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A Genome-Wide Linkage Scan for Cleft Lip and Cleft Palate Identifies a Novel Locus on 8p11-23

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Abstract

Isolated or nonsyndromic cleft lip and palate (NS CLP) is a complex disorder resulting from multiple genetic and environmental factors. NS CLP has a birth prevalence of 1 per 500 in the Philippines where large families provide an opportunity for gene localization. Genotyping of 392 microsatellite repeat markers at 10 cM intervals over the genome was performed by the Center for Inherited Disease Research (CIDR) on 220 Filipino families with 567 affected and 1,109 unaffected family members genotyped. Among the most statistically significant results from analysis of the genome-wide scan data was a 20 cM region at 8p11-23 in which markers had LODs ≥ 1.0 . This region on 8p11-23 has not been found in any previous genome wide scan nor does it contain any of the candidate genes widely studied in CLP. Fine mapping in 8p11-23 was done in the 220 families plus an additional 51 families, using SNP markers from 10 known genes (*FGFR1*, *NRG1*, *FZD3*, *SLC8A1*, *PPP3CC*, *EPHX2*, *BNIP3L*, *EGR3*, *PPP2R2A*, and *NAT1*) within the 20 cM region of 8p11-23. Linkage and association analyses of these SNPs yield suggestive results for markers in *FGFR1* (recessive multipoint HLOD 1.07) and *BAG4* (recessive multipoint HLOD 1.31).

Keywords

cleft lip and palate; linkage analysis; association analysis; fine mapping; SNP markers; FGFR1

Introduction

Nonsyndromic cleft lip and cleft palate (NS CLP) is a common birth defect, and the source of substantial morbidity and mortality worldwide [Murray, 2002]. With an average birth prevalence of 1/700 live births, there is remarkable population to population variation [Mossey and Little, 2002]. In general, Asian populations have a higher birth prevalence of clefting (1/500 births), Caucasians are intermediate (1/1,000 births), and African populations have the lowest

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(1/2,500 births). A long-term follow-up study of 5,331 Danish individuals with NS CLP found a standardized mortality ratio of 1.4 for males and 1.8 for females. This increased mortality ratio was present in infancy, childhood, and adulthood [Christensen et al., 2004]. Individuals with NS CLP had an increased rate of death from cardiovascular disease, suicide, and epilepsy, as well as a marginally increased rate of death from cancer. Infants with NS CLP also had increased deaths from prematurity, pneumonia, aspiration, and sepsis [Christensen et al., 2004].

An examination of familial recurrence patterns in NS CLP indicated that there may be anywhere from 2 to 14 interacting loci involved in clefting [Schliekelman and Slatkin, 2002]. The upper limit on this range represents more loci than was previously theorized, and indicates that very large sample sizes may be necessary to detect the loci involved in NS CLP [Mitchell and Risch, 1992]. For a complex genetic disorder such as NS CLP, several experimental techniques may be used. These include breakpoint mapping, deletion mapping, direct sequencing of candidate genes, linkage analysis, and linkage disequilibrium analysis [Lidral and Murray, 2004]. Genome wide linkage scans of complex traits can best succeed when heterogeneity is minimized and sample sizes are maximized. The Philippines provides an opportunity to base such a study with the common occurrence of isolated clefting, large average family sizes, and a motivated public health enterprise [Murray et al., 1997].

A study of van der Woude syndrome, a single-gene Mendelian disorder with cleft lip and/or cleft palate as part of the phenotype, identified disease-causing mutations in *IRF6* [Kondo et al., 2002]. An association study in Filipino, Caucasian, and South American NS CLP samples found a strong association of a particular V274I mutation with NS CLP [Zuccherro et al., 2004], that has since been confirmed in multiple other populations (Italy [Scapoli et al., 2005]; Belgium [Ghassibe et al., 2005]; US [Blanton et al., 2005]; Thailand [Srichomthong et al., 2005]). *IRF6* maps to 1q32, and there was also evidence supporting linkage to this region in a meta-analysis of CLP genome scans [Marazita et al., 2004].

In order to extend these earlier studies by identifying possible Filipino-population-specific clefting loci and to identify genes in addition to *IRF6* that contribute to NS CLP, we performed a genome scan in 220 extended Filipino families with at least two affected members. Fine mapping and mutation searches were carried out in a region on 8p11-23 where suggestive linkage results were obtained from the genome scan.

Materials and Methods

Sample Collection

Under the auspices of Operation Smile and Operation Smile-Philippines, 271 extended Filipino families were collected. Families had two or more affected members; unaffected family members were collected as well. Clinical aspects of sample collection have been previously described [Murray et al., 1997; Schultz et al., 2004]. The University of Iowa IRB gave approval for sample collection (approval numbers 9701068, 199804081, and 200003065) in conjunction with local approval in the Philippines. In addition, 90 DNA samples from cases in the Philippines and another 90 from cases born in Iowa were utilized to extend the sequencing studies. Whole blood samples were collected by venipuncture. Subjects were reviewed by JCM or SDH to exclude any with syndromic features. Sample and family characteristics are shown in Table I.

DNA Extraction

Stabilization buffer (AS1; Qiagen, Valencia, CA) was added to the whole blood samples for transport back to the United States. Samples were processed using QiaAmp DNA Blood Maxi

or Midi kits, depending on sample volume. The protocol was obtained from Qiagen, modified for AS1 use.

Genotyping

DNA samples from the initial 220 extended Filipino kindreds were sent to the Center for Inherited Disease Research (CIDR) for genotyping. Genotyping was completed on 392 markers from the Marshfield Genetics screening set 8. Further information on CIDR is available from their website <http://www.cidr.jhmi.edu/>.

Fine Mapping

Fine mapping was carried out using TaqMan™ Assays-on-Demand probes (Applied Biosystems, Foster City, CA) and done on an Applied Biosystems 7900 HT Sequence Detection System machine. Single-nucleotide polymorphism (SNP) markers were selected within candidate genes and/or at 1–4 Mb intervals across 8p.

Statistical Analyses

Preliminary steps—Each genome-scan and fine-mapping marker was assessed with PedCheck [O'Connell and Weeks, 1998] to test for inconsistencies due to non-paternity or other errors. For the parametric linkage analyses, allele frequencies were estimated from the unaffected founders in the study families and are available on our laboratory website at genetics.uiowa.edu. The genetic model parameters were taken from segregation analysis results in a sample of the Filipino families (unpublished results). The dominant model was a disease allele frequency of 0.002 with a penetrance of 0.30. The recessive model was a disease allele frequency of 0.04 with a penetrance 0.45.

Linkage calculations

Two-point and multipoint LOD scores: We calculated two-point LOD scores in the extended kindreds employing the LINKAGE program with recent updates to speed calculations (VITESSE and FAST-LINK) [Cottingham, 1993; Terwilliger and Ott, 1994; O'Connell and Weeks, 1995]. For multipoint LOD and heterogeneity LOD (HLOD) [Smith, 1963] calculations, the descent graph method [Sobel and Lange, 1996; Sobel et al., 1996; Lange, 2002] implemented in computer program SIMWALK2 was used. Given the probable complexities in the genetic model for NS CLP (e.g., heterogeneity), we also calculated model-free linkage statistics (both two-point and multipoint) using MERLIN [Abecasis et al., 2002].

Allelic association—Alleles at each genome-scan and fine-mapping marker were tested for association with CLP using the Family Based Association Test (FBAT) [Laird et al., 2000; Rabinowitz and Laird, 2000; Horvath et al., 2001].

Results

Three regions yielded suggestive positive results in the initial 220 multiplex extended Filipino kindreds: 2p21, 6q23, and 8p21. Figure 1 shows the results from the genome scan markers in the 8p11-23 region that was chosen for fine-mapping, the peak of significance ranges from approximately 45 cM to 55 cM. Power calculations using SIMLINK predict the mean maximum LODs for these families assuming 5% of families linked is 2.0 ± 0.10 under a dominant model, and 3.1 ± 0.13 under a recessive model. For 2% of families linked the mean max LODs are 1.3 ± 0.07 and 1.8 ± 0.09 respectively. Therefore, the HLODs for *FUT10*, *BAG4*, and *FGFR1* are close to the maximum expected under models of heterogeneity.

Figure 2 summarizes the genes genotyped in the region as well as the genome scan markers genotyped by CIDR. Table II summarizes the linkage and association results for the fine-mapping markers genotyped in the 271 multiplex extended Filipino kindreds (the 220 kindreds used for the genome scan plus an additional 54 extended kindreds). Both linkage and association results were positive for markers in *FGFR1* and *BAG4*, while either linkage or association showed positive results for markers in *SLC18A1*, *EPHX2*, and *FZD3*.

Discussion

In our genome-scan of 220 extended multiplex Filipino families, a 20 cM region on 8p11-23 had evidence for linkage with NS CLP. This region has not previously been identified in any of the five published genome-scans for NS CLP; therefore we targeted that region for fine-mapping studies in 271 extended multiplex Filipino families (the 220 genome-scan kindreds, plus an additional 54 extended kindreds). The association approach can be more powerful than linkage if there is a large degree of heterogeneity. Thirteen CLP candidate gene loci within the 8p11-23 region were assessed yielding positive results for five genes. As this study was exploratory in nature, we did not do any adjustment for multiple comparisons, instead using the usual alpha level of 0.05 as potentially statistically significant. Of these five genes with positive, only *FGFR1* has literature supporting its involvement in craniofacial development.

Fibroblast growth factor receptor 1 (FGFR1) is a transmembrane receptor tyrosine kinase involved in signal transduction. Signaling from FGFR1 follows homodimerization, which requires the binding of two FGF molecules to two FGFR molecules in the presence of heparan sulfate proteoglycans [Mohammadi et al., 2005]. In mice, FGFR1 is widely expressed in the branchial arches during development. Mice homozygous for the hypomorphic allele *Fgfr1*^{n7/n7} have clefts of the palate in 80% of cases, which appears to be secondary to a defect in neural crest migration into the second branchial arch during development [Trokovic et al., 2003].

A role for *FGFR1* in CLP has also been suggested by the report that loss-of-function mutations in *FGFR1* cause Kallmann syndrome with clefting as part of the phenotype [Dode et al., 2003; Kim et al., 2005]. In total, 25 novel nonsense, missense, splice-site, frameshift, deletion, and translocation mutations in *FGFR1* have been reported in Kallmann syndrome cases [Vermeulen et al., 2002; Dode et al., 2003; Sato et al., 2004; Albuissou et al., 2005; Kim et al., 2005; Pitteloud et al., 2005; Sato et al., 2005]. Seventeen familial and sporadic Kallmann cases have been reported with CLP or CPO, of those 9 have identified mutations in *FGFR1* [Lieblich et al., 1982; White et al., 1983; Tompach and Zeitler, 1995; Molsted et al., 1997; Dode et al., 2003; Albuissou et al., 2005; Jonklaas, 2005; Kim et al., 2005; Zenaty et al., 2006].

Interestingly, these families included a mixed clefting pattern, with some family members having cleft palate only (CPO) while other family members have CLP. These two types of clefts are not commonly seen together in families except in cases of *MSX1* mutations [van den Boogaard et al., 2000] and Van der Woude Syndrome *IRF6* mutations [Kondo et al., 2002]. Kallmann Syndrome is autosomal dominant when found with *FGFR1* mutations; the phenotype includes anosmia and hypogonadotrophic infertility as primary features, with CLP or CPO as part of the phenotype in about 5% of cases (M McCray and Jeff Murray, unpublished data).

In humans, a gain of function mutation in *FGFR1*, P252R, results in the craniosynostoses syndrome features, which can also be caused by mutations at the homologous position in *FGFR2* (P253S = Pfeiffer Syndrome; P253R = Apert Syndrome) [Slaney et al., 1996; Wilkie et al., 2002]. Three mutations in *FGFR2* (S252W, S252F, and P253R) account for most cases of Apert syndrome. Overall, about 44% of Apert syndrome cases have cleft palate, cases whose phenotype includes a cleft are more commonly associated with the S252W mutation (59%) rather than the P253R mutation (17%) [Slaney et al., 1996].

The genome scan data presented in this article were included in the summary results reported in the meta-analysis of 13 total genome scans for CLP [Marazita et al., 2004]. We report here the detailed analysis of one of these populations from the Philippines. This provides insights into a single, mostly homogeneous South Asian population with a high birth prevalence of clefting [Murray et al., 1997] and where the effects of specific genes and/or alleles are likely to be enriched. Notably, 8p21 had a nominally significant *P*-value of 0.027 in the meta-analysis. The meta-analysis identified multiple genome-wide significant regions that we are now pursuing with additional fine-mapping studies [Marazita et al., 2004].

In summary, we have identified a novel region (8p11-23) that is likely to be involved in nonsyndromic clefting; a search for disease-causing mutations in one of the genes within this region (*FGFR1*) is underway. Recent work has identified a significant role for the *FGF* and *FGFR* genes in NS CLP, including *FGFR1* [Riley et al., in press]. If the search for mutations within *FGFR1* is successful, then phenotypic expression due to *FGFR1* may be similar to *MSX1* and *IRF6*, genes in which multiple mutations and/or polymorphic variants lead to variable phenotypic expression, including both clinically defined syndromes and isolated cleft phenotypes. For example, Witkop syndrome [Jumlongras et al., 2001], hypodontia [van den Boogaard et al., 2000], and isolated CLP [Jezewski et al., 2003; Vieira et al., 2005] are all found with mutations in *MSX1*. Van der Woude and Popliteal Pterygium syndromes are both found with mutations in *IRF6* [Kondo et al., 2002], while NS CLP is associated with polymorphic variants in the same gene [Zuccherro et al., 2004]. Previous work has shown that genes that play a role in syndromic clefts may also have presumed hypomorphic variants that play a role in isolated clefting, therefore genetic variation in the *FGFR1* gene may contribute to syndromes as well as NS CLP. Finding mutations, despite the lack of complete penetrance for these mutations [Suzuki et al., 2004], can substantially alter recurrence risks and genetic counseling. These results demonstrate the power of a large study sample to further clarify the complex inheritance and etiology of NS CLP.

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Fig. 1. Plot of the results from the microsatellite markers in the 8p11-23 region genotyped by the Center for Inherited Disease Research for 220 Filipino extended multiplex kindreds (i.e., with two or more family members affected with NS CLP).

Fig. 2.

Map of 8p11-23. Above the chromosome are markers genotyped by the Center for Inherited Disease Research in the genome-wide linkage scan. D8S1771 (in bold) had the largest single-point LOD score and most significant NPL result. Below the chromosome are the SNP markers used for fine-mapping. In bold are those fine-mapping genes with the most significant FBAT *P*-values (*FGFR1*, *BAG4*, *EPHX2*). Distances in centimorgans between genes are shown in the intervals.

Table I

Sample Characteristics for 271 Filipino Families

Affected (NS CLP)	744
Unaffected	1,464
Phenotype unknown	28
Total	2,236
Number of family members for whom samples were unavailable	2,729

Table II
Results of 8p11-23 Fine-Mapping From 271 Filipino Multiplex NS CLP Families

Gene	Marker	Distance to next Marker	Singlepoint LOD	Multipoint HLOD	Multipoint NPL P-value	FBAT multiallele P-value
NAT1	rs3850751	1.97 Mb	No positives	Rec 0.64 α = 0.05	P = 0.22	P = 0.19
SLC18A1	rs227970	2.34 Mb	Dom 1.23 θ = 0.2	Dom 0.42 α = 0.05	P = 0.21	P = 0.36
PPP3CC	rs2461491	199 kb	Rec 0.53 θ = 0.3	Dom 0.42 α = 0.05	P = 0.11	P = 0.95
EGR3	rs12541654	3.6 Mb	Dom 0.60 θ = 0.3	Rec 0.41 α = 0.05	P = 0.14	P = 0.38
PPP2R2A	rs17055106	93 kb	Rec 0.68 θ = 0.3	Rec 0.37 α = 0.05	P = 0.11	P = 0.91
BNIP3L	rs3808579	1.13 Mb	No positives	Rec 0.29 α = 0.05	P = 0.11	P = 0.73
EPHX2	rs721619	1 Mb	Rec 0.31 θ = 0.4	Dom 0.45 α = 0.05	P = 0.10	P = 0.003
FZD3	rs2241802	2.65 Mb	Dom 1.28 θ = 0.3	Dom 0.66 α = 0.05	P = 0.13	P = 0.94
WRN	rs2725362	1.52 Mb	Rec 0.69 θ = 0.3	Dom 0.66 α = 0.05	P = 0.09	P = 0.85
NRG1	rs2466048	768 kb	Rec 0.81 θ = 0.3	Rec 0.63 α = 0.05	P = 0.11	P = 0.25
FUT10	rs2459516	4.66 Mb	Dom 0.15 θ = 0.4	Rec 1.31 α = 0.10	P = 0.11	P = 0.37
BAG4	rs7836805	2.23 Mb	Dom 0.34 θ = 0.4	Rec 1.31 α = 0.10	P = 0.07	P = 0.03
FGFR1 (1)	rs13317	36 kb	Dom 0.41 θ = 0.4	Rec 1.07 α = 0.05	P = 0.06	P = 0.01
FGFR1 (2)	rs6996321	5.9 kb	Rec 0.09 θ = 0.4	Rec 1.06 α = 0.05	P = 0.07	P = 0.40
FGFR1 (3)	rs10958704	4 kb	Rec 0.01 θ = 0.4	Rec 1.04 α = 0.05	P = 0.07	P = 0.36
FGFR1 (4)	rs881301	N/A	No positives	N/A	P = 0.07	P = 0.15

Mb, megabases; Dom, dominant; Rec, recessive; NPL, non-parametric linkage; FBAT, family based association test; N/A, not applicable; θ , recombination fraction; α , proportion of linked families.