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Developmental Biology: Frontiers for Clinical Genetics

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Gene/environment causes of cleft lip and/or palate

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Craniofacial anomalies, and in particular cleft lip and palate, are major human birth defects with a worldwide frequency of 1 in 700 and substantial clinical impact. A wide range of studies in developmental biology has contributed to a better knowledge of how both genes and environmental exposures impact head organogenesis. Specific causes have now been identified for some forms of cleft lip and palate, and we are at the beginning of a time in which the common nonsyndromic forms may also have specific etiologies identified. Mouse models have an especially important role in disclosing cleft etiologies and providing models for environmental cotriggers or interventions. An overview of the gene–environment contributions to nonsyndromic forms of clefting and their implications for developmental biology and clinical counseling is presented.

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Craniofacial anomalies comprise a significant component of morbid human birth defects. They require surgical, nutritional, dental, speech, medical and behavioral interventions and impose a substantial economic burden (1). Clefts of the lip and palate affect about 1/700 births with wide variability related to geographic origin (2) and socioeconomic status (3). In general, Asian or Amerindian populations have the highest frequencies, often at 1/500 or higher, with Caucasian populations intermediate, and African-derived populations the lowest at 1/2500. There are many exceptions to these summaries, however, with some particular geographic areas having high frequencies thought to be related to founder effects or environmental triggers. The complex etiology of clefting affords ample opportunities to identify genes and gene–environment interactions and to learn more about human embryology and its disturbances (4).

Fogh-Andersen (5) first defined genetic factors in clefting, which have been confirmed by segregation analysis (6). Genetics and embryology suggest that clefts of the primary (hard) palate that involve the lip and/or palate are different in mechanism from clefts affecting only the secondary (soft) palate (7). Following these general rules, clefts are most often discussed as either those that involve the lip with or without the palate (CL/P) or those that involve the palate only (CPO) (see Fig. 1). In addition, clefts can be divided into nonsyndromic and syndromic forms. In nonsyndromic clefts, affected individuals have no other physical or developmental anomalies. Most studies suggest that about 70% of cases of CL/P and 50% of CPO are nonsyndromic (8). The syndromic cases can be subdivided into chromosomal syndromes, more than 350 Mendelian disorders (Online Mendelian Inheritance in Man, 2002), teratogens (e.g. phenytoin or alcohol) and uncategorized syndromes.

Genetics

Advances in both quantitative and molecular analysis make linkage and association approaches to CL/P etiology practical (9). Animal models can provide genes and loci for studies in humans and can be used themselves to look at gene–gene and gene–environment interaction. Dense genetic maps (10) provide resources for family-based studies. Studies of twins have been particularly informative

regarding the genetics of clefting. Concordance in monozygotic (MZ) twins ranges between 40% and 60%, and is 5% in dizygotic twins. The lack of 100% concordance in monozygotic twins suggests that genetic events alone are not responsible for the clefting phenotype. Either some degree of non-penetrance, perhaps based around random developmental events, or the dissimilar environmental effects found in what might not be a homogeneous *in utero* environment must underlie this discordance. Nonetheless, the greatly increased MZ concordance does strongly support a major genetic component.

Genetic linkage studies of CL/P have been limited by insufficient numbers of families and genotyping resources (11). Studies (12, 13) using from one to 40 families suggest loci for clefts on chromosomes 4, 6, 17 and 19. Linkage has been excluded at these same loci in other datasets. Only loci on 6p have consistently shown linkage to CL/P in Denmark (14), Italy (15, 16) and the UK (17). One genome-wide screen has been carried out using approximately 100 sib-pairs from the UK (17). Although no highly significant loci were identified in this study, nine regions of interest were confirmed in a 5-cm scan. Three of these (1p36,

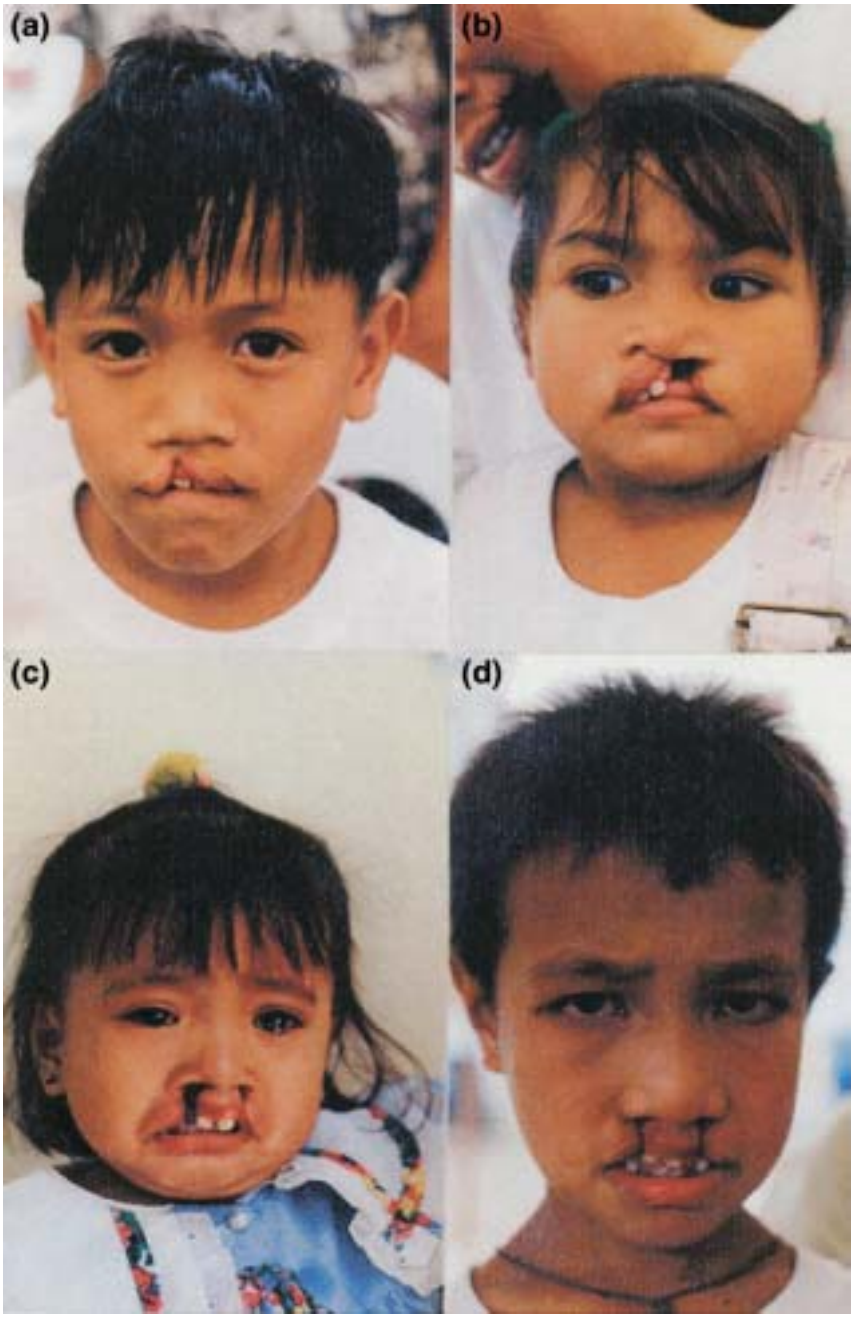

Fig. 1. Four children with unrepaired facial clefts: (a) unilateral cleft lip only; (b) unilateral cleft lip and palate; (c) bilateral cleft lip and palate; (d) Van der Woude syndrome with a lower lip pit and bilateral cleft lip and palate.

2p13 and 6p24) are near genes or loci suggested in other studies (Table 1). One region at 1p36 has at least three genes of interest (SKI, P73 and MTHFR) and deletions of 1p36 have an increased frequency of clefting (47). Future studies involving larger sets of families are likely to provide additional power to use the genome-wide search approach.

Association studies have also been used extensively 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 (48). 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 (49). Ardinger et al. (50) first reported a role for transforming growth factor alpha (TGFA) as contributing to CL/P. Although some studies have failed to replicate this association, a recent meta-analysis supports a role for TGFA as a modifying factor in cleft lip and palate (51), as does expression-based analysis (52). Other genes/loci showing association include D4S192, MSX1, TGFB3, RARA, MTHRF, GABRB3 and PVRL1, with the data summarized in Table 1. MSX1 is of particular interest in that a large pedigree published by van den Boogaard et al. (53) showed that a stop codon mutation in exon 1 cosegregated with the phenotype of cleft lip and/or palate in multiple family members. Hypodontia was also found in many affected family members, which is consistent with previous evidence that missense mutations in MSX1 can cause isolated dental anomalies (54). This family provides strong evidence that what appears to be nonsyndromic clefting (if the dental anomalies are overlooked, as might easily happen) can provide a candidate for other nonsyndromic forms as well. In addition, it suggests that mixed clefting types (CPO and CL/P) can occur secondary to the same mutation.

Chromosomal anomalies can also provide important clues for genes involved in clefting. A comprehensive survey of chromosomal deletions (55) and duplications (56) was done to identify phenotypes significantly associated with particular partial aneuploidies. Regions that were highly significantly associated with clefts were identified at 1q25, 3p21, 4p15, 4q32 and 10p15. The 4p15 region is of particular note in that it contains the MSX1 homeobox gene and is also the site of deletions causing the Wolf–Hirschhorn syndrome, which is commonly associated with orofacial clefting as well.

Several recent studies have also provided strong evidence that syndromic forms of clefting may provide insights into genetic etiologies in nonsyndromic forms. An autosomal recessive disorder, Margarita Island Ectodermal Dysplasia and Clefting syndrome, was shown to have mutations in the PVRL1 gene (57). Recent evidence from this group (58) suggests that heterozygotes for this mutation may also have an increase in nonsyndromic clefting. Although this study needs to be replicated (59), it opens an exciting door into additional genes and mechanisms for nonsyndromic clefting. As PVRL1 is a cell adhesion molecule with viral receptor homologies and additional family members, these molecules would serve as good candidates for investigation. Other disorders in which apparent nonsyndromic clefting may show up in extended pedigrees include the CPX (60) and EEC syndromes (61). Mutations in the P63 gene underlying EEC can occasionally be found in individuals in whom an isolated cleft may appear to be the only abnormality, and this is similarly true of cleft palate only for TBX22 mutations in CPX where the ankyloglossia may be mild or overlooked. Finally, the Van der Woude syndrome (VDWS), an autosomal dominant form of clefting on the long arm of chromosome 1 (62) in which lip pits and hypodontia are the only additional anom-

Table 1. Gene linkage/association studies of clefts

Gene	Locus	Linkage	LD/TDT	Other data	References
SKI/MTHFR	1p36	+	+ + / –	CH	18, 19, 20, 21, 22, 23, 24, 25
TGFB2	1q41	–	– / +	KO/EXP	26, 27
TGFA	2p13	–	+ + / –	EXP	26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 50, 51
MSX1	4p16	+	+ + / –	CH/KO/EXP	26, 40, 41, 42
	4q31	+ / –	+ / –	CH/KO/EXP	43
	6p23	+ + / –	–	CH/KO	15, 16, 33
PVRL1	11q23	–	+	EXP	58
TGFB3	14q24	–	+ + / –	KO/EXP	26, 27, 40, 41, 42
GABRB3	15q11	–	+	KO	27, 82
RARA	17q21	+ / –	+ / –	TG/EXP	29, 44
BCL3	19q13	+ / –	+ / –	CH	45, 46

Linkage disequilibrium/transmission disequilibrium test (LD/TDT); –, negative studies; +, one positive study; + +, more than one positive study; CH, chromosome deletion (recurrent) or translocation, mouse knockout (KO), transgenic (TG) or expression (EXP).

alies noted outside of isolated clefts of the lip or palate, must always be a consideration in families in which more than one individual is found with a cleft. Nonpenetrance for the lip pit phenotype is found in at least 10% of affected individuals and those without the pits are phenocopies for nonsyndromic clefting. VDWS also mimics the MSX1 mutations noted above in that isolated CPO and CL/P occur in the same family, suggesting that the VDWS gene may lie in the same developmental program as MSX1.

Animal models

Many mouse mutants include clefts of the lip or palate as part of the phenotype (Table 2). For human nonsyndromic clefting the best candidates are those in which clefts appear without other abnormalities, including Clf1 and Clf2. Two genome-wide searches for susceptibility loci in the mouse have been performed. One used the A strain derivative A/WySn to identify (69) two loci for cleft susceptibility – Clf1 and Clf2. A second scan used teratogen susceptibility in the AXB/BXA inbred strains (70) and identified 16 susceptibility regions, including one containing Msx1. Random insertions and targeted knockouts in the mouse have now been generated for over 10 years and more than 40 of these are listed in the transgenic databases as including cleft lip and/or palate. Although transgene phenotypes initially seemed 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 requires not only a clefting phenotype from the transgene but also that normal gene expression includes a critical time and tissue for lip and palate development. Four excellent examples are the Msx1, Tgfb3, Tfap2a and Gabrb3 in which gene expression supports their role in craniofacial development and the knockouts result in clefts. These four are also supported by genetic data summarized in Table 1 for humans.

For Msx1, two independent knockouts (71, 72) result in 100% cleft palate, and Msx1 is expressed in developing craniofacial structures. In the case of Tgfb3, two independent knockouts result in the phenotype of cleft palate (73, 74). Expression data (75, 76) and work showing that exogenous TGFβ3 can induce palate fusion in the chicken (77), where the palate is normally cleft (and TGFβ3 absent), further support a role for TGFB3 in clefting. Knockouts of the retinoic acid-dependent transcription factor Tfap2a resulted in extensive craniofacial and other structural disruptions (78). Chimeric knockouts for Tfap2a (64) suggest a more specific role for Tfap2a in clefting. Tfap2a also lies near the site of two balanced translocations that have CL/P phenotype (79, 80). The knockout of Gabrb3 has CP in a portion of animals and normal gross brain morphology but with seizures and abnormal behavior (81). A recent transmission disequilibrium test (TDT) study suggests GABRB3 may play a role in human clefting (82) and the association of clefting with functional brain anomalies is consistent with recent human studies of CL/P showing some cognitive deficits not previously recognized and accompanied by magnetic resonance imaging (MRI) differences (83).

Environmental studies

An environmental component to clefting was recognized when Warkany et al. (84) associated nutritional deficiencies with cleft palate. Recognized teratogens that cause clefts include rare exposures, such as phenytoin, valproic acid and Thalidomide,

Table 2. Mouse models relevant to human clefting

Gene/locus name	Mouse/human chromosomal location	Type	Phenotype	References
Msx1	5/4p16	KO	CP, D	71, 72
Tgfb3	12/14q24	KO	CP, D	73, 74
Tgfb2	1/1q41	KO	CP	63
Tfap2a	13/6p24	KO	CL, CP, D	64
Ryk	9/3q22	KO	CP	65
Lhx8	3/1p	KO	CP	122
Ski	4/1p36	KO	CL, CP	66
Gabrb3	7/15q11	KO	CP	81
Pax9	12/14q12	KO	CP, D	67
Dlx2	2/2q24	KO	CP, D	68
clf1	11/17q	SM	CL	69
clf2	13/5q or 9q	SM	CL	69

CL, cleft lip; CP, cleft palate; D, dental anomalies; KO, knockout; SM, spontaneous mutation.

and also common environmental exposures, such as maternal alcohol or cigarette use (85), herbicides such as dioxin (86), and altitude (87). The exposures are important in that they can suggest metabolic pathways whose disruption may play a role in the development of CL/P. Epidemiologic studies support a role for environmental factors in clefting, especially in regions of low socioeconomic status (SES). In the Philippines, three studies (3, 88, 89) report incidences of CLP 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 (89), Hawaii (90) and California (91). When SES does not change through a geographic move, no change in frequency was noted by Christensen et al. (92). 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. A summary of some recent environmental studies is presented in Table 3.

Gene–environment connections

Gene–environment interactions (103, 104) for nonsyndromic CL/P are summarized in Table 4. TGFA and smoking have been most widely studied, with an interaction suggested but not confirmed. Preliminary data also support interactions between alcohol, nutritional factors and the MSX1 and TGFB3 genes in addition to TGFA. Alcohol induces the fetal alcohol syndrome, which includes 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 anomalies (112). Folate-metabolizing enzymes are candidates based on preliminary (113) data that suggest that folic acid supplementation can reduce the incidence of clefting, but the data remain controversial (114). Gene associations for methylene tetrahydrofolate reductase (MTHFR) based on work in neural tube defects (115) are widely reported but again with no consensus (Table 4). Enzymatic pathways that are candidates for variation-induced clefting with common exposures include the genes for alcohol, vitamin A, smoking by-products, and folate metabolism.

Other risks include environmental estrogens or dioxins, which bind to endogenous nuclear receptors that also serve as transcription factors (116). This activity is mediated through the aryl hydrocarbon (Ah) receptor and the Ah receptor nuclear translocator (ARNT) genes, which are expressed in developing palate and have their expression altered by dioxins. Dioxin and retinoic acids also alter TGFB3 expression (117, 118), and there are strong teratogenic effects of dioxins (119) and retinoic acid (120) in the mouse and possibly human (86, 112). One path for gene–environment interactions might involve environmental effects (alcohol, dioxins, estrogens) mediated via the Ah–ARNT and retinoic acid pathways and disturbing the critical role of TGFA or TGFB3 in lip and palate formation.

Table 3. Environmental risks of clefting

Agent	Selected references (both positive and negative)
Infections	42, 93
Smoking	42, 94, 95, 96, 97, 98
Alcohol	42, 96, 99, 100
Vitamins	101, 102, 113, 121

Table 4. Gene–environment interactions in cleft lip and palate

Gene/Environmental	References
TGFA/Smoking	32, 35, 105, 106, 107
TGFA/Alcohol	106
TGFA/Vitamins	108
MSX1/Smoking	106, 109
MSX1/Alcohol	106, 109
TGFB3/Smoking	32, 106, 109
TGFB3/Alcohol	106, 109
RARA/Smoking	32
MTHFR/Vitamins	18, 20, 21, 22, 23, 24, 25
P450/Smoking	110
GST/Smoking	110, 111
EPHX1/Smoking	111

Diagnosis and prevention

Studies of genes and environmental interactions with orofacial clefting have started to provide insights into better diagnosis and prevention. Preventively, it is clear that avoiding common exposures in pregnancy of smoking and alcohol is likely to decrease the risk of having a child with a cleft. Other drugs for medical treatment, particularly anticonvulsant medications, need to be evaluated carefully, as they pose risks to the fetus but need to be balanced against the risk of withdrawal for a mother affected with a seizure disorder. While some particular environmental exposures may have their risks enhanced by pharmacogenetic variation identified in the mother or the fetus, we have not yet reached the stage where these assays can provide useful predictive information. With respect to genetic diagnosis, it is clear that syndromic evaluation needs to be carried out in great detail, and in particular looking for evidence of the hypodontia that may be associated with MSX1 mutations or the lip pits associated with Van der Woude syndrome, as well as the more apparent clinical

syndromes that cause clefting, needs to be a part of any evaluation. We may soon be at the stage at which molecular diagnosis of MSX1, the Van der Woude syndrome gene, or other gene mutations can provide useful data for recurrence risks. Finally, prevention may also benefit from maternal nutritional supplementation, in particular with folic acid, vitamin B_6 or other micronutrients (121). Although the evidence for the use of folate or vitamin B_6 is not yet confirmed, preliminary reports support these efforts, and at a minimum, all mothers should take the recommended prenatal vitamins, beginning preconceptually and continuing throughout pregnancy, which would include 400 μg of folic acid daily. Whether recurrences of clefts can be reduced within families with a history of clefting awaits the results of randomized clinical trials.

It seems likely that over the next decade, specific information regarding prevention using easily manipulable environmental agents, such as micronutrients, as well as far more explicit data about the specifics of recurrence risks will be a routine part of practice. In parallel with these important clinical advances, our understanding of the biology of clefting is also increasing at a dramatic rate, and we will soon be at the time when our understanding of craniofacial structure development has a sound, biological basis.

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