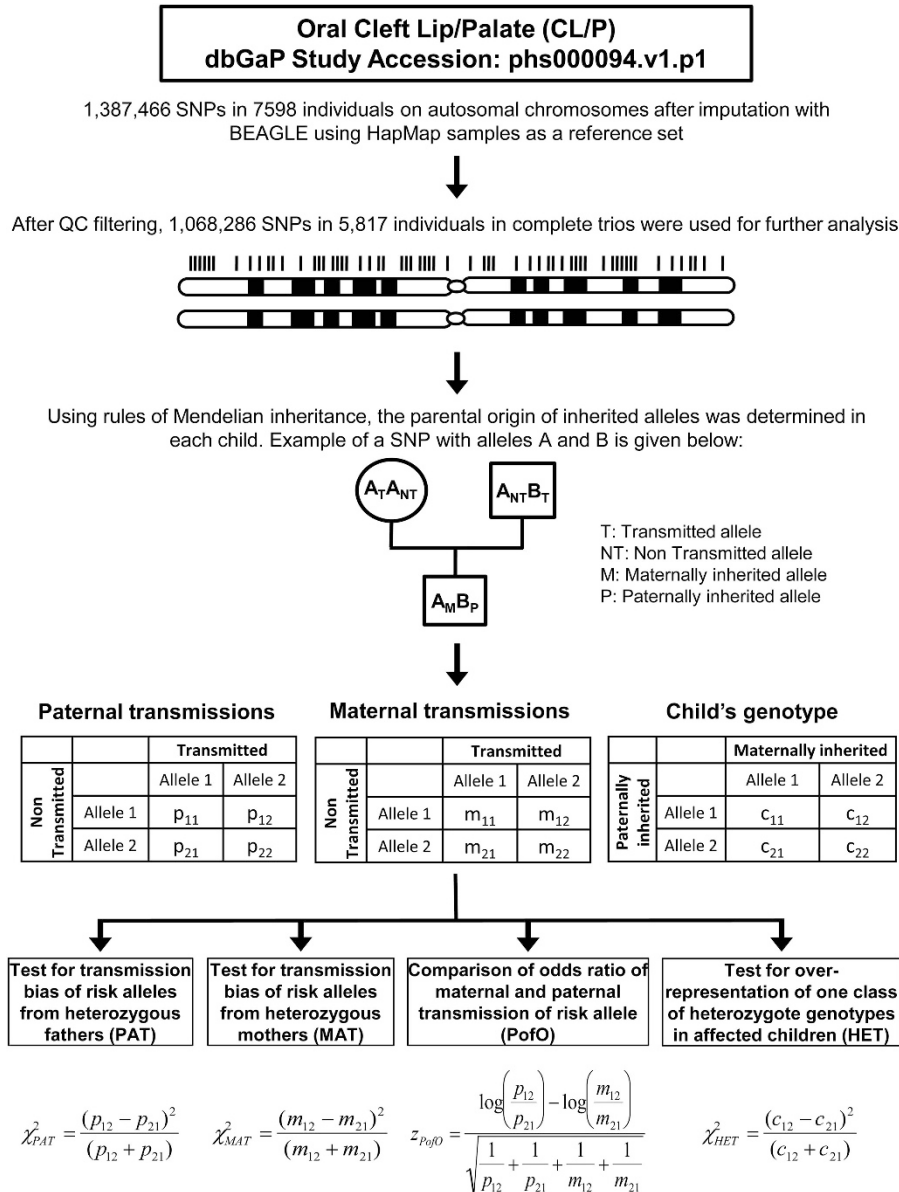
Oral Cleft Lip/Palate (CL/P)
dbGaP Study Accession: phs000094.v1.p1

1,387,466 SNPs in 7598 individuals on autosomal chromosomes after imputation with BEAGLE using HapMap samples as a reference set

After QC filtering, 1,068,286 SNPs in 5,817 individuals in complete trios were used for further analysis

Using rules of Mendelian inheritance, the parental origin of inherited alleles was determined in each child. Example of a SNP with alleles A and B is given below:

$A_T A_{NT}$ $A_{NT} B_T$

$A_M B_P$

T: Transmitted allele
NT: Non Transmitted allele
M: Maternally inherited allele
P: Paternally inherited allele

Paternal transmissions

Non Transmitted		Transmitted	
		Allele 1	Allele 2
Allele 1		p_{11}	p_{12}
Allele 2		p_{21}	p_{22}

Maternal transmissions

Non Transmitted		Transmitted	
		Allele 1	Allele 2
Allele 1		m_{11}	m_{12}
Allele 2		m_{21}	m_{22}

Child's genotype

Paternally inherited		Maternally inherited	
		Allele 1	Allele 2
Allele 1		c_{11}	c_{12}
Allele 2		c_{21}	c_{22}

Test for transmission bias of risk alleles from heterozygous fathers (PAT)	Test for transmission bias of risk alleles from heterozygous mothers (MAT)	Comparison of odds ratio of maternal and paternal transmission of risk allele (PofO)	Test for over-representation of one class of heterozygote genotypes in affected children (HET)

$$\chi^2_{PAT} = \frac{(p_{12} - p_{21})^2}{(p_{12} + p_{21})} \qquad \chi^2_{MAT} = \frac{(m_{12} - m_{21})^2}{(m_{12} + m_{21})} \qquad z_{PofO} = \frac{\log\left(\frac{p_{12}}{p_{21}}\right) - \log\left(\frac{m_{12}}{m_{21}}\right)}{\sqrt{\frac{1}{p_{12}} + \frac{1}{p_{21}} + \frac{1}{m_{12}} + \frac{1}{m_{21}}}} \qquad \chi^2_{HET} = \frac{(c_{12} - c_{21})^2}{(c_{12} + c_{21})}$$

Figure 1 Summary of the method used to screen for parent-of-origin effects in orofacial clefts.

(NSCL/P + NSCPO, $P = 0.013$), Europeans (NSCL/P + NSCPO, $P = 0.026$) and All (NSCPO, $P = 0.003$). A similar excess of loci showing maternal-specific transmission bias was also observed using SNPs with an increased significance threshold of $P < 1 \times 10^{-5}$ in the MAT and PAT tests, although due to the smaller number of loci, these differences did not reach statistical significance (data not shown).

We calculated the genomic inflation factors for the MAT and PAT tests (Supplementary Table 4). We observed a small increase in the genomic inflation factor for the MAT compared with the PAT in all categories, although the magnitude of this bias is small, with the maximum genomic inflation factor observed being 1.04. QQ plots for the MAT and PAT are shown in Supplementary Figure 2.

Replication study
The results of replication analysis using 48 SNPs passing all quality filtering steps are summarized in Table 2 and Supplementary Table 5.

Among the SNPs exceeding our low stringency discovery thresholds in the initial GWAS ($P \leq 1 \times 10^{-5}$), only two passed our thresholds for successful replication, showing $P \leq 0.01$ with the MAT or PAT in the replication cohort in the same direction as observed in the discovery phase. Considering the replication data alone, the most significant P-value observed was $P_{PAT} = 0.002$ at SNP rs719325 in NSCL/P (Figure 4). This same SNP yielded $P_{PAT} = 8.1 \times 10^{-6}$ in the discovery GWAS (using combined European and Asian samples), giving a combined $P_{PAT} = 5.4 \times 10^{-8}$. However, although in the discovery GWAS this SNP yielded $P_{PofO} = 0.005$ and $P_{MAT} = 0.69$ suggesting this locus shows a paternal-specific transmission bias, this effect was not seen in the replication cohort, with $P_{PofO} = 0.41$ and $P_{MAT} = 0.048$. Results using a conventional TDT test for this SNP were $P_{TDT} = 7.8 \times 10^{-4}$ in the discovery GWAS, $P_{TDT} = 0.002$ in the replication cohort, yielding a combined $P = 4.9 \times 10^{-6}$. A second SNP rs12543318, yielded $P_{MAT} = 2.2 \times 10^{-6}$ in the discovery GWAS (NSOFC in European samples), and $P_{MAT} = 0.004$ in the replication

Table 1 Summary of genome-wide screening for parent-of-origin effects in oral clefts

Cleft type	Ethnicity	Number of complete trios utilized in genome-wide analysis	Number of filtered SNPs used in genome-wide analysis	No. of SNPs (independent LD blocks) identified in genome-wide screen with $P < 1 \times 10^{-5}$ in MAT, PAT, PofO or HET tests	SNPs carried forward to replication study
NSCL/P and NSCPO	All individuals	1939	1 068 286	55 (13)	$11 + 2^a$
	Europeans	758	1 060 874	31 (21)	19
	Asians	949	952 138	9 (8)	—
NSCL/P	All individuals	1491	1 068 429	52 (14)	15
	Europeans	570	1 060 675	21 (17)	18
	Asians	742	952 438	16 (12)	—
NSCPO	All individuals	437	1 068 897	36 (19)	5

Abbreviations: HET, heterozygous; LD, linkage disequilibrium; MAT, maternal; NSCL/P, non-syndromic cleft lip with or without cleft palate; NSCPO, non-syndromic cleft palate only; PAT, paternal; PofO, parent-of-origin; SNP, single-nucleotide polymorphism.
[a]Two SNPs overlapping the *SEMA4D* gene were added to our replication set (see Methods).
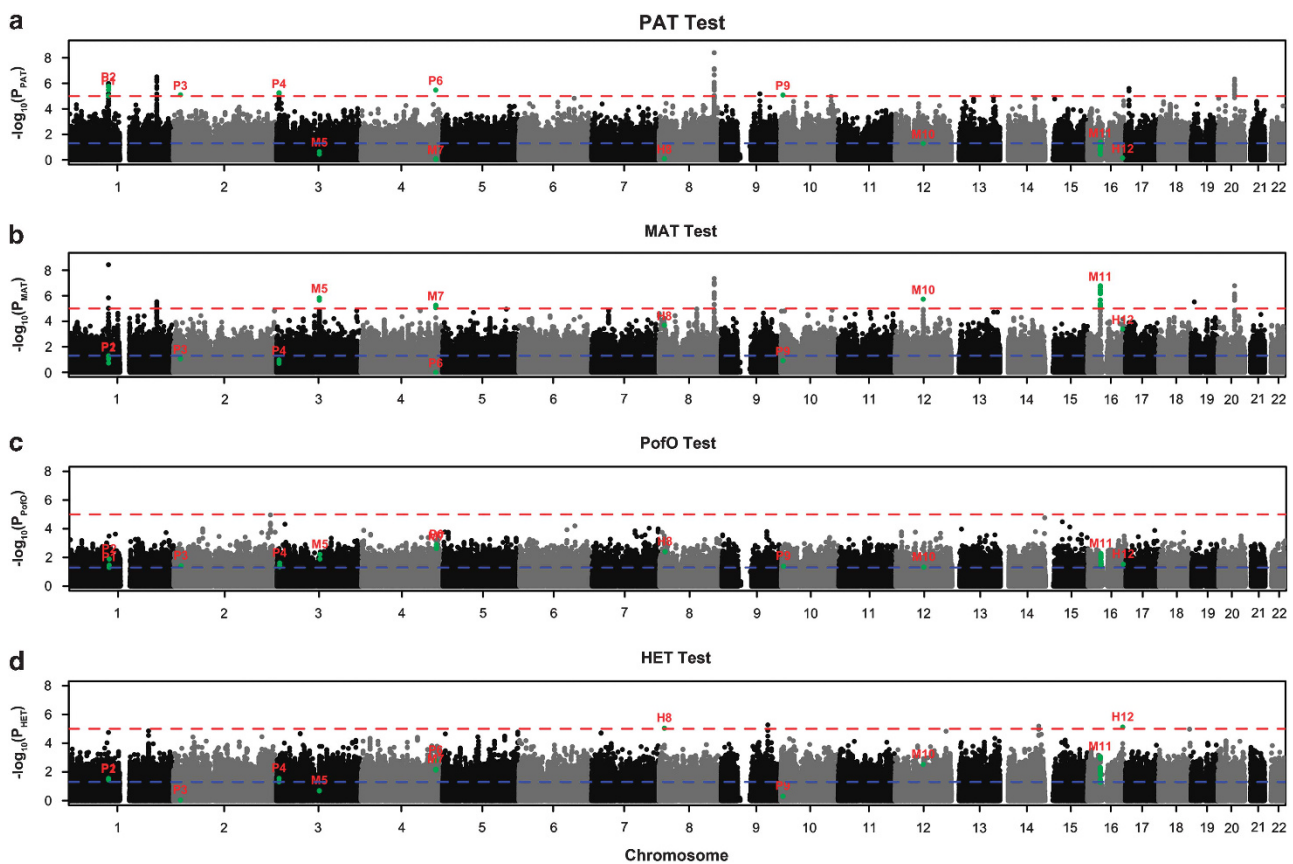
Figure 2 Manhattan plot showing the results of four tests for 22 autosomes performed using all ethnic groups (Europeans and Asians) and disease subtypes (CL/P and CPO) combined. The y axis shows the $-\log_{10}$ P-value for each test and the red and blue lines represent thresholds of $P < 1 \times 10^{-5}$ (suggestive association) and 0.05 (nominal significance), respectively. Green dots represent loci showing suggestive associations identified by the four tests (see Methods). Each locus is labeled based on the test showing $P < 1 \times 10^{-5}$: P# for PAT, M# for MAT, PofO# for PofO test and H# for HET. The P-values generated by four tests for each locus are shown in all four panels using the same label. For example, all SNPs at M11 loci have $P < 10^{-5}$ in MAT (above red line), while the same SNPs show $P > 0.05$ in PAT (below blue line). These SNPs show nominal significance in PofO and HET (between blue and red line).

cohort, giving a combined $P_{MAT} = 1.5 \times 10^{-7}$ (Figure 4). However, although this SNP yielded $P_{PofO} = 0.009$ in the GWAS PofO test, and $P_{PAT} = 0.24$ and 0.43 in the discovery and replication cohorts, respectively, suggesting a putative maternal-specific transmission bias, results for the PofO test in the replication cohort were not clear ($P = 0.12$). Results using a conventional TDT test for this SNP were $P_{TDT} = 1.2 \times 10^{-4}$ in the discovery GWAS, $P_{TDT} = 0.022$ in the

replication cohort, yielding a combined $P_{TDT} = 2.4 \times 10^{-5}$. This same SNP also yielded very similar results in NSCL/P using both European alone, and the combined European and Asian GWAS and replication samples.

Results for the 48 SNPs using a conventional TDT (which does not consider parental origin) are listed in Supplementary Table 6. We observed four SNPs (corresponding to three LD blocks) yielding both
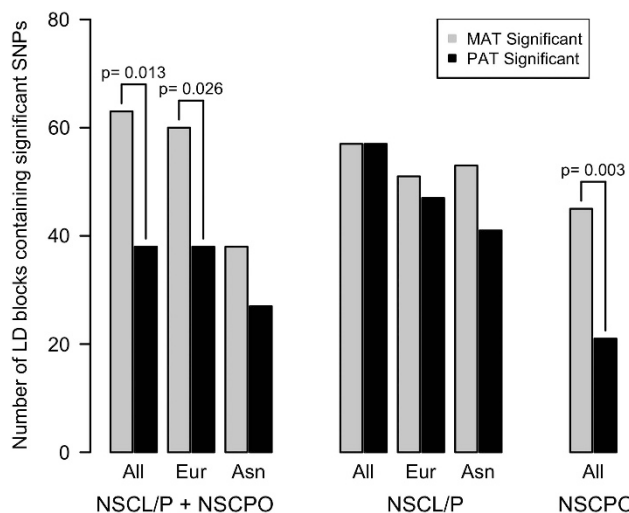
Figure 3 A global bias for maternal genetic effects in the etiology of OFCs. In every comparison using different subtypes of orofacial clefts and ethnicities (All: all ethnic groups, Eur: Europeans and Asn: Asians), we observed that the number of maternal-specific signals equals or exceeds the number of paternal-specific signals. A significant excess ($P<0.05$) of maternal-specific signals is observed in three categories, as indicated. Bar plots show the number of independent loci (LD blocks) with $P<1\times10^{-4}$ identified using the MAT (gray) and PAT (black) tests. A similar excess of maternal associations was also observed using a more stringent significance threshold of $P<10^{-5}$, although due to the smaller number of loci, these differences did not reach statistical significance (data not shown).

$P_{TDT}<0.01$ in the discovery cohort and $P_{TDT}<0.05$ in the replication cohort.

DISCUSSION

In this study, we have applied modified versions of the Transmission Asymmetry Test and Parental Asymmetry Test to perform genome-wide screening for PofO effects associated with non-syndromic oral cleft in mother/father/affected child trios. We used a previously published genome-wide SNP data from ~2500 trios for discovery,[7] followed by genotyping of selected SNPs in a replication cohort of ~1200 trios. Using four tests (PAT, MAT, PofO and HET – see Methods), a low stringency threshold in the discovery cohort identified a total of 210 SNPs (corresponding to 88 independent regions) showing evidence of possible parental-specific transmission bias associated with risk of non-syndromic oral clefts (NSCL/P or NSCPO) in various ethnic groups (all, European or Asian populations). Of these discovery SNPs, 64 were carried forward for testing in the replication cohort.

Although in our replication analysis we observed several loci reaching nominal significance in the same direction as the discovery GWAS data, none of the markers tested achieved generally accepted definitions of genome-wide significance ($P<5\times10^{-8}$) in the combined sample. However, there are several possible explanations for failure to replicate GWAS signals, including the 'winners curse' phenomenon.[27] Of particular note, our replication cohort was approximately half the size of our discovery cohort, and as a result is underpowered to detect small effects on disease risk detected in our discovery phase.

We identified two loci that passed our thresholds for successful replication ($P\leq1\times10^{-5}$ in the discovery cohort and $P\leq0.01$ in replication with either the MAT or PAT), raising the potential for PofO effects tagged by these loci. Paternally biased transmission was

detected for rs719325 at 2q25, and this result approached genome-wide significance in the combined analysis ($P_{PAT}=5.4\times10^{-8}$). This marker is located ~164 kb upstream of *SLC4A3*, a gene which encodes a trans-membrane transport protein involved in regulation of intracellular pH. *SLC4A3* has been described as a candidate gene for human retinal degeneration,[28] however, no role in craniofacial development has been described so far. Aside from genomic imprinting, mechanistically it is harder to conceptualize how paternal-specific effects on disease risk might operate, although some have been recognized.[9]

We also identified a possible maternal-specific transmission bias associated with rs12543318 located within 8q21.3. This same SNP was recently identified as a susceptibility locus for NSCL/P in a recent meta-analysis.[29] This marker maps to an intergenic region for which, so far, no functional information related to OFC is available. Although the meta-analysis of Ludwig *et al.*[29] and the current study utilized many of the same individuals, this previous study did not test for PofO effects. Comparison of the TDT results for the NSCL/P group in the present and the trio-part of Ludwig *et al*[29] reveals they are in the same range (with the slight difference being attributed to different quality controls on samples). However, the present PofO analysis suggests the signal at rs12543318 is largely attributable to maternally derived alleles, as our MAT test yielded a combined $P=1.5\times10^{-7}$, while the corresponding PAT test gave a combined $P=0.17$, with a combined $P_{PofO}=0.004$. No known imprinting effects map to this region. Thus, the 8q21.3 region might contain a genetic element which in some way interacts with other maternal-specific risk effects.

Two other loci that achieved suggestive evidence of replication ($P\leq1\times10^{-5}$ in discovery and $P\leq0.1$ in replication with the MAT) are worthy of mention. rs17447439 maps within an intron of the *TP63* gene at 3q28, and showed weak evidence of a maternal-specific transmission bias. Several studies provide evidence linking *TP63* with the development of OFC. For example, a homozygous mutation in *TP63* has been suggested to have a causative role in NSCL/P.[30] Further, a recent genome-wide analysis of p63-binding sites identified the AP-2α transcription factor as target site.[31] AP-2α is known to be essential for craniofacial development and cranial closure[32] and has been implemented in NSCL/P.[33] Similarly in the analysis of NSCPO, a suggestive bias for maternal over-transmission was observed for rs6539608 located on chromosome 12q21. No genes or transcripts map to this region, making an interpretation of functional relevance difficult. Notably, however, Koillinen *et al*[34] found suggestive linkage to this locus in a study comprising nine Finnish multiplex families affected with NSCPO.[34]

On balance our analysis finds only weak evidence for specific genetic loci individually contributing to PofO effects in OFC. After performing replication analysis of 48 SNPs showing suggestive evidence of PofO effects in the discovery cohort, individually none of these showed unambiguous confirmation of statistical signals that would allow us to define any PofO effects with confidence. These results are broadly consistent with the study of Shi *et al*,[20] which utilized an alternative method to analyze the same discovery cohort. One of the limitations of our method compared with the method used in Shi *et al*[20] is the inability to differentiate between different types of parental biases, such as imprinting or maternal effects. Our analysis approach also does not directly consider environmental effects such as the maternal intrauterine environment. One advantage of our method, however, is that by performing independent tests of the maternally and paternally derived alleles (MAT and PAT tests) we are able to directly compare the relative

Table 2 Associations results for parent-of-origin effects in oral clefts, showing the most significant SNP per locus in both discovery and replication cohorts

SNP ID	Chromosome	Position, hg18	GWAS data				Replication data				Combined data			
			P_{PAT}	P_{MAT}	P_{PotO}	P_{HET}	P_{PAT}	P_{MAT}	P_{PotO}	P_{HET}	P_{PAT}	P_{MAT}	P_{PotO}	P_{HET}
SNPs significant in NSOFC in the combined European and Asian GWAS sample analysis														
rs1394156	Chr3	6199872	5.57E-06	0.198	0.024	0.028	0.668	0.630	0.520	0.144	0.001	0.193	0.169	0.008
rs704570	Chr3	100959832	0.225	2.21E-06	0.013	0.213	0.170	0.186	0.997	0.345	0.070	5.60E-06	0.049	0.119
rs4627838	Chr4	175839337	0.885	5.53E-06	1.72E-03	0.007	0.479	0.116	0.519	0.568	0.545	1.41E-05	0.007	0.133
rs11999884	Chr9	91260211	6.68E-06	0.049	0.072	0.439	Failed QC in replication				Failed QC in replication			
rs1920435	Chr12	66081140	0.051	1.85E-06	0.047	0.003	Failed QC in replication				Failed QC in replication			
rs3814878	Chr16	29949467	0.052	1.69E-07	0.018	0.006	0.272	0.725	0.616	0.411	0.393	7.42E-05	0.025	0.007
SNPs significant in NSOFC in the European GWAS sample analysis														
rs4925671	Chr1	245689497	0.816	5.65E-06	0.002	0.005	0.034	0.458	0.043	0.626	0.158	3.87E-04	4.36E-04	0.026
rs10208086	Chr2	3154999	0.287	2.81E-06	0.015	0.070	0.255	0.170	0.873	0.738	0.121	4.57E-05	0.085	0.166
rs820042	Chr2	173853723	5.67E-06	0.751	0.003	0.008	0.414	0.110	0.560	0.405	0.001	0.147	0.166	0.410
rs12543318	Chr8	88937456	0.237	**2.24E-06**	0.009	0.109	0.433	0.004	0.122	0.462	0.174	**1.54E-07**	0.004	0.106
rs1519466	Chr9	110090618	0.198	0.075	0.034	6.61E-06	0.209	0.030	0.016	0.317	0.905	0.792	0.792	0.019
rs12264639	Chr10	15368599	0.034	3.44E-05	9.46E-06	0.004	0.386	0.257	0.156	0.024	0.523	0.085	0.091	0.968
rs1533763	Chr11	75578175	0.630	7.66E-06	3.41E-04	0.002	0.411	0.695	0.768	0.620	0.732	0.002	0.050	0.129
rs4944925	Chr11	74037177	0.082	0.022	0.004	5.97E-06	0.817	0.275	0.351	0.765	0.189	0.022	0.011	0.001
rs927053	Chr14	58524433	0.783	6.11E-06	0.001	0.535	0.932	0.697	0.841	0.125	0.901	0.014	0.100	0.473
rs7173522	Chr15	99135612	2.21E-06	0.141	0.025	0.008	0.530	0.107	0.461	0.788	0.001	0.029	0.417	0.057
rs11644607	Chr16	74806998	0.004	0.905	0.028	1.97E-07	0.061	1.000	0.172	0.095	0.001	0.937	0.013	1.72E-06
rs3852922	Chr20	53443077	0.382	7.24E-06	0.010	0.056	0.387	0.274	0.865	0.438	0.899	0.046	0.133	0.525
SNPs significant in NSCLP in the combined European and Asian GWAS sample analysis														
rs719325	Chr2	220376177	**8.07E-06**	0.692	0.005	0.353	0.002	0.048	0.412	0.403	**5.43E-08**	0.118	0.007	0.835
rs7618286	Chr3	6194615	2.24E-06	0.654	0.003	0.003	0.060	0.489	0.392	0.356	0.012	0.918	0.067	0.004
rs704570	Chr3	100959832	0.018	3.30E-06	0.119	0.960	0.218	0.304	0.906	0.282	0.009	1.67E-05	0.233	0.462
rs13317017	Chr3	101148211	0.109	4.14E-07	0.016	0.196	0.110	0.262	0.757	0.955	0.024	3.85E-06	0.094	0.319
rs17447439	Chr3	191032117	0.191	6.94E-06	0.049	0.001	1.000	0.062	0.202	0.131	0.320	2.63E-06	0.016	4.61E-04
rs12543318	Chr8	88937456	0.005	4.30E-06	0.176	0.207	0.548	0.010	0.143	0.336	0.010	**1.80E-07**	0.047	0.113
rs543704	Chr11	91869646	5.40E-06	0.147	0.033	0.031	0.481	0.098	0.092	0.206	7.90E-05	0.942	0.007	0.014
rs2196457	Chr12	94798821	0.269	9.20E-06	9.84E-05	0.001	Failed QC in replication				Failed QC in replication			
rs2948150	Chr12	119645972	0.002	0.149	0.001	3.36E-06	0.583	0.729	0.899	0.222	0.161	0.200	0.058	0.035
rs4884077	Chr13	77441896	0.059	0.313	0.040	9.94E-06	0.812	0.172	0.420	0.388	0.081	0.842	0.166	0.001
rs1777697	Chr14	82206283	7.58E-06	0.320	1.20E-04	0.004	0.520	0.894	0.588	0.080	5.11E-05	0.370	0.001	0.001
rs6011617	Chr20	61209037	9.05E-06	0.424	0.009	0.139	Failed QC in replication				Failed QC in replication			
SNPs significant in NSCLP in the European GWAS sample analysis														
rs12132001	Chr1	161092955	7.86E-06	0.746	0.003	0.041	0.467	0.648	0.402	0.604	0.026	0.576	0.233	0.409
rs10208086	Chr2	3154999	0.224	4.66E-06	0.024	0.035	0.243	0.166	0.879	0.914	0.095	6.31E-05	0.111	0.231
rs1513121	Chr3	103247757	0.633	3.31E-06	0.001	0.869	0.795	1.000	0.860	0.199	0.615	0.006	0.085	0.264
rs26883	Chr5	4959824	0.349	1.62E-06	0.007	0.035	0.382	0.559	0.853	0.899	0.962	0.004	0.040	0.132
rs9687030	Chr5	76663192	0.221	7.82E-06	0.020	0.134	0.391	0.684	0.732	0.113	0.855	0.009	0.092	0.029

Table 2 (Continued)

SNP ID	Chromosome	Position, hg18	GWAS data				Replication data				Combined data			
			P_{PAT}	P_{MAT}	P_{PofO}	P_{HET}	P_{PAT}	P_{MAT}	P_{PofO}	P_{HET}	P_{PAT}	P_{MAT}	P_{PofO}	P_{HET}
rs12543318	Chr8	88937456	0.311	**2.89E-06**	0.006	0.103	0.548	**0.010**	0.143	0.336	0.269	**7.85E-07**	0.004	0.071
rs2948372	Chr8	12802775	0.146	0.016	0.006	6.13E-06	0.736	1.000	0.812	0.655	0.216	0.209	0.079	0.002
rs890519	Chr8	18949329	5.63E-06	0.776	0.003	0.085	0.494	0.119	0.554	0.267	4.18E-04	0.169	0.120	0.821
rs7922467	Chr10	129643702	0.013	0.025	0.001	7.10E-06	0.083	0.001	0.291	0.918	0.003	0.467	0.089	0.001
rs12426170	Chr12	119597971	0.001	0.365	0.003	8.60E-06	0.473	0.532	0.954	0.356	0.210	0.283	0.099	0.050
rs7988514	Chr13	19763521	0.221	2.89E-06	0.013	0.013	0.860	0.516	0.737	0.610	0.362	0.001	0.069	0.054
rs4429270	Chr15	89828778	0.944	5.82E-06	0.002	0.122	0.462	0.700	0.431	0.663	0.527	0.011	0.171	0.525
rs1610093	Chr19	4505774	0.051	0.028	0.004	8.81E-06	0.790	0.728	0.957	0.715	0.187	0.255	0.082	0.013
rs3852922	Chr20	53443077	0.139	6.67E-07	0.013	0.033	0.852	0.660	0.657	0.289	0.285	0.005	0.215	0.568
SNPs significant in NSCPO in the combined European and Asian GWAS sample analysis														
rs11678000	Chr2	229965612	9.43E-08	0.414	0.001	2.56E-04	0.858	0.869	0.808	0.739	7.74E-05	0.463	0.012	0.008
rs512778	Chr6	79401865	0.249	5.93E-06	0.014	0.250	0.056	0.317	0.543	1.000	0.045	1.24E-05	0.078	0.329
rs1483757	Chr12	116245923	3.82E-06	0.115	0.022	0.076	0.448	0.917	0.636	0.414	2.78E-05	0.179	0.034	0.058
rs6539608	Chr12	80976335	1.000	4.50E-06	0.001	0.039	0.768	0.069	0.294	0.639	0.864	1.56E-06	0.001	0.050

Abbreviations: GWAS, genome-wide association studies; HET, heterozygous; LD, linkage disequilibrium; MAT, maternal; NSCL/P, non-syndromic cleft lip with or without cleft palate; NSCPO, non-syndromic cleft palate only; NSOFC, non-syndromic orofacial clefting; PAT, paternal; PofO, parent-of-origin; SNP, single-nucleotide polymorphism.
In bold: SNPs that pass our discovery criteria of $P < 1 \times 10^{-5}$ and show and $P \leq 0.01$ in the replication cohort.

influence of the two parental genomes on risk of OFCs in the child. Our stratified analyses by ethnic groups and type of OFC showed the number of loci showing a maternal-specific transmission bias equaled or exceeded the number of paternal-specific signals, with a significant excess of maternal-specific signals observed in three categories. Although this approach cannot discern the underlying mechanism, this observation suggests overall maternally inherited alleles exert a more significant effect on the risk of OFCs in offspring than do paternally derived alleles. In this regard, it is interesting to note effects of maternal genotype and maternal-specific environmental modifiers such as smoking, drinking and vitamin intake during pregnancy on oral cleft susceptibility have been previously reported in various studies.[2,10–19] However, it should be noted that the parental asymmetry tests we applied here do not specifically test for maternal effects which would be expected to occur via alterations of the *in utero* environment. Calculation of genomic inflation factors for the MAT and PAT tests showed a small increase for the MAT compared with the PAT in all categories, this increased inflation factor in the MAT does not discern the underlying cause, being consistent with either a true excess of biological associations with the maternally inherited alleles, or alternatively differing population structure between the two parental populations.[35] In addition, comparison of the 64 SNPs used in replication analysis with two online catalogs of known imprinted loci (http://igc.otago.ac.nz/home.html and http://www.geneimprint.com/) showed that none are located within 1 Mb of any known imprinted loci. However, we do note that one study recently reported *TBX6*, the most significant locus identified in our genome-wide screen for PofO effects in OFCs, as showing evidence of imprinting in placental tissue.[36] As a result, we suggest that further studies of this locus in OFCs are warranted.

Although we utilized four different tests (MAT, PAT, PofO and HET), we did not apply any multiple testing correction for several reasons. First, as the PofO and HET tests share the same underlying data used by the MAT and PAT these four tests are not independent. Second, although we utilized a combined significance threshold of $P < 5 \times 10^{-8}$, our primary filter in the discovery cohort was to select SNPs showing $P_{PofO} < 0.05$, and thus the MAT and PAT tests were only applied to a relatively small fraction of the total genotypes available. Third, the use of a two-stage discovery and replication design in an additional cohort of ~1200 trios of European descent, should be considerably more robust than any previous studies of PofO effects in OFC.

Both the method we have used, and that used by Shi et al[20] are heavily dependent on SNPs that tag loci showing PofO-specific transmission biases. Using this assumption, we can identify some types of PofO effects, but not all. We also analyzed our discovery and replication data using a conventional TDT which does not consider parental origin. In this analysis, we observed three loci yielding nominal replication with both $P_{TDT} < 0.01$ in the discovery cohort and $P_{TDT} < 0.05$ in the replication cohort (combined discovery plus replication TDT P-values ranging from 2.4×10^{-4} to 3.3×10^{-7}). It should be noted that our MAT and PAT tests are essentially a modification of the TDT in which only one half of the genotypes inherited by each child are considered. When considered as individual tests, they are also capable of detecting risk alleles that do not exhibit PofO effects, although with reduced power compared with the conventional TDT. For example, as can be seen in Figure 2, previously identified OFC risk loci such as the 8q24 locus[5] were detected, but as these show significant P-values in both the MAT and PAT tests strong PofO effects at these markers can be excluded. Therefore, a reasonable
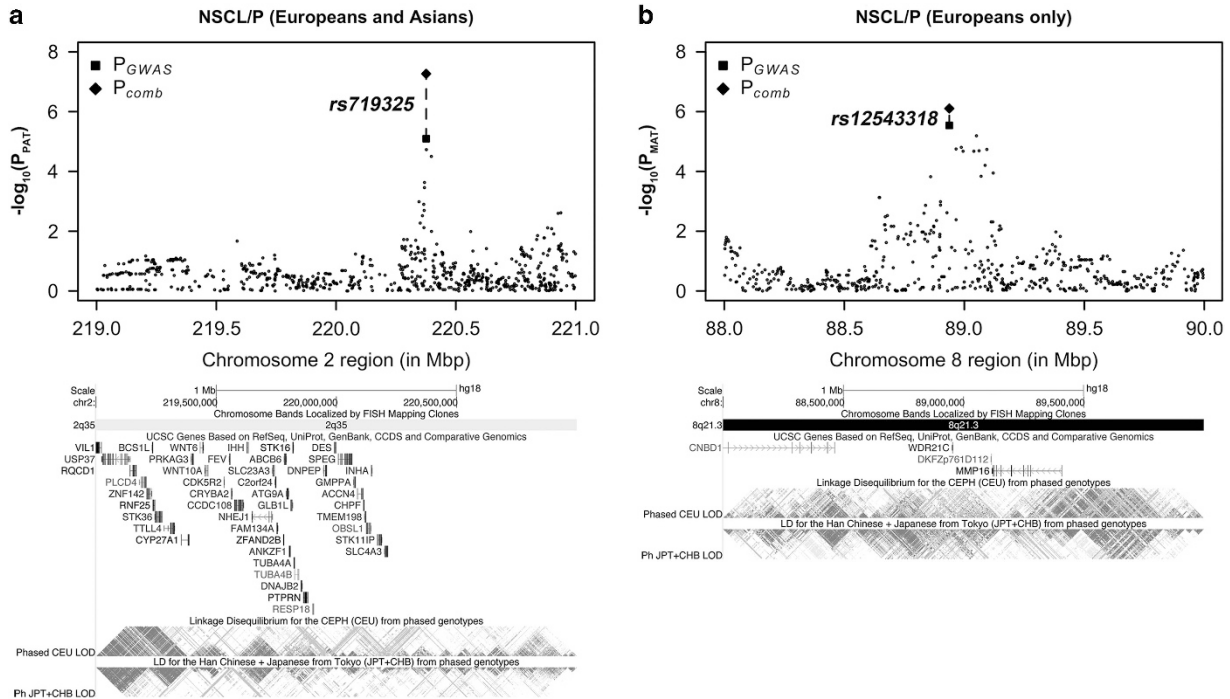
Figure 4 Loci on 2q35 and 8q21.3 show suggestive evidence of PofO effects in NSCL/P in both discovery and replication cohorts. (**a**) A paternal-specific signal at SNP rs719325 on chromosome 2q35 in combined European and Asian analysis (P_{GWAS}: $P_{PAT} = 8.07 \times 10^{-6}$, $P_{MAT} = 0.69$, P_{comb}: $P_{PAT} = 5.43 \times 10^{-8}$, $P_{MAT} = 0.12$). (**b**) A maternal-specific signal at SNP rs12543318 on chromosome 8q21.3 in European-only analysis (P_{GWAS}: $P_{PAT} = 0.31$, $P_{MAT} = 2.89 \times 10^{-6}$, P_{comb}: $P_{PAT} = 0.27$, $P_{MAT} = 7.85 \times 10^{-7}$). The squares and diamond represent P-values from the discovery GWAS and combined analysis, respectively.

alternative interpretation of our results is that some or all of the loci we detected with suggestive PofO effects might simply represent weak risk loci for OFC, and which manifest in our data in only one of the two parental tests by chance due to insufficient power to reliably detect these effects in both MAT and PAT tests. As such, we suggest some of the loci we identify represent interesting candidate regions for future studies of OFC.

In conclusion, our study provides suggestive evidence for PofO effects in susceptibility to OFC, identifying several loci showing a parental transmission bias, and an overall excess of maternal-specific association signals in the genome. Given abundant evidence supporting a role for non-Mendelian and transgenerational inheritance patterns in a variety of different diseases and conditions,[9] we suggest similar analyses considering parental origin of risk alleles will likely reveal novel PofO effects contributing to many human phenotypes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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