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Genetics of Nonsyndromic Orofacial Clefts

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Abstract

With an average worldwide prevalence of approximately 1.2/1000 live births, orofacial clefts are the most common craniofacial birth defects in humans. Like other complex disorders, these birth defects are thought to result from the complex interplay of multiple genes and environmental factors. Significant progress in the identification of underlying genes and pathways has benefited from large populations available for study, increased international collaboration, rapid advances in genotyping technology, and major improvements in analytic approaches. Here we review recent advances in genetic epidemiological approaches to complex traits and their applications to studies of nonsyndromic orofacial clefts. Our main aim is to bring together a discussion of new and previously identified candidate genes to create a more cohesive picture of interacting pathways that shape the human craniofacial region. In future directions, we highlight the need to search for copy number variants that affect gene dosage and rare variants that are possibly associated with a higher disease penetrance. In addition, sequencing of protein-coding regions in candidate genes and screening for genetic variation in non-coding regulatory elements will help advance this important area of research.

Keywords

Nonsyndromic orofacial clefts; candidate gene; sequencing; single nucleotide polymorphism; association study; genome-wide association study

Orofacial clefts include cleft lip only (CLO), cleft lip and palate (CLP) and cleft palate only (CPO). Collectively, these are the most common craniofacial birth defects in humans, affecting approximately 1/800 live births worldwide. Extensive medical and behavioral interventions are needed to treat these common structural birth defects and they impose substantial economic and personal health burden (Wehby and Cassell, 2010) which can persist from infancy to childhood and throughout life. Mounting evidence suggests that multiple genes and environmental factors influence the risk of orofacial clefts, either

individually or through their interactions in complex biological pathways. Technological advances and collaborative efforts have led to major advances in gene-mapping for clefts, with the first wave of genome-wide association (GWA) studies identifying several key candidate genes and loci (Birnbaum et al., 2009; Grant et al., 2009; Beaty et al., 2010; Mangold et al., 2010). By contrast, efforts to identify gene-environment (GxE) interactions have not been as successful, most likely because of a combination of insufficient sample size, study heterogeneity, differential assessment of environmental exposures, and a lack of robust methodology to detect these higher-order interactions.

Several reviews have recently been published on general aspects of craniofacial development and disorders affecting the craniofacial complex (Jugessur and Murray, 2005; Lidral and Moreno, 2005; Jiang et al., 2006; Gritli-Linde, 2007; Jugessur et al., 2009b; Meng et al., 2009; Mossey et al., 2009; Dixon et al., 2011). Rapid advances in high-throughput genetic technologies, coupled with more accurate phenotypic ascertainment and increased international collaboration, have led to significant progress in mapping genes for orofacial clefts. We review here key developments in candidate gene identification, new animal models, gene-expression studies, functional characterization of candidate genes, and GWA studies of clefts. We start by outlining classic genetic approaches that have mostly been used to identify candidate genes, followed by the application of new genomic technologies in the field. Finally, we discuss some of the more established environmental risk factors known to influence the risk of clefts and their interactions with specific genetic variants.

Overview of orofacial development

Orofacial clefts are caused by a failure of complete fusion between any of the independently derived facial primordia that collectively form the orofacial complex. Insights into the developmental circuitry controlling cell migration, proliferation, differentiation and apoptosis are necessary to understand how a particular cleft arises. Development of the human face begins with the migration of cranial neural crest cells from the dorsal region of the anterior neural tube into the facial region where they establish five distinct facial primordia during the 4th week of gestation. These primordia consist of the unpaired frontonasal prominence that gives rise to a pair of lateral and medial nasal processes during the 5th week, the paired bilateral maxillary prominences that form the upper jaw, and the paired mandibular prominences that develop into the lower jaw (Figure 1). During the following two weeks, the medial nasal processes enlarge and merge with each other to form the intermaxillary segment, providing the basis for the primary palate. Subsequently, the intermaxillary segment fuses with the flanking maxillary prominences, completing the formation of the upper lip [(Sadler, 2006); Figure 1]. Early development of these structures is mediated by epithelial-mesenchymal interactions and depends on a wide range of signaling molecules, including fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and transforming growth factors (TGFs). Also involved are various developmental transcription factors belonging to the msh homeobox (MSX), distal-less homeobox (DLX), paired box (PAX), and T-Box (TBX) gene families.

The palatal shelves consist of neural crest-derived mesenchymal cells. They grow vertically from the bilateral maxillary processes during the 6th week and elevate to a horizontal position above the tongue during the 7th week of gestation. The palatal shelves continue to grow towards each other during the 8th week until the medial edge epithelia (MEE) covering the opposing palatal shelves fuse at the midline to form a continuous epithelial seam which subsequently disintegrates (Sadler, 2006). There has been some controversy over the mechanism by which the palatal seam disappears; epithelial-mesenchymal transformation (EMT) initially received widespread acceptance as the mechanism for palatal fusion (Sun et

al., 2000; Nawshad and Hay, 2003), but others have later argued that programmed cell death is the major mechanism for shelf fusion (Cuervo and Covarrubias, 2004; Vaziri Sani et al., 2005). More recently, the work of Ahmed and colleagues showed that both migration and apoptosis are necessary for palatal confluence (Ahmed et al., 2007). What is incontrovertible, however, is that orofacial development is a rigidly coordinated spatio-temporal sequence of events involving cell migration, proliferation, differentiation, fusion and apoptosis. Disruption of any of these processes can result in an orofacial cleft.

Epidemiology of cleft lip and palate

Because lip formation precedes palatogenesis, failure of proper lip fusion has been suggested to interfere with palatal shelf contact. Genetic and embryologic studies suggest that distinct etiologic mechanisms underlie clefts of the lip with or without the primary palate (CL/P) and clefts of the secondary palate only (CPO) (Fraser, 1955; Sivertsen et al., 2008b). In some instances, both CL/P and CPO can be seen to segregate in a single family in which a single allele of an autosomal dominant form of clefting is present, suggesting some overlap in etiologies. This observation, termed ‘mixed clefting’, is best described in syndromic forms of clefts, such as those caused by mutations in *IRF6* (Kondo et al., 2002), *MSX1* (van den Boogaard et al., 2000), and *FGFR1* (Dode et al., 2003).

Orofacial clefts are categorized as syndromic if they are accompanied by additional structural and/or developmental abnormalities; nonsyndromic if they occur in isolation without other apparent abnormalities. The majority of CL/P cases are nonsyndromic (NS) (~70%), as are about half of CPO cases (Tolarova and Cervenka, 1998). The prevalence of NS CL/P varies according to ancestral origin (Vanderas, 1987) and socioeconomic status (Murray et al., 1997), ranging from ~1/500 in individuals of Asian and Amerindian origin to ~1/1000 in European and ~1/2500 in African populations (Mossey and Little, 2002). Some exceptions are observed in isolated geographic regions where the prevalence differs from that of the surrounding ancestral background—a discrepancy generally attributed to founder effects or specific environmental factors. Scandinavian populations, for example, tend to have a higher prevalence of cleft lip defects than most other European populations (Christensen, 1999; Mossey and Little, 2002; Harville et al., 2005).

A recent epidemiological assessment of a Norwegian cleft cohort suggested that at least a subset of CLO cases may have a separate etiology than CLP (Harville et al., 2005). When hospital and national birth registry data were combined, 17% of infants with CLP had at least one other non-cleft defect compared with only 9% of those with CLO. Although this supports the notion that CLP is simply a more severe version of CLO, the data also highlighted several qualitative differences not easily explained by disease severity alone. There was for instance a stronger male predominance among CLP infants compared with CLO infants. Furthermore, twins and cases born to consanguineous parents had a stronger risk of CLO than CLP. A study based on the US National Birth Defects Prevention Study (NBDPS) also showed that the association of CLO with associated anomalies was far lower than for cases of CLP with other anomalies (Genisca et al., 2009).

Molecular data also support etiologic differences between a subset of CLO and CLP. Rahimov and colleagues identified a common SNP (rs642961) within a highly conserved enhancer element for the interferon regulatory factor 6 (*IRF6*) gene that is strongly associated with NS CL/P (Rahimov et al., 2008). In that study, there was a clear separation of risk and transmission pattern with this SNP for NS CLO compared with NS CLP. Of note, the results for rs642961 were most significant for families in which affected individuals had NS CLO. A similar phenotype specificity was observed in a recent genome-wide linkage scan, where results for the *IRF6* region were most significant for NS CLO

(Marazita et al., 2009), and in a comprehensive search for fetal genetic effects among NS CLO and NS CLP trios in two Scandinavian population-based cleft cohorts (Jugessur et al., 2011).

Massive efforts have been undertaken to identify genes and loci underlying the isolated forms of clefting. This has largely been driven by several lines of evidence pointing to a strong genetic component to isolated clefts. Heritability estimates for both CL/P and CPO are above 90% (Grosen et al., 2010), and the risk of recurrence is 30–40 times higher among those with an affected first-degree relative compared to the population prevalence (Sivertsen et al., 2008a; Grosen et al., 2010). In the next section, we provide an overview of classic genetic approaches used to identify candidate genes/loci for clefts, followed by a review of recent applications of new genomic technologies that have significantly advanced the field.

Genetic approaches to identifying genes for orofacial clefts

Since the initial recognition of a strong genetic contribution to orofacial clefting (Fogh-Andersen, 1942), a variety of genetic approaches have been employed to identify genes and loci implicated in clefting. Animal models, chromosomal rearrangements, linkage studies, candidate gene based association studies, candidate gene sequencing and GWA studies have all had successful applications.

Linkage studies

Linkage studies assess the cosegregation of marker alleles with a disease phenotype in extended families with multiple affected individuals or in affected relative pairs. Because markers closest to the disease-causing mutation tend to cosegregate with the disease more often than expected by chance, the chromosomal location of the responsible gene can be refined to a narrow critical interval by tracking recombination events or assessing increased allele-sharing in affected relatives (Dawn Teare and Barrett, 2005). This approach has been particularly successful in mapping genes for rare, monogenic disorders, including syndromes with orofacial clefting such as Treacher Collins (The Treacher Collins Syndrome Collaborative Group, 1996), Van der Woude (Kondo et al., 2002), and cleft palate with ankyloglossia (Braybrook et al., 2001) syndromes.

In contrast, linkage studies for complex disorders such as NS CL/P have met with limited success due to the high degree of genetic and phenotypic heterogeneity commonly observed in these disorders. Whole genome-wide linkage scans using polymorphic microsatellite markers spread uniformly across the entire genome have identified possible susceptibility loci for NS CL/P on nearly every human chromosome in families from different populations (Mitchell et al., 1995; Prescott et al., 2000; Marazita et al., 2002; Radhakrishna et al., 2006; Beiraghi et al., 2007). As most of these findings have not been replicated in subsequent studies, it is plausible that no major gene is responsible for this condition, or that the genes observed have small, population-specific effects. Alternatively, these studies may have had insufficient power and/or marker resolution to detect variants of more modest effects (e.g. those increasing clefting risk by 10–50%). A meta-analysis of 13 genome-wide linkage scans revealed significant heterogeneity LOD scores for several loci on chromosome 1p, 6p, 6q, 14q, 15q, and 9q (Marazita et al., 2004). One candidate gene, *FOXE1* on 9q, has been identified (Moreno et al., 2009a), while fine-mapping studies for others are currently underway.

A major limitation of linkage studies of isolated clefts is the presence of extensive genetic and phenotypic heterogeneity. The phenotypic spectrum of orofacial clefting ranges from microforms and minimal clefts of the upper lip to complete bilateral clefts of the lip and palate. To date, most studies have assigned affected status only to those individuals who

exhibit overt features of clefting. However, there is growing consensus in the field that a number of subtle, subclinical features may be part of an ‘extended’ isolated cleft phenotype. For example, occult defects in the superior *orbicularis oris* (OO) muscle of the upper lip (also termed “subepithelial cleft lip”) may represent the mildest form of an isolated cleft (Martin et al., 2000). Indeed, unaffected relatives of individuals with overt CL/P were twice as likely to have defects in the OO muscle as individuals with no family history of orofacial clefting (Neiswanger et al., 2007). Interestingly, a clear pattern of Mendelian inheritance emerged when an extended family member with OO muscle defects, initially diagnosed as unaffected, was later included in the pedigree as affected (Marazita, 2007; Neiswanger et al., 2007). These findings are already having an impact on clinical risk assessments (Klotz et al., 2010), and underscore the need for more accurate phenotypic ascertainment in future studies of clefts. Besides OO muscle defects, the role of dental anomalies (Letra et al., 2007; Vieira et al., 2008), structural brain abnormalities (Nopoulos et al., 2002; Conrad et al., 2010), craniofacial morphology (Weinberg et al., 2008; Weinberg et al., 2009), and whorl patterns on the lower lip (Neiswanger et al., 2009) as proxies for an underlying genetic risk shows great promise in refining linkage analysis. Broadening the phenotypic spectrum of isolated clefts to include these subphenotypes (Figure 2) may help explain incomplete penetrance issues that have been observed in several families where a seemingly etiologic mutation is transmitted from a parent without overt CL/P (Weinberg et al., 2006; Jugessur et al., 2009b).

Candidate gene association studies

Until recently, limitations in technology and a lack of extended families resulted in candidate gene association studies being the primary approach for genetic dissection of NS CL/P. In contrast to linkage analysis, association studies can be carried out on the large number of sporadic cleft cases that occur in isolation without affected relatives to detect alleles with minor effects. (As noted above, however, these apparently ‘unaffected’ relatives may be harboring a range of subclinical features that are yet to be diagnosed). In essence, association studies look for significantly altered frequency of an allele or haplotype in affected individuals as differing from what would be expected by chance if there were no association between the marker(s) and the phenotype of interest (Martin, 2006). The associated marker, currently with an emphasis on single nucleotide polymorphisms (SNPs) and copy number variants (CNVs), may either be causative itself or in strong linkage disequilibrium (LD) with the true etiologic variant. Association studies of isolated orofacial clefts have been carried out on cohorts of affected individuals and unrelated matched controls, or collections of offspring-parent trios. Linkage studies often locate large genomic intervals that house many candidate genes, requiring population-based fine-mapping studies to pinpoint the exact location of the responsible genes. The *FOXE1* gene was identified by using this combination of initial broad linkage-mapping with subsequent fine-mapping by association (Moreno et al., 2009a). Candidate genes have also been suggested by animal models that exhibit a clefting phenotype due to a targeted gene deletion or spontaneous mutation, even before the functional relevance of these genes in humans is known. For example, mice null for the homeobox msh-like 1 (*Msx1*) gene exhibit cleft palate (Satokata and Maas, 1994), which was subsequently shown to be involved in human nonsyndromic clefting as well (Lidral et al., 1998; Jezewski et al., 2003).

Gene-expression studies that demonstrate strong and restricted expression in the orofacial structures are also valuable for candidate gene selection. One important resource for prioritizing candidate genes, based on their expression patterns during early stages of craniofacial development, is the Craniofacial and Oral Gene Expression Network (COGENE) database (<http://hg.wustl.edu/cogene>). COGENE catalogs expression profiles of a large number of genes in the craniofacial region (Cai et al., 2005). EMAGE (<http://genex.hgu.mrc.ac.uk/Emage/database>) is another example of a curated database of

gene-expression patterns in the developing mouse embryo. It provides standardized spatial representations of the sites of gene-expression, including those that are expressed in the craniofacial region, and is thus a valuable tool for selecting candidate genes for further evaluation (Christiansen et al., 2006). Expression data are particularly useful for follow-up studies of genes in critical linkage intervals and genes that cause clefts in transgenic mouse models. Chromosomal rearrangements that result in clefting, such as those that disrupt *IRF6* (Sander et al., 1994), *SATB2* (FitzPatrick et al., 2003) and *SUMO1* (Alkuraya et al., 2006), can be used as a primary source to determine the association of these genes with NS CL/P.

Perhaps the most fruitful approach that has led to the identification of two key genes for NS CL/P is the use of cleft syndromes as a model for isolated clefts [*IRF6* for Van der Woude syndrome (Zuccherro et al., 2004) and *FOXE1* for Bamforth-Lazarus syndrome (Clifton-Bligh et al., 1998)]. Genes that cause Mendelian disorders with orofacial clefting have been widely assessed as potential candidates for NS CL/P based on the rationale that less deleterious common variants in these genes would contribute to the pathogenesis of relatively less severe isolated forms of clefting (Stanier and Moore, 2004). However, most studies to date that have shown significant associations with NS CL/P have failed to demonstrate obvious pathogenic changes in the coding sequences of these genes, leaving open the possibility that common etiologic variants in the regulatory elements of these genes might confer susceptibility to NS CL/P by altering the expression level of the gene in combination with additional genetic and/or environmental risk factors, consistent with the proposed etiologic mechanism underlying common, complex diseases (Lander and Schork, 1994).

Direct sequencing

Attempts to identify common causative variants in associated candidate genes have often led to the detection of multiple rare variants in individual families. Direct sequencing of protein-coding and regulatory regions has become a standard strategy to identify causal variants in candidate genes for NS CL/P (Jezewski et al., 2003; Marciano et al., 2004; Vieira et al., 2005; Watanabe et al., 2006; Riley et al., 2007; Riley and Murray, 2007; Suzuki et al., 2009). A mutation is considered pathogenic if it occurs *de novo* in the parental germline cells or if it is nonsense and absent in unaffected individuals. Only a handful of mutations detected in NS CL/P patients have met these stringent criteria (Riley et al., 2007). Many identified variants are either silent or missense, and are usually inherited from unaffected parents and sometimes shared with the unaffected siblings. This emphasizes one of the complicated issues that has yet to be resolved in complex disease genetics—the non-penetrance and expression variability of amino acid-changing mutations (Hindorff et al., 2009). Bioinformatic programs such as PolyPhen (Ramensky et al., 2002) and SIFT (Ng and Henikoff, 2003) have been informative in predicting the likely effects of missense variants based on their evolutionary conservation and their effects on the 3D structure of the protein. Given the vast majority of associated variants in GWA studies are not within protein-coding regions (Manolio et al., 2009), it is important to consider both coding and non-coding regions when searching for causal variants. Recent developments in massively parallel sequencing now make it practical to do whole-exome (the protein-coding part of the genome) or even whole-genome sequencing for Mendelian disorders (Ng et al., 2009; Lupski et al., 2010; Ng et al., 2010a), providing impetus to apply this strategy to complex traits such as NS CL/P, particularly in large and/or inbred families where linkage can assist in gene prioritization.

Array comparative genomic hybridization

Submicroscopic deletions and duplications are associated with numerous congenital anomalies (Mefford et al., 2007). Array comparative genomic hybridization (CGH)

measures DNA copy number differences between a reference genome from an unaffected individual and a patient's DNA. Over the past several years, array-CGH has proven to be a powerful approach to identify genes involved in various congenital anomalies (Vissers et al., 2005). Array-CGH based deletion analyses have recently been performed on NS CL/P subjects using whole-genome BAC clone arrays (Osoegawa et al., 2008). In a combined sample of 104 NS CL/P and NS CPO cases, one subject was identified with a 3.2 Mb deletion at chromosome 6q25.1-25.2 and another with a 2.2 Mb deletion at 10q26.11-26.13. These regions contain the genes for estrogen receptor 1 (*ESR1*) and fibroblast growth factor receptor 2 (*FGFR2*), respectively, which were subsequently identified as likely causative genes using a gene prioritization software. In another study, a low resolution scan for Mendelian allele loss in case-parent trios identified a 3.2 Mb *de novo* deletion at 6q25.1-25.2 and a 2.2 Mb deletion at 10q26.11-26.13 inherited from an affected mother (Shi et al., 2009). In addition, deletions involving the genes for transcription factor AP-2α (*TFAP2A*) and SMT3 suppressor of mif two 3 homolog 1 (*SUMO1*) were detected in four unrelated individuals with NS CL/P. These findings further prove the applicability of the array-CGH approach and the use of Mendelian allele loss in discovering novel genes affecting complex birth defects such as NS CL/P. These same approaches can be applied to candidate genes in order to detect deletions/duplications in the coding and non-coding regulatory elements that may affect a subset of NS CL/P cases.

Genome-wide association (GWA) studies

With recent advances in high-density SNP genotyping arrays and statistical methodology, GWA studies have heralded a new era of gene-discovery for complex diseases. Unlike the hypothesis-driven candidate gene approach, GWA studies do not have an *a priori* hypothesis and can thus identify novel genes and loci contributing to the trait of interest. More importantly, despite the relatively small increments in risk often identified, these studies can be instrumental in discovering new biological pathways involved in the disease etiology (Christensen and Murray, 2007; Hirschhorn, 2009). On the downside, the large number of tests and small odds ratios associated with risk alleles require very large sample sizes, often necessitating collaboration between different groups and the establishment of a reliable platform for genotype/phenotype harmonization (Manolio et al., 2007). To date, many GWA studies have been applied to complex diseases with a high prevalence in the population, such as cancer, diabetes, cardiovascular disorders, and obesity (Pearson and Manolio, 2008). But, like the candidate gene-based efforts, most of the identified common variants tend to confer relatively small increases in risk (1.1–1.5-fold), explaining only a small fraction of the overall phenotypic variance attributable to additive genetic factors (Manolio et al., 2009).

The first GWA study on NS CL/P was performed in individuals of Central European ancestry and identified a susceptibility locus on chromosome 8q24 (Birnbaum et al., 2009), which was subsequently replicated in three independent GWA studies (Grant et al., 2009; Beaty et al., 2010; Mangold et al., 2010). Table 1 summarizes the most salient findings from these four GWA studies. The most significant genes/loci demonstrated by these studies are: interferon regulatory factor 6 (*IRF6*); v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian) (*MAFB*); ATP-binding cassette, sub-family A (ABC1), member 4 (*ABCA4*); noggin (*NOG*); ventral anterior homeobox 1 (*VAX1*); Pvt1 oncogene (*PVT1*); gasdermin C (*GSDMC*); coiled-coil domain containing 26 (*CCDC26*); paired box 7 (*PAX7*); netrin 1 (*NTN1*); and *KIAA1598* (undefined). A detailed description of these genes is beyond the scope of this review, but more information is available in the references cited in Table 1.

Genes and pathways implicated in orofacial clefting

As with other complex traits, multiple genes and genetic pathways are likely to be implicated in craniofacial development and NS CL/P. In this section, we review several genes and pathways that are recognized as key players in NS CL/P based on their association in multiple studies and from evidence in animal model and *in vitro* studies.

Interferon Regulatory Factor 6 (IRF6)

Of the large number of candidate genes thought to contribute to orofacial clefting, *IRF6* is in a class of its own in that it is the only gene that has shown a convincing degree of consistency across studies. Mutations in *IRF6* cause two allelic autosomal-dominant clefting disorders known as Van der Woude (VWS) and popliteal pterygium (PPS) syndromes (Kondo et al., 2002). *IRF6* is strongly expressed in the ectoderm covering the developing facial primordia. Mice deficient for both *Ir6* alleles develop abnormally thick skin with severe limb and craniofacial abnormalities, including cleft of the secondary palate (Kondo et al., 2002; Knight et al., 2006). The lack of a normally stratified epidermis in *Ir6*-null mice, due to a defect in keratinocyte proliferation and differentiation, confirms an important role for *Ir6* in epidermal development (Ingraham et al., 2006; Richardson et al., 2006). Significant associations between *IRF6* and NS CL/P have been reported in multiple populations (Zuccherro et al., 2004; Scapoli et al., 2005; Blanton et al., 2005; Ghassibe et al., 2005; Srichomthong et al., 2005; Park et al., 2007; Jugessur et al., 2008; Huang et al., 2009). In a follow-up study, a common etiologic variant (rs642961) in a highly conserved *IRF6* enhancer element was responsible for 18% of cleft lip occurrence in Northern European populations (Rahimov et al., 2008). The strongest observed association with this SNP was with NS CLO, with a relative risk of 2.4 for the homozygous genotype. However, no association was found with NS CPO. The associated A-allele of this SNP disrupts an AP-2 α binding motif—a transcription factor required for craniofacial development (Schorle et al., 1996). Mutations in *TFAP2A* (the gene encoding AP-2 α) cause branchio-oculo-facial syndrome (Milunsky et al., 2008) which is characterized by some of the same features observed in VWS (occasional lip pits and orofacial clefts), potentially linking these important pathways during craniofacial development.

More recently, Little and colleagues determined the DNA sequence to which wild-type IRF6 binds and used this sequence to show that IRF6 functions as a co-operative transcriptional activator (Little et al., 2009). Furthermore, VWS-causing mutations in the protein interaction domain of IRF6 disrupted this activity. Finally, in the GWA study by Beaty et al. (2010) (Table 1), four SNPs in *IRF6* showed genome-wide significance ($P < 5 \times 10^{-8}$) with clefting.

Forkhead box E1 (FOXE1)

FOXE1 is a member of the forkhead/winged helix-domain transcription factor family whose members are primarily involved in embryonic development. Targeted disruption of mouse *Foxe1* results in cleft palate and thyroid malformation (De Felice et al., 1998). Loss-of-function mutations within its forkhead DNA-binding domain cause Bamforth-Lazarus syndrome, which is characterized by thyroid agenesis, choanal atresia, bifid epiglottis, spiky hair and cleft palate (Clifton-Bligh et al., 1998; Castanet et al., 2002). A meta-analysis of 13 genome-wide linkage scans revealed a susceptibility locus on chromosome 9q22, in the vicinity of *FOXE1* and other potential candidate genes (Marazita et al., 2004). In addition, a recent mutation screening detected missense mutations in three unrelated NS CL/P patients (Vieira et al., 2005; Venza et al., 2006). Subsequently, significant associations were reported between *FOXE1* and NS CL/P in multiple populations, although no common coding variants were identified (Venza et al., 2006; Moreno et al., 2009b; Castanet et al., 2010).

Poliovirus receptor-related 1 (PVRL1)

Nectin-1, encoded by *PVRL1*, is an immunoglobulin related transmembrane cell-cell adhesion molecule. Homozygosity for a common nonsense mutation in *PVRL1* (Trp185Stop) causes the autosomal recessive CL/P-ectodermal dysplasia in the indigenous population of Margarita Island (the largest island of the Nueva Esparta State in Venezuela) where there is an unusually high incidence of clefting (5.4/1000) (Suzuki et al., 2000). Heterozygous carriers of the same mutation from a neighboring region in Northern Venezuela were also shown to be at high risk for NS CL/P (Sozen et al., 2001). Rare mutations in sporadic cases and a statistically significant association between a common coding variant (G361V) in *PVRL1* and NS CL/P were found in multiple population (Avila et al., 2006). In addition, mutations in *PVRL1* homologue genes *PVR* and *PVRL2*, and significant associations between a *PVR* variant and NS CL/P were reported in multiple populations from South America (Warrington et al., 2006).

Msh homeobox 1 (MSX1)

Ablation of the murine muscle-segment homeobox 1 (*Msx1*) gene causes a complete cleft of the secondary palate and a variety of other craniofacial defects (Satokata and Maas, 1994). In humans, a nonsense mutation segregating with tooth agenesis and mixed clefting was reported in an extended Dutch family, suggesting an important role for *MSX1* in human clefting (van den Boogaard et al., 2000). Significant associations were reported between SNPs in *MSX1* and both NS CL/P and NS CPO in different populations (Lidral et al., 1998; Suzuki et al., 2004; Tongkobetch et al., 2006). In addition, direct sequencing of the *MSX1* coding regions showed that mutations in *MSX1* could account for approximately 2% of NS CL/P cases (Jezewski et al., 2003). However, no common variants affecting the coding sequence have thus far been identified.

SMT3 suppressor of mif two 3 homolog 1 (SUMO1)

SUMO1 is a 101-amino acid polypeptide involved in posttranslational modification of a variety of proteins. A balanced reciprocal translocation resulting in *SUMO1* haploinsufficiency was identified in a patient with isolated unilateral CLP (Alkuraya et al., 2006). *Sumo1* showed strong expression in the upper lip, primary palate and medial edge epithelium (MEE) of the secondary palate in the mouse at E13.5. Mice with a *Sumo1*-hypomorphic allele had cleft palate (Alkuraya et al., 2006). However, a knockout of *Sumo1* was viable and did not have a cleft phenotype, nor did the heterozygotes (Zhang et al., 2008). A microdeletion encompassing *SUMO1* in a cleft patient supports its role in human clefting (Shi et al., 2008), although other genes near *SUMO1* also warrant continued consideration as genes potentially implicated in clefting. Finally, genetic associations between NS CL/P and *SUMO1* variants have been reported in two ancestrally diverse populations, China (Song et al., 2008) and Ireland (Carter et al., 2010).

T-box 22 (TBX22)

Function-impairing mutations in the T-box DNA-binding domain of the transcription factor gene *TBX22* cause X-linked cleft of the secondary palate (CPX), usually associated with ankyloglossia or tongue-tie (Braybrook et al., 2001). Its expression is localized to the developing palatal shelves and the base of the tongue. Consistent with the human findings, *Tbx22*-null mice also present with submucous cleft palate and ankyloglossia (Braybrook et al., 2002; Pauws et al., 2009). A genome-wide linkage analysis of NS CL/P families identified a susceptibility locus in the vicinity of *TBX22*, suggesting that the linkage signal may emanate from this gene (Prescott et al., 2000). In addition, mutations in *TBX22* were found in individuals with isolated CPO (Marcano et al., 2004; Suphapeetiporn et al., 2007). Using an array of *in vitro* functional assays, Andreou et al. demonstrated that TBX22

functions as a transcriptional repressor and that pathogenic missense mutations in the DNA-binding domain disrupt its DNA-binding affinity and impair its repression ability (Andreou et al., 2007).

Special AT-rich sequence binding protein 2 (SATB2)

SATB2 belongs to a small family of DNA-binding proteins that specifically bind to nuclear matrix-attachment regions (MARs) to regulate gene transcription in a tissue-specific manner through chromatin remodeling (Britanova et al., 2005). An important role for *SATB2* in orofacial clefting was discovered after fine-mapping of translocation breakpoints in the 2q32-q33 region in two unrelated cases with NS CPO (FitzPatrick et al., 2003). The *de novo* translocation breakpoints disrupted the *SATB2* transcriptional unit and separated a putative regulatory element from the gene in these patients. Subsequently, significant associations were reported between genetic variants in *SATB2* and NS CL/P in two Asian populations (Beaty et al., 2006). Mouse *Satb2* is strongly expressed in the developing palate and is 99.6% identical to the human *SATB2* at the protein level (FitzPatrick et al., 2003). The biological significance of this gene was not known until two animal models lacking a functional *Satb2* were reported to have severe craniofacial malformations (Britanova et al., 2006; Dobрева et al., 2006). Notably, mice with a single functional copy of *Satb2* developed various craniofacial defects including cleft palate, closely mimicking the human phenotypes resulting from *SATB2* haploinsufficiency. Losing the second allele severely augmented these phenotypes. In addition, significant changes in the expression patterns of three essential genes involved in craniofacial development and diseases—*Msx1*, *Pax9* and *Alx4*—were observed in *Satb2*^{-/-} mice (Britanova et al., 2006).

Fibroblast Growth Factor (FGF) signaling pathway

The FGF signaling pathway plays a central role in craniofacial development, essentially through induction and migration of cranial neural crest cells and regulation of epithelial-mesenchymal interactions during fusion of the facial prominences (Nie et al., 2006). To date, 23 mammalian FGF proteins have been recognized along with their seven main receptors (Ornitz and Marie, 2002). The majority of the FGF ligands and the receptors FGFR1 and FGFR2 are broadly expressed in the developing facial primordia (Bachler and Neubuser, 2001). Several members of this family of signaling molecules have been implicated in various birth defects that also afflict craniofacial structures. For example, gain-of-function mutations in FGF receptor genes 1, 2 and 3 are commonly associated with craniosynostosis syndromes (Marie et al., 2005).

Mutations in the FGF receptor 2 gene (*FGFR2*) cause Apert syndrome, a craniosynostosis syndrome characterized by cleft palate in 76% of cases and a Byzantine arch-shaped palate in nearly all cases. Mice deficient for *Fgfr2b* and one of its ligand genes, *Fgf10*, exhibit clefts of the secondary palate (De Moerloose et al., 2000; Rice et al., 2004). Recently, two potentially etiologic missense mutations in the functional domains of FGFR2 (Riley et al., 2007) and a microdeletion in a region containing *FGFR2* (Osoegawa et al., 2008) were identified in patients with NS CL/P. In addition, statistically significant associations were detected between a common genetic variant in *FGF10* and NS CL/P (Riley et al., 2007), further highlighting the significance of the FGF10-FGFR2 pathway in human orofacial development (Pauws and Stanier, 2007).

Cleft lip and palate are occasionally associated with the autosomal-dominant form of Kallmann syndrome (KAL2), which is characterized by hypogonadotropic hypogonadism with anosmia. KAL2 is caused by loss-of-function mutations in *FGFR1* (Dode et al., 2003). Mice homozygous for a hypomorphic allele of *Fgfr1* exhibit craniofacial defects, including cleft of the secondary palate, and, remarkably, this phenotype can be rescued by restoring

Fgfr1 function specifically in neural crest cells (Trokovic et al., 2003). Mutations in *FGFR1* can also cause nonsyndromic forms of clefting, as evidenced by a Kallmann syndrome patient inheriting a protein truncating mutation from a father with an isolated CL/P, and missense mutations disrupting conserved residues of the FGFR1 protein in two unrelated patients with NS CL/P (Riley et al., 2007).

FGF8 is an important ligand of this pathway and is required for patterning of the first pharyngeal arch under the control of sonic hedgehog (SHH) (Trumpp et al., 1999; Haworth et al., 2007). It is expressed in the mandibular and maxillary ectoderms and induces the expression of LIM homeobox protein 6 (*Lhx6*), distal-less homeobox 1 (*Dlx1*), distal-less homeobox 2 (*Dlx2*) and BarH-like homeobox 1 (*Barx1*). A *de novo* mutation that changes a highly conserved amino acid residue of FGF8, with a predicted loss-of-function, was found in a case with NS CL/P (Riley et al., 2007). In addition to potentially etiologic rare mutations in the above-mentioned FGF signaling members, the Riley et al. study also reported significant associations between SNPs in *FGF3*, *FGF7*, *FGF18* and *FGFR1* and isolated clefts. Finally, there is a potential connection between alleles in *FGFR2* causing CL/P and alleles contributing to breast cancer. Data from a large Danish cohort study revealed an odds ratio of 1.3 for women born with a cleft to later develop breast cancer (Bille et al., 2005). Several recent studies have also shown a highly significant association of alleles in *FGFR2* with breast cancer (Katoh, 2008).

Transforming growth factor (TGF) signaling pathway

The TGF superfamily of growth factors and their binding receptors play significant roles in craniofacial development. Transforming growth factor α (*TGFA*) was one of the first genes reported to be associated with nonsyndromic clefting (Ardinger et al., 1989; Shiang et al., 1993; Jugessur et al., 2003b). It is strongly expressed in the MEE of fusing palatal shelves and promotes extracellular matrix biosynthesis (Dixon and Ferguson, 1992). In addition, *TGFA* alleles are among the few genetic factors that have shown significant interactions with various environmental factors, including maternal smoking and vitamin use (Shaw et al., 1996; Shaw et al., 1998; Jugessur et al., 2003a; Zeiger et al., 2005; Sull et al., 2009).

Most of our knowledge of the role of TGF signaling in orofacial development stems from animal models and *in vivo* organ culture studies. Two independent mouse knockouts for *Tgfb3* both manifested cleft palate phenotypes (Kaartinen et al., 1995; Proetzel et al., 1995). Targeted overexpression of the Tgf- β 3 signal mediator Smad2 in MEE of these mice partially rescued the cleft palate phenotype, indicating a specific requirement for this growth factor in palatal closure (Cui et al., 2005). Interestingly, exogenous application of TGF- β 3 also induced palatal fusion in chickens, a species born with a natural cleft palate (Sun et al., 1998). In addition, TGF- β 3 signaling is central to MEE disintegration as well as sequential induction of cell cycle arrest in MEE, cell migration and apoptosis at advanced stages of palatal development (Ahmed et al., 2007). Finally, genetic variants in *TGFB3* have been associated with NS CL/P in multiple populations (Lidral et al., 1998; Reutter et al., 2008; Suazo et al., 2010a; Zhu et al., 2010).

Bone morphogenetic protein (BMP) signaling pathway

The BMPs are a collection of secreted cell signaling molecules of the TGF- β superfamily of growth factors. They regulate important developmental processes, including cell proliferation, differentiation and apoptosis (Srichomthong et al., 2005). Members of this signaling pathway are expressed throughout the orofacial primordia in a strictly regulated spatio-temporal pattern, and outgrowth and patterning of the facial primordia are BMP-dosage sensitive (Barlow and Francis-West, 1997; Ashique et al., 2002). Conditional inactivation of the type 1 Bmp receptor gene (*Bmpr1a*) in the orofacial primordia causes

bilateral CL/P with tooth agenesis, whereas conditional deletion of its ligand *Bmp4* in the same tissue results in isolated cleft lip only (Liu et al., 2005). The cleft lip accompanied by cleft palate was attributed to increased apoptosis in the ectodermal and mesenchymal tissues of the medial nasal prominence. Interestingly, the cleft lip in the *Bmp4*-mutant mouse was repaired at advanced stages of embryogenesis, perhaps owing to redundancy among BMP factors.

This wound healing property of *BMP4* may also have a parallel in humans. It was recently proposed that *BMP4* may be involved in mild forms of cleft lip, as defects in the *orbicularis oris* (OO) muscle in the mouth may be a manifestation of *in utero* healed cleft lip (Marazita, 2007). Supportive of this hypothesis are the reported potential etiologic mutations in *BMP4* in two individuals with OO muscle defects and one with a microform cleft lip (Suzuki et al., 2009). Additional mutations were also detected in five cases with NS CL/P. Further, pathogenic mutations in *BMP4* have recently been associated with eye, brain and digit abnormalities with no apparent facial dysmorphologies (Bakrania et al., 2008). It is plausible that an ultrasound examination of these patients might reveal OO muscle defects.

Thus far, only two association studies have been published on genetic variants in *BMP4* and risk of NS CL/P. The first investigated three SNPs in *BMP4* (rs762642, rs2855532 and rs1957860) in 150 unrelated NS CL/P trios from Chile (Suazo et al., 2010b). An association was found with haplotypes of rs1957860-rs762642, but not with any individual SNP. The second study reported an association between a single non-synonymous SNP (rs17563) in *BMP4* among 184 patients with NS CL/P and 205 controls from China (Lin et al., 2008).

Sonic Hedgehog (SHH) pathway

The sonic hedgehog (SHH) signaling pathway is involved in various aspects of embryonic development and craniofacial morphogenesis, as illustrated by a wide spectrum of craniofacial defects caused by perturbations in this evolutionarily conserved signaling pathway. Mutations in human *SHH* are responsible for a subset of holoprosencephaly cases, a congenital birth defect characterized by a spectrum of brain and facial abnormalities (Roessler et al., 1996). Mice null for *Shh* die prenatally due to severe midline defects (Chiang et al., 1996). *Shh* is broadly expressed in the ectoderm covering primordial facial structures (Jeong et al., 2004). SHH initiates its signaling by binding to its cell surface receptor Patched to relieve its inhibition of the transmembrane protein Smoothened, which in turn activates the GLI family of zinc-finger transcription factors that regulate the expression of downstream target genes such as *FOXE1* (Brancaccio et al., 2004; Eichberger et al., 2004).

One of the downstream target genes is the Patched receptor gene (*PTCH*) itself, whose induction ensures an autoregulation of the pathway. Mutations in *PTCH* are associated with nevoid basal cell carcinoma (also known as Gorlin syndrome), which includes cleft palate in ~4% of cases. A mutation screen of the *PTCH* coding sequences in 220 multiplex families with NS CL/P revealed missense mutations in its predicted extracellular SHH-binding domain, which may interfere with its binding activity (Mansilla et al., 2006). However, no disease-causing mutations in *SHH* have yet been found in isolated clefts (Orioli et al., 2002).

Loss-of-function mutations in *GLI2*, one of the three canonical transcription factors that convey intracellular SHH messaging, are associated with holoprosencephaly-like features with various combinations of cleft lip/palate (Roessler et al., 2003). Linkage and significant association between SNPs in the *GLI2* region and NS CL/P have been reported in various populations (Beatty et al., 2006). Furthermore, potentially pathogenic missense mutations that disrupt conserved residues in *GLI2* were found in sporadic individuals with NS CL/P

(Vieira et al., 2005). Overall, these findings demonstrate that members of the SHH signaling pathway are involved in the pathogenesis of NS CL/P.

Other candidate genes

In addition to these extensively studied genes and pathways, preliminary evidence for a number of other genes exists, supporting their role in NS CL/P. One recently identified gene associated with NS CL/P is cysteine-rich secretory protein LCCL domain containing 2 (*CRISPLD2*), with strong expression in the fusing palatal shelves (Chiquet et al., 2007). Ectopic expression of *Tbx10* (a member of the T-box gene family of transcription factors) in transgenic mice resulted in cleft lip and palate (Bush et al., 2004), and mutations in *TBX10* were also detected in individuals with NS CL/P (Vieira et al., 2005). Similarly, overexpression of the gene for sprouty homolog 2 (*Spry2*) causes stage-dependent craniofacial defects in transgenic mice (Goodnough et al., 2007). Mutations in human *Sprouty2* (*SPRY2*) further delineates its role in clefting (Vieira et al., 2005).

Heterozygous mutations in the tumor protein p63 gene (*TP63*) are responsible for the autosomal dominant ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome (Celli et al., 1999). A *de novo* missense mutation that disrupts a functional domain of p63 was reported in a NS CL/P patient (Leoyklang et al., 2006). Further, a missense mutation in the gene for RYK receptor-like tyrosine kinase (*RYK*) was also found in a case with isolated clefting (Watanabe et al., 2006). Lastly, significant association between different members of the wingless-type MMTV integration site family (WNT) signaling pathway and isolated clefting have also been reported in various populations (Chiquet et al., 2008), with mouse model studies providing further confirmation (Juriloff et al., 2006; Juriloff and Harris, 2008). A list of NS CL/P candidate gene is provided in Table 2.

Gene-environment (GxE) interactions

It has long been hypothesized that orofacial clefts result from the complex interplay of multiple genes and environmental factors, but only recently have practical approaches become available for a robust investigation of this hypothesis at the genome-wide level (Engelman et al., 2009; Murcray et al., 2009; Gauderman et al., 2010). Analyses of GxE interaction are important, because a failure to incorporate genetic and environmental exposures in a joint analysis of a population composed of both susceptible and non-susceptible individuals will bias observed associations toward the null (Khoury and Wacholder, 2009). Furthermore, they are important in determining the potential for public health intervention on environmental factor(s) which alone could reduce the occurrence/recurrence of clefts, particularly in genetically-susceptible subgroups of the population. This rationale is supported by animal models; the spontaneous CL/P rate among the cleft-susceptible CL/Fr mouse is about 20% compared to <10% in the normal C57BL/6J strain (Juriloff, 2002), but this can be easily increased to almost 100% at dosages of 6-aminonicotinamide (a vitamin B3 inhibitor). Just as some mouse strains are more susceptible to external teratogens (Millicovsky and Johnston, 1981; Juriloff, 2002), human fetuses carrying specific high-risk alleles may be more sensitive to particular teratogenic agents. Thus, identifying teratogens that interact with specific genetic factors will deepen our understanding of the biological mechanisms leading to orofacial clefts.

Although a large number of maternal exposures have been reported to influence the risk of orofacial clefts at critical stages of development, only a handful have survived scrutiny after being tested in large, well-characterized populations. Maternal smoking (Zeiger and Beaty, 2002; Little et al., 2004; Lie et al., 2008); alcohol consumption (especially at binge levels) (Deroo et al., 2008); folic acid and other B-complex vitamin supplementation (Munger, 2002; Hayes, 2002; Wilcox et al., 2007); use of anti-folate medication (Hernandez-Diaz et

al., 2000; Holmes et al., 2001); and specific exposures related to particular parental occupations (Nguyen et al., 2007) are all relevant environmental factors. In addition, the role of maternal illnesses such as hyperthermia (Peterka et al., 1994; Botto et al., 2002; Shahrukh Hashmi et al., 2010), diabetes and obesity (Cedergren and Kallen, 2005; Stothard et al., 2009) have recently been identified as important research gaps for additional public health research (Yazdy et al., 2007).

Maternal risk factors such as cigarette smoking, alcohol consumption, nutritional deficiencies and infectious diseases during pregnancy may adversely affect the intrauterine environment in which the embryo grows. Although both the mother and fetus have an inborn capacity to cope with diverse environmental insults through the action of detoxification enzymes, deleterious variants in these detoxification genes may reduce this ability to biotransform toxic components, rendering the fetus more vulnerable to teratogenic exposures. Below, we briefly review three environmental factors, maternal cigarette smoking, alcohol consumption and folic acid/multivitamin supplement use, for which the accumulated evidence is the most consistent across studies.

Cigarette smoking during the first trimester of pregnancy has been repeatedly associated with an increased risk of clefting (Shi et al., 2008). A meta-analysis strongly supports an odds ratio of ~1.3 for smoking with clefting (Little et al., 2004). Increased risks from exposures can suggest metabolic pathways in which disruptions may play a key role in the pathogenesis of CL/P, with recent evidence of a particularly compelling GxE interaction between fetal glutathione S-transferase theta 1 (*GSTT1*) gene variants and maternal smoking (Shi et al., 2007), and between variants in the gene for alcohol dehydrogenase 1C (class I), gamma polypeptide (*ADH1C*) and alcohol metabolism (Jugessur et al., 2009a; Boyles et al., 2010). In the Boyles et al study, heavy alcohol drinking was associated with risk of clefts *only* if either the mother or the baby carried the slow-metabolizing *ADH1C* variant (Boyles et al., 2010). This finding supports the hypothesis that genetic susceptibility in detoxification genes increases vulnerability of the fetus to alcohol-related orofacial clefts (Shi et al., 2007; Shi et al., 2008).

Through genome-wide expression analyses of B-lymphoblasts derived from NS CL/P patients, Blik and co-workers identified a large number of folate responsive genes and showed that folate deficiency perturbs normal cell development (Blik et al., 2008). Several studies have shown that folic acid lowers the risk of orofacial clefts (Badovinac et al., 2007; Wilcox et al., 2007). Whether this is mediated through the action of genes involved in folic acid metabolism has not yet been established. A pathway-wide analysis of 108 SNPs and one insertion polymorphism in 29 genes involved in folate/one-carbon metabolism found no convincing evidence that genetic variants in these folate metabolism genes play a causal role in orofacial clefting (Boyles et al., 2009). A second, more recent analysis of 97 SNPs in 14 genes in or interacting with the folate pathway found suggestive evidence of association with six genes in the folate pathway only when a less conservative approach was used for correcting for multiple testing than the stricter Bonferroni correction (Blanton et al., 2011). These findings suggests that the genetic contribution to orofacial clefts may be independent of pathways by which multivitamin supplementation provides protection from clefts.

In summary, only a few GxE interactions have been conclusive despite the well-recognized role of environmental factors in clefting (Mossey et al., 2009). Known challenges are related to exposure assessment, sample size/statistical power, and study heterogeneity (Clayton and McKeigue, 2001; Akey et al., 2004; Weinberg, 2009; Thomas, 2010). Indeed, most cleft studies are not sufficiently large or phenotypically well-characterized to provide the level of statistical power necessary to tease out GxE interactions. The emergence of collaborative networks such as the Gene, Environment Association Studies (GENEVA) consortium

[(Cornelis et al., 2010); <http://www.genevastudy.org>] may help resolve some of the sample size issues. Concurrently, novel study designs that increase statistical power and are not prone to confounding from population stratification are needed to advance the study of GxE interactions in clefting (Engelman et al., 2009; Murcray et al., 2009; Shi et al., 2010; Gauderman et al., 2010).

Future directions

Linkage and candidate gene approaches have had their fair share of success in identifying genes and genetic pathways involved in orofacial clefts. Because of the high prevalence and genetic complexity of this disorder, most of these studies have assessed association with common variants, under the assumption that a combination of common susceptibility alleles increases the risk of the disease. As an alternative to the common disease-common variant hypothesis, accumulation of mildly deleterious, low-frequency missense mutations may represent a potential mechanism underlying the pathogenesis of common, complex diseases (Pritchard, 2001; Pritchard and Cox, 2002). A recent quantitative study, supplemented with empirical data from mutation screening of candidate genes involved in obesity, provides a rough estimation of the degree to which such rare variants contribute to complex diseases (Ahituv et al., 2007; Kryukov et al., 2007). These low-frequency variants can only be detected when a sufficiently large number of individuals are sequenced. Indeed, case-control candidate gene resequencing efforts in NS CL/P have revealed rare missense variants with a statistically significant excess in affected individuals when large numbers of cases are sequenced (Jezewski et al., 2003; Riley et al., 2007). Recent advances in massively parallel sequencing technologies will enable us to sequence at least all protein-coding and highly conserved non-coding functional portions of the human genome, if not the entire genome, quickly and more affordably. Several recent studies clearly illustrate the power of targeted exome sequencing in detecting rare, disease-causing mutations (Ng et al., 2009; Ng et al., 2010b). A few studies have already applied whole-genome sequencing approaches to personalized medicine (Ashley et al., 2010; Lupski et al., 2010; Sobreira et al., 2010). In addition, the inclusion of rare variants with minor allele frequency typically below 0.5% in both candidate gene and GWA studies may help account for some of the missing heritability in complex disorders (Gorlov et al., 2008).

With the increasing number of associated genes without obvious amino-acid changing mutations, the significance of genetic variation in non-coding regulatory elements is becoming more recognized. Therefore, mutation screening of highly conserved, potential regulatory elements flanking candidate genes for NS CL/P should be carried out in order to identify previously undetected pathogenic variants. An excellent resource for selecting regulatory element is the genome-wide enhancer screening initiative (Pennacchio et al., 2006), which harnesses the evolutionary conservation of non-coding regions in the human genome. Enhancer activities of randomly selected, highly conserved regions are assessed in transgenic mouse assays and the data are deposited into the VISTA Enhancer database (<http://enhancer.lbl.gov>), which can then be queried based on their expression patterns in specific tissues or organs (Visel et al., 2007). Enhancer activities of highly conserved regions near candidate genes can facilitate the discovery of regulatory elements of genes expressed in the orofacial region. This can then be followed up by resequencing in NS CL/P populations to search for both rare and common etiologic variants. Again, next generation sequencing technologies will provide an opportunity to perform deep sequencing of potential regulatory elements on a much larger scale than previously possible.

Characterizing the role of regulatory RNAs in craniofacial morphogenesis provides another exciting new avenue for gene discovery, as exemplified by a recent study demonstrating, for the first time, a role for microRNA in palatogenesis (Eberhart et al., 2008). Interestingly,

disruption of a signaling pathway mediated by a member of the platelet-derived growth factor (Pdgf) family had previously been shown to result in palatal clefting in knockout mice (Ding et al., 2004). Further, targeted deletion of the Pdgf receptor alpha gene (*Pdgfra*) leads to neural tube closure defects, including midfacial and palatal clefting (Soriano, 1997). The microRNA in the Eberhart study negatively regulated translation of zebrafish *Pdgfra* mRNA via its 3'UTR region. Since spatio-temporal downregulation of *Pdgfra* is required for the proper migration of a specific subset of neural crest cells into craniofacial structures (Eberhart et al., 2008), these findings provide novel non-protein coding target genes for further evaluation in human orofacial clefting.

Conclusion

Significant knowledge of the risk factors for a range of birth defects has already been gained and is being used to guide public health recommendations for pregnancy planning in order to reduce the overall health burden of these defects. However, further research is needed on environmental and genetic factors to improve preventive measures and risk assessment. To date, smoking, alcohol and folic acid use during the first trimester of pregnancy appear to be the most consistent environmental factors for clefting, although other exposures have also been suggested. Identification of specific genetic and environmental causes of clefting could enable major changes in genetic counseling, improved programs for personalized medicine applications and aid in identifying new biological pathways and gene networks for investigation of the underlying biology. Genetic and epidemiologic studies of NS CL/P currently underway hold the promise of refining our ability for more accurate diagnosis, recurrence risk estimation, and eventually the prevention of cleft lip and palate. Information derived from GxE interaction studies will provide insights into efficient preventive interventions in individuals with susceptible genotypes. Furthermore, the inclusion of relevant subphenotypes may reveal hitherto unrecognized patterns of Mendelian inheritance, raising the exciting possibility of using standard genetic techniques to identify causative genes for isolated clefts. As we are entering the age of personalized medicine, individually tailored medications and vitamin supplementation regimen based on a thorough assessment of the genetic risk profiles of pregnant mothers may become an important measure in reducing the overall health burden of orofacial clefts and other similar birth defects.

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Figure 1. Development of the orofacial structures

The medial nasal prominences enlarge and merge with each other during the 6th–7th weeks of gestation to form the intermaxillary segment providing the basis for both the philtrum and primary palate. The intermaxillary segment fuses with the flanking maxillary prominences giving rise to the upper lip. The lateral nasal prominences form the sides of the nose. The paired bilateral maxillary prominences form the upper jaw, and the paired mandibular prominences develop into the lower jaw. Adapted from (Helms et al., 2005).

Figure 2. Refining the isolated orofacial cleft phenotype for enhanced linkage analysis

Subclinical features (or ‘subphenotypes’) such as defects in the muscle that surrounds the mouth (*Orbicularis Oris*), dental anomalies, craniofacial morphology and speech-related disorders may be part of the overall spectrum of isolated clefting. Therefore, their inclusion in classic linkage approaches may boost the search for causal genetic variants. “Others?” in the figure refers to additional subphenotypes, such as variations in brain morphology; lip pits/prints, dermatoglyphic patterns, etc. A circle inside a pedigree symbol refers to an apparently ‘unaffected’ individual who harbors at least one of the subphenotypes and is therefore categorized as a potential gene-carrier; filled symbols refer to individuals with an overt cleft.

Review of GWA studies of nonsyndromic orofacial clefts.

Table 1

Study	Study population and origin	Study design	Genotyping platform	Locus/gene with hit	Candidate gene in region *	Most significant SNP	P-value associated with SNP	OR/RR (95% CI)	Attributable risk (AR) for SNP	Results *
Birnbaum <i>et al.</i> 2009	Discovery sample: 224 NS CL/P cases and 383 controls of Central European Origin; Confirmation sample: 462 NS CL/P cases and 954 controls. SNP association study: 295 NS CPO cases.	Case-control; case-parent trio	Illumina BeadChip HumanHap550k	8q24.21	<i>PVT1</i> ; <i>GSDMC</i> ; <i>CCDC26</i>	rs987525	3.34×10 ⁻²⁴	OR=2.57 (2.02–3.26) for heterozygous genotype; OR=6.05 (3.88–9.43) for homozygous genotype.	41%	No association detected between rs987525 and NS CPO (<i>P</i> = 0.788).
Grant <i>et al.</i> 2009	111 NS CL/P cases and 5951 controls from the US, of European descent.	Case-control	Illumina Infinium II HumanHap550k BeadChip	8q24 18q22	None suggested	rs987525 on 8q24; rs17085106 on 18q22	9.18×10 ⁻⁸ ; 3.78×10 ⁻⁸	OR=2.09 (1.59–2.76) for rs987525; OR=4.07 (2.37–7.00) for rs17085106.	NA	Like the 8q24 signal, the 18q22 signal also resides in a gene-poor region. No association found with <i>IRF6</i> .
Mangold <i>et al.</i> 2010	401 NS CL/P cases and 1323 controls of Central European origin.	Case-control; Case-parent trio	Illumina BeadChips (Human610-Quad and HumanHap550k)	17q22 10q25.3	<i>NOG</i> (17q22); <i>KIAA1598</i> and <i>VAX1</i> (10q25.3)	rs227731 on 17q22; rs7078160 on 10q25.3	1.07×10 ⁻⁸ ; 1.92×10 ⁻⁸	RR=1.84 (1.34–2.53) for rs227731 in homozygotes; RR=2.17 (1.32–3.56) for rs7078160 in homozygotes.	23.9% for rs227731; 12.3% for rs7078160; joint population AR=54.6%	The study population is an extension of the Birnbaum <i>et al.</i> 2009 study. 177 additional NS CL/P cases and 940 controls were genotyped. Three was suggestive evidence of association with three other loci at 13q31.1, 15q13.3, and 2p21, respectively.
Beaty <i>et al.</i> 2010	NS CLO and NS CLP trios from Europe, US, China, Taiwan, Singapore, Korea and the Philippines. For specific numbers, see Supplementary Table 1 of the article.	Case-parent trio	Illumina Human660W-Quad v1 DNA Analysis BeadChip Kit	8q24 1q32 20q12 1p22.1	<i>IRF6</i> (1q32); <i>MAFB</i> (20q12); <i>ABCA4</i> (1p22.1)	rs13041247 in <i>MAFB</i> ; rs560426 in <i>ABCA4</i>	1.44×10 ⁻¹¹ for rs13041247; 5.01×10 ⁻¹² for rs560426	OR per minor allele=0.70 (0.64–0.78) for rs13041247 and 1.43 (1.29–1.59) for rs560426.	Three genes (<i>PAX7</i> on 1p36, <i>VAX1</i> on 10q25.3, and <i>NTN1</i> on 17p13) had one or more SNPs near genome-wide significance.	

* Gene acronyms from Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/gene>): *PVT1*, Pvt1 oncogene (non-protein coding); *GSDMC*, gastermin C; *CCDC26*, coiled-coil domain containing 26; *NOG*, noggin; *KIAA1598*, not available; *IRF6*, interferon regulatory factor 6; *MAFB*, v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian); *ABCA4*, ATP-binding cassette, subfamily A (ABC1), member 4; *PAX7*, paired box 7; *VAX1*, ventral anterior homeobox 1; *NTN1*, netrin 1

Table 2

Genes implicated in nonsyndromic cleft lip with or without cleft palate (NS CL/P)

Gene	Chromosomal location	Linkage	Association	Mutations	Deletions	Animal models
<i>IRF6</i>	1q32.2	+	+	-	-	+
<i>FOXE1</i>	9q22.3	+	+	+	-	+
<i>MSX1</i>	4p16.2	-	+	+	-	+
<i>FGFR1</i>	8p12	-	-	+	+	+
<i>BMP4</i>	14q22.2	-	-	+	-	+
<i>SUMO1</i>	2q33.1	-	-	-	+	+
<i>TBX22</i>	Xq21.1	-	+	+	-	-
<i>TP63</i>	3q28	-	-	+	-	+
<i>PVRL1</i>	11q23.3	+	+	+	-	-
<i>TGFB3</i>	14q24.3	-	+	-	-	+
<i>TGFA</i>	2p13.3	-	+	-	-	-
<i>TFAP2A</i>	6p24.3	-	-	-	+	+
<i>CRISPLD2</i>	16q24.1	-	+	-	-	-
<i>RYK</i>	3q22.1	-	-	+	-	+
<i>TBX10</i>	11q13.2	-	-	+	-	+
<i>SPRY2</i>	13q31.1	-	-	+	-	+
<i>GABRB3</i>	15q12	-	+	-	-	+