HHS Public Access

Author manuscript

Birth Defects Res A Clin Mol Teratol. Author manuscript; available in PMC 2016 December 23.

Published in final edited form as:

Birth Defects Res A Clin Mol Teratol. 2015 October ; 103(10): 857–862. doi:10.1002/bdra.23413.

Novel evidence of association with NSCL/P was shown for SNPs in *FOXF2* gene in an Asian population

Dr. Lingxue Bu,
Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Qingdao University, Qingdao, China

Ms. Qianqian Chen,
Department of Epidemiology and Biostatistics, School of Public Health, Peking University, Beijing, China. National Center for Public Health Surveillance and Information Services, Chinese Center for Disease Control and Prevention, Beijing, China

Dr. Hong Wang,
Department of Epidemiology and Biostatistics, School of Public Health, Peking University; Ministry of Health Key Laboratory of Reproductive Health, Beijing, China

Mr. Tianxiao Zhang,
Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri, USA

Ms. Jacqueline B. Hetmanski,
Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

Dr. Holger Schwender,
Mathematical Institute, Heinrich Heine University Duesseldorf, Duesseldorf, Germany

Ms. Margaret Parker,
Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

Dr. Yah-Huei Wu Chou,
Department of Medical Research, Chang Gung Memorial Hospital, Taipei, Taiwan

Dr. Vincent Yeow,
Department of Plastic Surgery, K K Women's and Children's Hospital, Singapore

Dr. Samuel S. Chong,

Correspondence: Hong Wang, Department of Epidemiology & Biostatistics, School of Public Health, Peking University Health Science Center, Phone: (8610) 82801619, (86)13651015152, hwang2010@aliyun.com.
Dr. Lingxue Bu and Miss Qianqian Chen are joint first authors on this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: HW QC EWJ AFS THB. Performed the experiments: SSC AFS. Analyzed the data: HW QC JBH HS TZ MP BZ. Wrote the paper: LB QC HW TZ EWJ AFS THB JBH MP. Subject recruitment and/or diagnosis: Y-HWC SSC VY EWJ AFS THB JBH.

Department of Pediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; Khoo Teck Puat – National University Children's Medical Institute, National University Health System, Singapore

Ms. Bo Zhang,
Department of Biomedical Engineering, Xi'an JiaoTong University, Xi'an, China

Dr. Ethylin Wang Jabs,
Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York City, New York, USA. Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Dr. Alan F. Scott, and
Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Dr. Terri H. Beaty
Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

Abstract

Background—The forkhead box F2 gene (*FOXF2*) located in chromosome 6p25.3 has been shown to play a crucial role in palatal development in mouse and rat models. To date, no evidence of linkage or association has been reported for this gene in humans with oral clefts.

Methods—Allelic transmission disequilibrium tests were used to robustly assess evidence of linkage and association with nonsyndromic cleft lip with or without cleft palate (NSCL/P) for 9 SNPs in and around *FOXF2* in both Asian and European trios using PLINK.

Results—Statistically significant evidence of linkage and association was shown for two SNPs (*rs1711968,* and *rs732835*) in 216 Asian trios where the empiric *P* values with permutation tests were 0.0016 and 0.005, respectively. The corresponding estimated odds ratios for carrying the minor allele at these SNPs were 2.05 (95%CI=1.41, 2.98) and 1.77 (95%CI=1.26, 2.49), respectively.

Conclusions—Our results provided statistical evidence of linkage and association between *FOXF2* and NSCL/P.

Keywords

FBAT; *FOXF2*; nonsyndromic cleft lip with or without cleft palate; PLINK; SNP; transmission disequilibrium test

INTRODUCTION

Oral clefts (which include cleft lip, cleft lip and palate, and cleft palate) represent a collection of common birth defects that severely influence quality of life of the affected and their families (Wehby et al., 2010). There is strong evidence for some genetic etiology for oral clefts from many family, twin and population studies (Marazita, 2012), and a large number of candidate genes have been suggested as important in the etiology of these

complex and heterogeneous malformations (Jugessur et al., 2009; Dixon et al., 2011). Genome-wide association studies (GWAS) have confirmed at least a dozen genes playing some role in the etiology of oral clefts in recent years (Birnbaum et al., 2009; Beaty et al., 2010; Mangold et al., 2010; Grant et al., 2009), although specific causal variants and biological mechanisms remain unclear.

The human forkhead box F2 gene (*FOXF2*), located in chromosome 6p25.3, has been shown to play a crucial role in palatal development in mouse models (Wang et al., 2003). However, no evidence of linkage or association has been reported for this gene in humans including linkage studies of multiplex NSCL/P families recruited from various populations (Marazita et al., 2004 &2009), two candidate gene studies of population-based Scandinavia NSCL/P case-parent trios (Jugessur et al., 2009&2010) and several GWAS (Birnbaum et al., 2009; Beaty et al., 2010; Mangold et al., 2010; Grant et al., 2009).

To clarify the potential role that *FOXF2* may play in the etiology of this common and complex disorder in humans, we performed allelic transmission disequilibrium tests (TDT) for NSCL/P with markers in and near *FOXF2* gene using both Asian and European case-parent trios. Our results provide statistical evidence of association between markers in *FOXF2* and NSCL/P in Asian trios, and provide new insight to the etiology of this birth defect.

MATERIALS AND METHODS

Ethics statement

Institutional review boards (IRB) or ethical committees from the following institutions reviewed and approved the study: KK Women's and Children's Hospital in Singapore, National University of Singapore, Chang Gung Memorial Hospital in Taiwan, Yonsei University in South Korea, and Johns Hopkins University in the US. Written informed consent was obtained from adult participants (including biological parents of all probands and probands old enough to give their own consent/assent) and parents or guardians of the minor participants.

Sample description

This study includes 216 Asian NSCL/P probands and their biological parents, recruited from cleft lip and palate treatment centers in Taiwan, Singapore, Korea, and 75 European NSCL/P trios, recruited from Maryland (US) and Singapore (n=1) (Table 1). All probands received clinical genetic assessment by a health professional to check for other birth defects or developmental delays and were diagnosed as having a non-syndromic, isolated oral cleft.

SNP selection, DNA, and genotyping

Nine SNPs spanning 7 691 base pairs (bps) in physical distance were genotyped in and near *FOXF2* for the current study. SNPs were chosen with a goal of identifying an average of one SNP per 5 kilo bps of physical distance. The physical distance between adjacent genotyped SNPs ranged from 56 to 2909 bps. The target region, represented by the 9 genotyped SNPs, covered the whole gene from its 5′end (*rs1711971*) to its 3′ end (*rs1737762*) involving all

the two exons (exon 1: *rs11759800*; exon 2:*rs2293783*) and the only intron (*rs3799321*, *rs1711968*, *rs9405473*, *rs732835*, and *rs3823148*) basing on variant NM_1400521 (Fig. 1, http://www.ncbi.nlm.nih.gov/gene/2295). SNPs with "SNP scores" >0.6, high validation levels in dbSNP, and high heterozygosity levels were given priority in selection. Three (*rs1711968*, *rs732835*, and *rs2293783*) of the four SNPs released by the HapMap project (HapMap Data Rel 24/phaseII Nov08, on NCBI B36 assembly, dbSNP b126) were included in the marker panel for the current study. Among these 9 SNPs, *rs1711971* was the only SNP genotyped for both the current study and the published GWAS panel (Birnbaum et al., 2009; Beaty et al., 2010; Mangold et al., 2010; Grant et al., 2009).

Genomic DNA was extracted from peripheral blood using protein precipitation method and stored at −20°C. Aliquots (4μg) of each genomic DNA sample were dispensed into a bar-coded 96-well microtiter plate at a concentration of 100ng/μl and genotyped by Illumina's GoldenGate chemistry (Oliphant et al., 2002) at the Genetic Resources Core Facility (GRCF) of Johns Hopkins University. Two duplicates and four controls from the Centre d'Etude du Polymorphisme Humain (CEPH) collection were included in each plate to evaluate genotyping consistency within and between plates.

Statistical analysis

(1) SNP screening and preliminary analysis—We first screened SNPs for minor allele frequency (MAF) and Hardy-Weinberg equilibrium (HWE) using parents' genotypes, for Asian and European trios, respectively. SNPs with MAF>1% and showing adherence to HWE (P>0.01) were eligible for association analysis. This screening procedure resulted in 6 and 5 eligible SNPs in Asian and European NSCL/P trios, respectively. Further, one additional SNP was excluded due to very low number of informative families in European trios. As a result, six SNPs in Asian and four SNPs in European trios were finally used in association analysis. SNP screening analysis and pairwise linkage disequilibrium (LD, measured as r^2) estimation for eligible SNPs were all carried out using Haploview (v4.2, http://www.broadinstitute.org/haploview/haploview) (Barrett et al., 2005).

In addition, data from the 1000 genome project (Genomes Project Consortium, 2010) for Asian samples (Han Chinese in Beijing, CHB; Han Chinese South, CHS; Japanese individuals, JPT; as well as the combined Asian sample, Asian), and the CEPH collection were used to estimate LD patterns among SNPs genotyped for the current study and those genotyped in the GWAS panel using Haploview.

(2) Association analysis—We performed an allelic TDT analysis, originally proposed by Spielman et al., (1993) on each eligible marker using PLINK (v1.07; http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007) and used Family Based Association Test (FBAT; http://www.biostat.harvard.edu/~fbat/fbat.htm) to calculate the number of informative families (Laird et al., 2006). In addition to the Bonferroni correction to each P-value generated from allelic TDT analysis, an overall empiric P value for each SNP was also generated from 10,000 permutations in Haploview to correct for multiple comparisons.

RESULTS

Preliminary analysis

Three and four monomorphic SNPs among the 9 genotyped SNPs in or near *FOXF2* for the current study were dropped from association analysis in the Asian NSCL/P trios (*rs1711971*, *rs1737762* and *rs11759800*) and in the European NSCL/P trios *(rs11759800*, *rs3799321*, *rs9405473* and *rs3823148*), respectively. The number of informative European trios (n=3) for *rs1711971* was too low for meaningful association analysis, and was excluded as well. The remaining 6 SNPs in Asian parents and 4 SNPs in European parents were all compatible with HWE (at p>0.01). Genotyping call rates in Asian and European founders were no lower than 98.3% and 97.8%, respectively (Table 2).

Among the Asian founders, SNPs *rs1711968*, *rs9405473* and *rs732835* formed the only linkage disequilibrium (LD) block where *rs1711968* and *rs732835* were in strong LD with r^2= 0.72. Among European founders, the only LD block was formed by SNPs *rs732835*, *rs2293783* and *rs1737762* where *rs1711968* was independent and r^2 between *rs1711968* and *rs732835* was 0.31 (Fig. 2).

No evidence of strong LD was shown for SNPs eligible for the current study with SNPs included in the GWAS panel (r2 ranged from 0 to 34) using data from 1000 genomes for CHB, CHS, JPT, and the combined Asian group (Fig. S1).

Association analysis

Statistically significant linkage and association was shown for three SNPs (*rs1711968*, *rs732835* and *rs2293783*) in the NSCL/P Asian trios by allelic TDT analysis for each marker individually using PLINK. The corresponding Bonferroni corrected *P*-values were 0.00072, 0.0043 and 0.032. The overall empiric *P* values for these three SNPs over 10,000 permutations (using Haploview) were 0.0016, 0.005 and 0.054, respectively. The corresponding estimated odds ratios (OR) for carrying the minor allele for *rs1711968* and *rs732835* were 2.05 (1.41, 2.98) and 1.77 (1.26, 2.49), respectively (Table 3). Significance held for *rs1711968* and *rs732835* for Taiwan trios (Table S1).

Among the European trios, nominally significant evidence in the allelic TDT analysis was seen for two adjacent SNPs (*rs732835* and *rs2293783*) which were in strong LD with one another (r^2 = 0.87). However, after Bonferroni correction they were no longer significant (Table 3).

DISCUSSION

The *FOXF2* gene is one of the important candidate genes for oral clefts. It has 1 intron and 2 exons and encodes a transcription factor protein which belongs to a large family of evolutionarily conserved DNA-binding proteins (http://genome.ucsc.edu/cgi-bin) (Clark et al., 1993). It was found to be expressed in developing palate, tongue, oral cavity as well as other organs by in situ hybridization in mouse (Wang et al., 2003; Aitola et al., 2000) and rat (Aitola et al., 2000). The embryonic expression levels in FOXF2-deficient homozygous mutant mouse were the same in the wild-type mouse for several other candidate genes for

oral clefts including *MSX1*, *MSX2*, *TBX1*, *FOXC2* (*MFH1*), and *FOXF1*. All the 34 examined FOXF2-deficient mice were born with cleft palate without any combination of other craniofacial malformations (Wang et al., 2003). In addition, heterozygous DNA sequence variations in *FOXF2* were identified in 2 of the 18 patients with both disorder of sex development and cleft palate but not in any of the 20 normal controls (Jochumsen et al., 2008).

In the current analysis, statistically significant evidence of linkage and association was shown for NSCL/P with two adjacent intronic SNPs (*rs1711968* and *rs732835*) in *FOXF2* in Asian case-parent trios after correction for multiple comparison. These statistical results are consistent with the findings in animal models and provide further support for *FOXF2* as a potential susceptibility gene for NSCL/P in humans. The failure to replicate these significant findings in our European trios may be explained by etiologic heterogeneity, the small sample size available for analysis or missing of important SNPs in the marker panel.

In analyses of Scandinavian nationwide population-based NSCL/P and NSCP case-parent trios, none of the three tested SNPs (*rs1711970*, *rs732835*, *rs2293783*) in or near *FOXF2* in that study showed evidence of linkage or association with either NSCL/P or NSCP (Jugessur et al., 2009&2010). However, our significant findings for *rs732835* in Asians do not necessarily conflict with this Scandinavian study because different population origins may well explain the inconsistency.

Significant linkage or association identified with *rs1711968* for Asian NSCL/P in the current study does not conflict with the published NSCL/P GWAS either (Birnbaum et al., 2009; Beaty et al., 2010; Mangold et al., 2010; Grant et al., 2009). In one of these GWAS for NSCL/P, *rs1711971* is the only SNP tested in *FOXF2* (Grant et al., 2009). For the other 3 GWAS for NSCL/P, *rs1711971* is the only SNP in common (Birnbaum et al., 2009; Beaty et al., 2010; Mangold et al., 2010) in *FOXF2* with those in the marker panel for the current study. None of the other 32 SNPs genotyped in the GWAS panel for *FOXF2* fell into the 7 691 base pair region where the 9 SNPs tested in this study are located. Also, because neither of the statistically significant SNPs (*rs1711968* and *rs732835*) identified in the current study has been included in the previous NSCL/P GWAS, and they are unlikely in tight LD with SNPs genotyped in the GWAS panel, our study can be seen as an independent analysis although 58.3% (126 trios) of our Asian sample went into one of these GWAS where only one SNP (*rs11753773*, P=0.01) reached nominal significance of linkage or association with NSCL/P (Beaty et al., 2010). Because *rs1711968* is a common SNP and located in the intronic region of the gene, the statistically significant association seen between NSCL/P and *rs1711968* in our Asian trios could possibly be explained by LD with an unobserved causal variant.

This study reported the first significant linkage and association between markers in *FOXF2* and NSCL/P in humans, building upon previous experimental animal studies. Further exploration is warranted to replicate these findings in other populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by grants from The National Natural Science Foundation of China (No. 81273164, HW), the National Institute of Dental & Cranial Facial Research (No. R21-DE-013707 and R01-DE-014581, TH Beaty), and Fogarty Institution (No. D43-TW006176, EW Jabs). The funders had no role in study design, data collection and data analysis, decision for publication, or preparation of the manuscript. We thank all participants who donated samples for this multi-center study of oral clefts, as well as staff at each participating site and institution.

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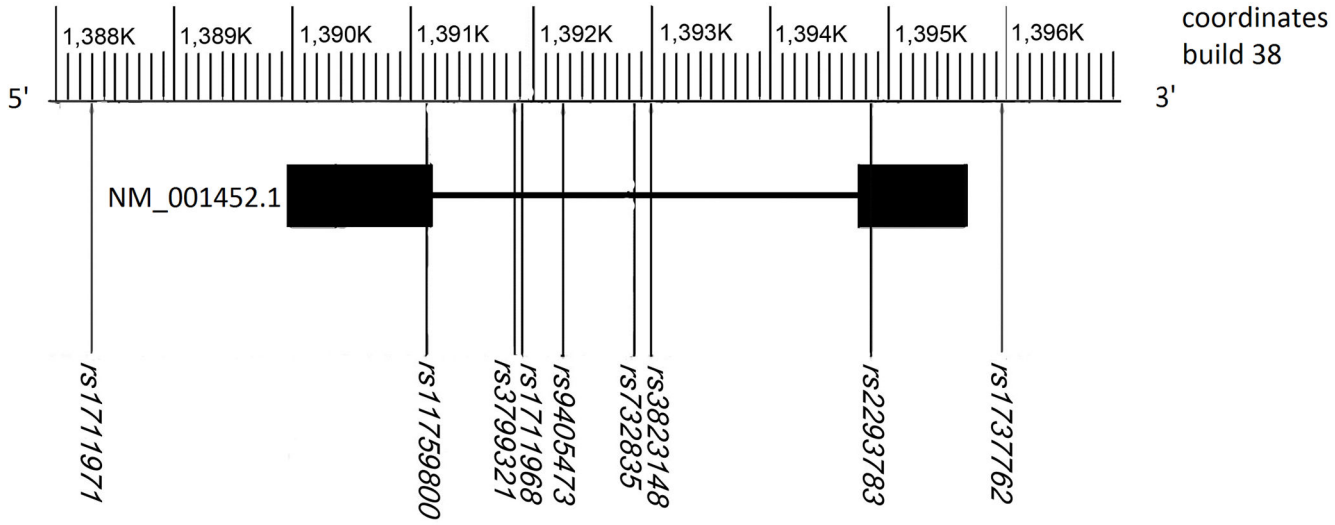

Fig. 1.

Schematic genomic structure of *FOXF2* and coordinates of 9 SNPs genotyped in this study and SNPs representing those genotyped using Illumina 610-Chips for NSCL/P GWAS. Variant NM_001452.1 of the human *FOXF2* gene is shown in the figure with 2 exons aligned from left to right. Filled black boxes represent exons. Scale on top of the figure shows position of the gene and coordinates for the 9 SNPs (black) genotyped in this study and some of the 32 SNPs (gray) genotyped using Illumina 610-Chips in NSCL/P GWAS basing on GRCh build 38. There are 5 SNPs (*rs1737753*, *rs10498654*, *rs1711969*, *rs1878475* and *rs1711970*) genotyped between *rs12524544* and *rs1711971* at the 5′ of the gene, and there are another 24 SNPs (*rs4959557*, *rs1711973*, *rs746095*, *rs731394*, *rs9501718*, *rs9328051*, *rs11753773*, *rs9378623*, *rs1711959*, *rs1711960*, *rs9392288*, *rs1555110*, *rs1737786*, *rs9502924*, *rs11242687*, *rs9405475*, *rs2493158*, *rs9502928*, *rs1922932*, *rs10458124*, *rs7774941*, *rs17202895*, *rs9328053*, *rs932410*) genotyped between *rs6596817* and *rs2816251* at the 3′ of the gene in the NSCL/P GWAS panel.

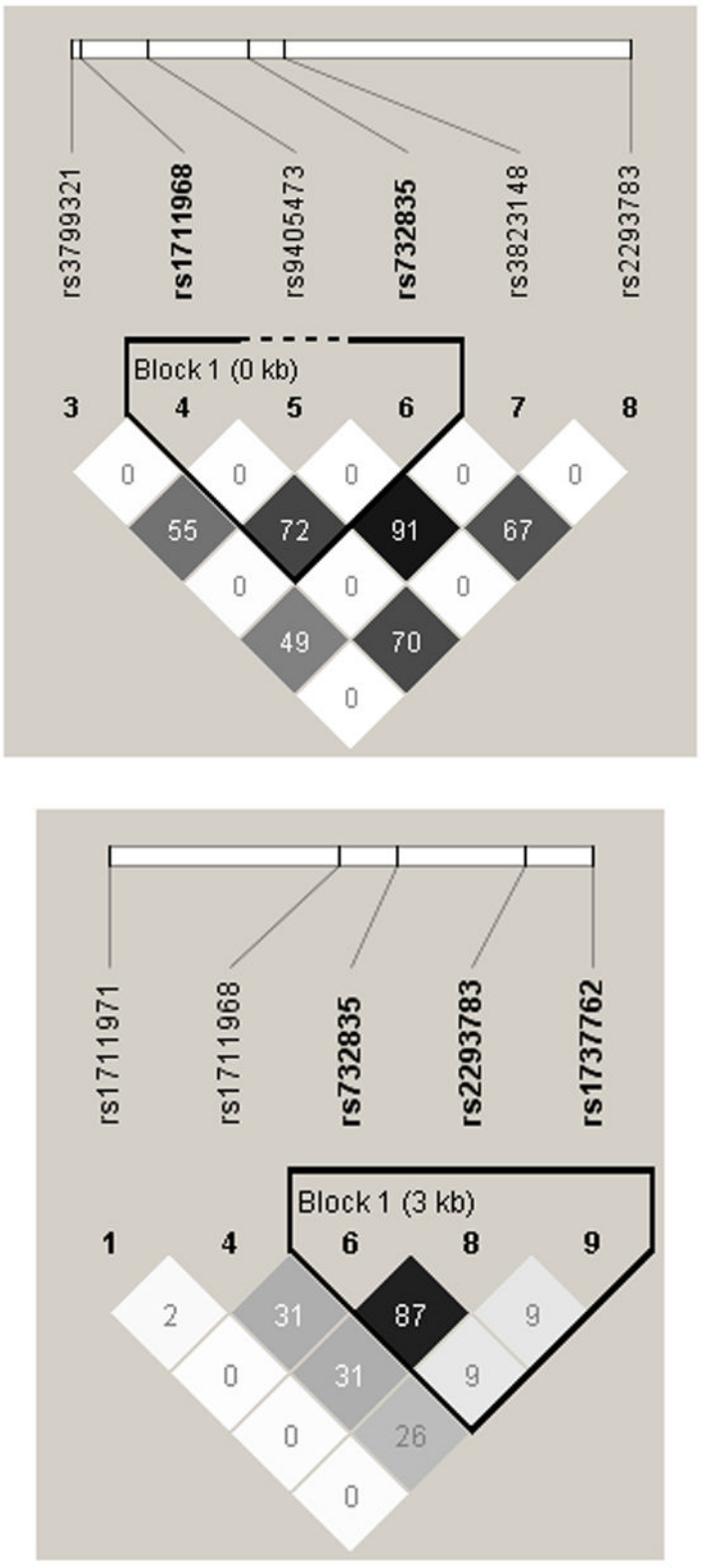

Fig. 2.

The LD patterns in founders for Asian (left) and European (right) NSCL/P probands. Numbers shown on the figure represent r^2 between SNPs.

Table 1

Race and gender for 297 NSCL/P trios

Race&Site	NSCL/P		
	Male	Female	Total
Asian			
Korea	22	18	40
Singapore	21	9	30
Taiwan	95	51	146
Subtotal	138	78	216
European			
Maryland	43	30	73
Singapore	1	1	2
Subtotal	44	31	75
AA			
Maryland	1	2	3
Other			
Singapore	2	1	3
Total	185	112	297

AA: African American

Table 2

MAF for SNPs in and near *FOXF2* gene in Asian and European NSCL/P trios

SNPs	Position build 38	Asian				European			
		HW pval	% Geno	MAF (%)	Minor allele	HW pval	% Geno	MAF (%)	Minor allele
rs1711971	1388187	1.00	100.0	0.0	T	1.00	99.3	2.6	T
rs11759800	1391096	1.00	100.0	0.0	G	1.00	100.0	0.0	G
rs3799321	1391817	0.99	98.3	4.1	C	1.00	74.6	0.0	C
rs1711968	1391873	0.12	99.8	22.4	C	0.24	98.6	45.6	C
rs9405473	1392224	1.00	100.0	2.9	T	1.00	100.0	0.0	T
rs732835	1392765	0.40	99.8	26.0	C	0.05	97.8	20.7	C
rs3823148	1392958	1.00	99.8	2.6	G	1.00	99.3	0.0	G
rs2293783	1394808	0.62	99.8	23.8	C	0.36	100.0	22.1	C
rs1737762	1395878	1.00	99.3	0.0	A	1.00	97.8	25.2	A

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Table 3

TDT analysis for SNPs in and near *FOXF2* gene in 216 Asian and 75 European NSCL/P trios

SNPs	Position build 38	Minor allele	MAF (%)	FAM	T	NT	OR (95%CI)	*P* Value	Bonferroni *P* Value	Empiric *P* Value
Asian										
rs3799321	1391817	C	4.1	32	18	16	1.13 (0.57, 2.21)	0.73	1	1
rs1711968	1391873	C	22.4	101	84	41	2.05 (1.41, 2.98)	0.00012	0.00072	0.0016
rs9405473	1392224	T	2.9	21	14	8	1.75 (0.73, 4.17)	0.2	1	0.85
rs732835	1392765	C	26.0	119	92	52	1.77 (1.26, 2.49)	0.00086	0.0043	0.005
rs3823148	1392958	G	2.6	19	13	7	1.86 (0.74, 4.66)	0.18	1	0.75
rs2293783	1394808	C	23.8	114	85	53	1.60 (1.14, 2.26)	0.00645	0.032	0.054
European										
rs1711968	1391873	C	45.6	32	17	22	0.77 (0.41, 1.46)	0.42	1	0.98
rs732835	1392765	C	20.7	25	9	20	0.45 (0.20, 0.99)	0.041	0.16	0.063
rs2293783	1394808	C	22.1	27	10	23	0.43 (0.21, 0.91)	0.024	0.1	0.093
rs1737762	1395878	A	25.2	27	20	14	1.43 (0.72, 2.83)	0.3	1	0.95

*FAM: number of informative families, T: number of transmitted alleles, NT: number of un-transmitted alleles; Empiric *P* Value: *P* values for 10,000 permutations in Haploview

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