The many faces and factors of orofacial clefts

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Orofacial clefts are congenital structural anomalies of the lip and/or palate that affect ~1/1000 live births. Their frequent occurrence as well as their extensive psychological, surgical, speech and dental involvement emphasize the importance of understanding the underlying causes. The etiology of orofacial clefts is complex, including multiple genetic and environmental factors. Rare forms, where they occur as one component of multiple congenital anomaly syndromes, have Mendelian or teratogenic origins; the non-syndromic forms of orofacial clefts are more common and are likely due to secondary gene–environment interactions. Recent advances in both molecular and quantitative approaches have begun to identify the genes responsible for the rare syndromic forms of cleft and have also identified both candidate genes and loci for the more common and complex non-syndromic variants. Animal models, in particular the mouse, have also contributed greatly to an understanding of these disorders. This review describes genes that are involved in orofacial clefts in humans and animal models and explores genetic approaches to identifying additional genes and gene–environment interactions that constitute the many factors of orofacial clefts.

INTRODUCTION

Cleft lip and palate is a major congenital structural anomaly that is notable for significant lifelong morbidity and complex etiology. The prevalence of orofacial clefts varies from 1/500 to 1/2500 births depending on geographic origin (1), racial and ethnic backgrounds (2,3) and socioeconomic status (4). In many parts of the world orofacial clefts go unrepaired. Individuals who do have their clefts repaired undergo surgical, speech, dental and psychological therapies. These outcomes, along with the relatively high prevalence of orofacial clefts, emphasize the importance of understanding the underlying causes of orofacial clefts.

The causes of orofacial clefts are complex, involving both genetic and environmental factors. That genes play an almost deterministic role in the development of normal craniofacial structures is evident from observations of monozygotic twins, where the majority are phenotypically indistinguishable. At the same time, this genetic program is exquisitely sensitive to post-conception disturbances in genes or the environment, as evidenced by the identification of numerous teratogens that lead to orofacial clefts. Thus, the complex etiology of clefts affords ample opportunities to identify genes, explore gene–environment interactions and learn more about human embryology and its disturbances.

FACIAL DEVELOPMENT

Normal facial development begins with migrating neural crest cells that combine with mesodermal cells to establish the facial primordia. The growth of facial primordia from undifferentiated mesenchymal cells into the finely detailed structures of the mature head and face is largely determined genetically. These processes are known to be dependent on a spectrum of signaling molecules, transcription factors and growth factors. A subset of genes already shown to play an important role in the development of the head and with particular relevance to development of the lips and palate is listed in Table 1.

Additional growth and signaling factors (5) that play a role in facial development include JAGGED1, sonic hedgehog, patched, CREB-binding protein, GLI3, FGFR1, CASK, treacle and FGFR2. Other transcription factors involved include DLX5/6 and PAX3. Extracellular matrix proteins such as COL2A1, COL1A2, COL11A2, PIGA, αv integrin, glypican 3, fibrilllin 3 and aggrecan are essential as well. This ever-expanding catalog of molecules highlights the complex genetics of facial clefts.

When the structure or expression of these genes is altered, a cleft of some type may occur (Fig. 1). Orofacial clefts can be divided into four groups: non-syndromic cleft lip with or without cleft palate, non-syndromic cleft palate only, syndromic cleft lip with or without cleft palate and syndromic cleft palate only. The term ‘non-syndromic’ is restricted to cleft cases where the affected individuals have no other physical or developmental anomalies and no recognized maternal environmental exposures (6). Cleft cases are further divided by which palate is affected. Genetics and embryology suggest that clefts of the primary (hard) palate that involve the lip and/or palate are different in mechanism from clefts that affect the secondary (soft) palate (7).

At the present time, most studies suggest that ~70% of cleft lip with or without cleft palate (CL/P) cases are non-syndromic (8). The remaining 30% of syndromic cases can be subdivided into chromosomal anomalies, >300 different recognizable chromosomal anomalies, >300 different recognizable

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Mendelian syndromes (http://www.ncbi.nlm.nih.gov/Omim/), teratogens and uncategorized syndromes. Recent reviews of birth defects secondary to chromosomal anomalies identified five regions in which there was a significantly higher frequency of clefting associated with either specific deletions (4p16–14, 4q31–35 or 1q25) or duplications (3p26–21 or 10p15–11) than would be expected from the background frequency (9,10). Nonetheless, it is also apparent that deletions or duplications of portions of every chromosomal arm, including the X chromosome, have been associated with clefts, suggesting that many genes are involved in facial development and that cleft lip and palate can represent a common end-point for disruption of facial processes (9,10). It is likely that in the future CL/P will be subdivided further based on underlying genetic etiologies or better phenotypic descriptors. Some attempts at clinical distinction already include associations with hypoplasia of the orbicularis oris muscle or handedness (11).

### GENETICS OF OROFACIAL CLEFTS

The study of orofacial clefts has a rich history in human genetics and provides a model for complex disease study in general. An inherited component for clefts was first widely recognized through the work of Fogh-Andersen in his thesis of 1942 (12). Genetic factors in clefting are now well established through segregation analysis (13,14) as well as through twin studies (15). Additional genetic linkage and association studies are now being used to identify these genetic factors.

Although genetic linkage studies of CL/P have been limited by insufficient numbers of families and genotyping resources, a few efforts using candidate genes or loci have been reported. Studies (reviewed in refs 6,16) using from 1 to 40 families have suggested loci for clefts on chromosomes 2, 4, 6, 17 and 19. Linkage has been excluded at these same loci in other data sets. These inconsistent linkage results reflect the small number of families studied and probable locus heterogeneity. Thus, while the studies are useful for preliminary data, they need to be replicated on far larger sample sets. Only loci on 6p have consistently shown linkage to CL/P, beginning with studies from Denmark (17) and subsequently in Italy (18–20).

Association studies have also been used to examine candidate genes in CL/P. Association studies have the advantage over linkage in that they use the large number of cases that occur in isolation without affected relatives (21). In addition, association studies exploit a wealth of literature in developmental biology that identifies specific genes expressed during critical phases of lip or palate formation (22). Ardinger et al. (23) first reported a role for transforming growth factor-α (TGFA) as contributing to CL/P. Since that time, five studies have confirmed this result and, although several other studies have failed to replicate the association, a recent meta-analysis supports a role for TGFA as a modifying factor in cleft lip and palate (24). Other genes/loci showing association include D4S192, MSX1, TGFB3 and RARA (16,25–29), with only the TGFB3 finding replicated. A summary of human studies is shown in Table 1.

### ANIMAL MODELS

Candidate loci for CL/P have also been proposed based on spontaneously arising or transgenic mouse models with clefts. While there are many mouse mutants that include clefts of the lip or palate as part of the phenotype, the best candidates for human clefting are those in which clefts appear without other abnormalities, including Cfl1, Cfl2, Cps-1, Dep-1 and Dep-2. Two genome-wide searches for susceptibility loci in the mouse have also been performed. One used the A strain derivative A/WySn to identify two loci for cleft susceptibility, Cfl1 and Cfl2 (30,31). A second scan used teratogen susceptibility in the AXB/BXA inbred strains and identified 16 susceptibility regions, including one near Msx1 (32).

Random insertions and targeted knockouts in the mouse have now been generated for >10 years and 30 of these are listed in the transgenic database (http://tbase.jax.org) as including cleft lip and/or palate. Although, initially, transgene phenotypes appeared to support a role for certain genes in cleft causation, it is now apparent that clefts are a frequent end-point of knockout and insertion experiments. For a gene to be a strong cleft candidate, it must provide a model for complex disease study in general. An
candidate, it must result in a clefting phenotype in the transgene and be expressed at a critical time and in a tissue relevant to lip and palate development. The three best examples to date are the Msx1, Tgfb3 and Ap-2 knockouts, in which gene expression supports a role in craniofacial development and the knockouts result in clefts.

For Msx1, two independent knockouts (33,34) result in 100% cleft palate and Msx1 is expressed in developing craniofacial structures (35), including the palate (P. Sharpe, personal communication). MSX1 is deleted in cases of the human 4p– syndrome, which commonly includes clefts. In the case of Tgfb3, two independent knockouts result in the phenotype of cleft palate (36,37). Expression data (38,39) and recent work showing that exogenous TGFβ3 can induce palate fusion in the chicken, where the palate is normally cleft (40), further support a role for TGFβ3 in clefting.

Figure 1. A photomontage of children with various forms of orofacial clefts. (A) Right-sided unilateral cleft lip and palate; (B) left-sided unilateral cleft lip only; (C) bilateral cleft lip and palate with right lower paramedian lip pit, indicative of Van der Woude syndrome; (D) isolated cleft of the soft palate.
phenotype. Of the ∼Mendelian pattern of inheritance and a closely related syndromic clefting is to study a disorder that exhibits a clear

alternative approach for identifying genetic factors in non-syndromic clefting (43,44).

near the site of two balanced translocations at 6p that have CL/P (42) suggest a more specific role for Ap-2 in clefting. Ap-2 also lies

and palate is the Van der Woude syndrome, an autosomal dominant disorder characterized by paramedian lip pits of the lower lip, 50% are autosomal recessive, 40% autosomal dominant and 10% X-linked. Approximately 30 genes have been cloned from humans wherein gene disruptions can include a cleft as part of the phenotype. Gene classes represented in this group include extracellular matrix proteins (COL2A1, COL11A2 and GPC3), transcription factors (GLI3, PAX3, SIX3 and SOX9) and cell signaling molecules (FGFR2, PTCH and SHH). Two mapped disorders, X-linked cleft palate (OMIM 303400) and Van der Woude syndrome (OMIM 119300), are particularly attractive models because their phenotypes are so similar to non-syndromic forms of clefting. One of the first birth defect loci mapped using DNA-based polymorphisms was that for the X-linked cleft palate and ankyloglossia locus (CPX), mapped by linkage to Xq21-q22 (45). The CPX phenotype is exclusively confined to the palate, which may be cleft or insufficient, and to the tongue, resulting in ankyloglossia (tongue-tied). Although the gene associated with this phenotype is not yet identified, high resolution mapping of the X chromosome will likely soon disclose its nature (46) and lead to a better understanding of palate development. Another good Mendelian model for non-syndromic cleft lip and palate is the Van der Woude syndrome, an autosomal dominant disorder characterized by paramedian lip pits of the lower lip (Fig. 1C), cleft lip with or without cleft palate, isolated cleft palate alone and occasional hypodontia. The disorder has no other craniofacial anomalies and is associated with normal intelligence. The condition received its descriptive eponym following Anne Van der Woude’s description of the disorder in 1954 (47) but has been recognized as a common form of clefting with multiple families reported for well over a century (48). Burdick et al. (49) have reported an extensive summary of

families with the disorder and it is particularly remarkable in that it is one of the very rare disorders in which clefts of the primary palate can be found in the same family and segregating with the identical allele as isolated secondary palate clefts. This suggests that a very early stage of embryogenesis is likely being affected.

Linkage for Van der Woude syndrome and chromosomal localization was first suggested by Wienker et al. (50) and by the report of Bocian and Walker (51) of a large, cyogenetically visible deletion on the long arm of chromosome 1. In 1990, genetic linkage was established (52) to 1q32 and, subsequent to this, linkage has been confirmed by other groups more finely defined through the use of recombinants (53,54) and microdeletion patients (55,56) that have confirmed a relatively narrow region in which the gene is likely to reside. Efforts at identifying genes within this region are currently under way and a complete BAC contig and sequence from this region is now under analysis. The identification of the Van der Woude gene itself is likely to contribute greatly to a better understanding of other genes and gene paths likely to play a role in craniofacial development. A substantial number of such genes that include other complex anomalies are shown in Table 1. However, Van der Woude syndrome is of particular interest because the phenotype is so similar, with the exception of the lip pits, to the appearance of non-syndromic cleft lip and palate.

ENVIRONMENTAL INTERACTIONS
An environmental component to clefting was recognized when Warkany et al. (57) associated nutritional deficiencies with cleft palate. Recognized teratogens that cause clefts include rare exposures, such as phenytoin, valproic acid and thalidomide, and also common environmental exposures, such as maternal alcohol or cigarette use (58) and, more recently, pesticides such as dioxin (59). The exposures are important in that they can suggest metabolic pathways whose disruption may play a role in the development of CL/P. Epidemiological studies support a role for environmental factors in clefting, especially in regions of low socioeconomic status (SES). In the Philippines, three studies (4,60,61) all report incidences of CL/P of 2/1000 in indigent populations, while complementary studies show an incidence of 1.2/1000 in native Filipinos living in areas of higher SES, including Manila (60), Hawaii (62) and California (2). When SES did not change

Table 2. Studies of candidate genes for clefting

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Linkage¹</th>
<th>Association¹</th>
<th>TDT²</th>
<th>Other data²</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFα</td>
<td>2p13</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
<td>–</td>
<td>23,24,26</td>
</tr>
<tr>
<td>MSX1</td>
<td>4p16</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
<td>CH/KO/EXP</td>
<td>26,95</td>
</tr>
<tr>
<td>N/A³</td>
<td>4q31</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>25,96</td>
</tr>
<tr>
<td>N/A³</td>
<td>6p23</td>
<td>++/–</td>
<td>++/–</td>
<td>–</td>
<td>CH/KO</td>
<td>17–19</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>1q24</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
<td>KO/EXP</td>
<td>26,28</td>
</tr>
<tr>
<td>RARA</td>
<td>17q21</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
<td>TG/EXP</td>
<td>27,97–99</td>
</tr>
<tr>
<td>BCL3</td>
<td>19q13</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
<td>CH</td>
<td>26,29,100</td>
</tr>
</tbody>
</table>

¹–, one or more negative studies; +, single positive study; ++, more than one positive study.

²CH, chromosome deletion (recurrent) or translocation; KO, mouse knockout; TG, transgene; EXP, expression.

³No candidate genes have been identified at this map locus.

Knockouts of the retinoic acid-dependent transcription factor Ap-2 resulted in extensive craniofacial and more generalized structural disruptions (41). Recently, chimeric knockouts for Ap-2 (42) suggest a more specific role for Ap-2 in clefting. Ap-2 also lies near the site of two balanced translocations at 6p that have CL/P phenotypes (43,44).

GENE IDENTIFICATION
An alternative approach for identifying genetic factors in non-syndromic clefting is to study a disorder that exhibits a clear Mendelian pattern of inheritance and a closely related phenotype. Of the ∼150 Mendelian disorders that include cleft lip, 50% are autosomal recessive, 40% autosomal dominant and 10% X-linked. Approximately 30 genes have been cloned from humans wherein gene disruptions can include a cleft as part of the phenotype. Gene classes represented in this group include extracellular matrix proteins (COL2A1, COL11A2 and GPC3), transcription factors (GLI3, PAX3, SIX3 and SOX9) and cell signaling molecules (FGFR2, PTCH and SHH). Two mapped disorders, X-linked cleft palate (OMIM 303400) and Van der Woude syndrome (OMIM 119300), are particularly attractive models because their phenotypes are so similar to non-syndromic forms of clefting.

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through a geographic move, no change in frequency was noted by Christensen et al. (63). Thus, nutritional or toxic environmental exposures may contribute directly to as much as one third of cleft cases and etiologies will be most identifiable in indigent populations.

Evaluation of gene–environment interaction (64) is still at a preliminary stage (28,65), but studies have looked at the role of smoking with TGFA acting as a covariate (66–68). An interaction is suggested, although not confirmed, in all studies (69,70). Preliminary data (68,70–72) support interactions between alcohol, nutritional factors and the MSX1 and TGFB3 genes, in addition to TGFA. Fetal alcohol syndrome can include clefts of the lip and/or palate as part of the phenotype. Vitamin A and its congeners, such as Accutane, are known to induce craniofacial abnormalities (73). Folate-metabolizing enzymes are candidates based on preliminary data that suggest that folate acid supplementation can reduce the incidence of clefting (74,75). However, the data remain controversial (76), with no gene supplementation can reduce the incidence of clefting (74,75). However, the data remain controversial (76), with no gene association yet found for methylene tetrahydrofolate reductase (77), a key player in the folate pathway in neural tube defects. Pyridoxine (vitamin B₆) may also play a role in facial development and protective effects have been suggested in studies in rats (78) and humans (79). Enzymatic pathways that are candidates for variation-induced clefting thus include the genes for alcohol, vitamin A, vitamin B₆ and folate metabolism.

Other risks include environmental estrogens or dioxins (TCDD) which bind to endogenous nuclear receptors that also serve as transcription factors (80,81). This activity is mediated through the aryl hydrocarbon receptor (AhR) and the AhR nuclear translocator (ARNT) genes, which are expressed in developing palate (82) and have their expression altered by dioxins (83). Dioxin and retinoic acids also alter TGFB3 expression (84,85) and there are strong teratogenic effects of dioxins (86) and retinoic acid (87) in the mouse and possibly human (59,73). Thus, a plausible path for gene–environment interactions might involve environmental effects (alcohol, dioxins and estrogens) mediated via the AhR/ARNT and retinoic acid pathways and disturbing the critical role of TGFA or TGFB3 in lip and palate formation.

SUMMARY

Studies of orofacial clefting are valuable, both for the morbidity of the defect itself and for providing a model to examine a complex human birth defect. Identification of specific genes and environmental factors will immediately provide for better risk counseling and holds the promise of preventive strategies and improved therapeutics. As a model for complex traits, cleft lip and palate is ideal, with a high frequency in global populations, so that collections of large numbers of isolated and familial cases will have the power to determine genetic and environmental etiology.

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