Orofacial clefting: recent insights into a complex trait
Astanand Jugessur¹,² and Jeffrey C Murray¹,³

Orofacial clefts are common birth defects of multifactorial etiology. Several novel approaches have recently been applied to investigate the causes of clefts. These include examining Mendelian forms of clefting to identify genes that might also be implicated in isolated clefting, analyzing chromosomal rearrangements in which clefting is part of the resultant phenotype, studying animal models in which clefts arise either spontaneously or as a result of mutagenesis experiments, exploring how expression patterns correlate with gene function and examining the effects of gene–environment interactions. Together, these complementary strategies are providing researchers with new clues as to what mechanisms underlie orofacial clefting.

Addresses
¹ Department of Paediatrics, University of Iowa, Iowa City, IA 52242, USA
² Section for Epidemiology and Medical Statistics, Department of Public Health and Primary Health Care, University of Bergen, Norway
³ The Institute of Public Health, University of Southern Denmark, Odense, Denmark

Corresponding author: Murray, Jeffrey C (jeff-murray@uiowa.edu)

Introduction
Orofacial clefts comprise a large fraction of all human birth defects and are notable for their significant lifelong morbidity and complex etiology. On the basis of anatomical, genetic and embryological findings, orofacial clefts are commonly subdivided into those affecting the lip and/or palate (CL/P) and those involving the palate only (CPO) [1]. Clefts can be further categorized into syndromic (see Glossary) and isolated forms, according to whether affected individuals have other physical and developmental anomalies. Because the great majority of clefts appear to be isolated (~70% CL/P and ~50% CPO) [2], understanding the causes of these forms of clefts has long been a focus of research.

Many aspects of clefting, including epidemiology, clinical care, and genetic and environmental risks, have been recently reviewed [3]. In this overview, we focus on recent developments in genetics [4], animal models [5] and gene–environment interactions [6].

Development of the lip and palate
After conception, a precisely coordinated cascade of developmental processes involving cell migration, growth, differentiation and apoptosis results in the development of craniofacial structures from the originating oropharyngeal membrane [7]. Early in the sixth week, the medial nasal prominences merge with each other and the bilateral maxillary processes to form the primary palate and the upper lip. The lower lip and jaw are produced by the mandibular prominences, which merge across the midline. The secondary palate begins to develop early in the sixth week from the two palatal shelves, which extend from internal aspects of the maxillary prominences. During weeks 7–8, apoptosis and epithelial–mesenchymal transformation (EMT) at the medial edges enable the palatal shelves to fuse after the shelves have ascended to an appropriate position above the tongue. Proteins such as integrins, matrix metalloproteinases, microtubules and actin cytoskeletons are involved in the EMT process [8].

The molecular events that underlie the formation of orofacial structures are under the strict control of an array of genes that includes the fibroblast growth factors (Fgfs), sonic hedgehog (Shh), bone morphogenetic proteins (Bmps), members of the transforming growth factor β (Tgf-β) superfamily, and transcription factors such as Dlx, Pitx, Hox, Gli and T-box families [2]. Hydration of extracellular matrix components (principally hyaluronan) in the shelf mesenchyme is thought to provide the necessary intrinsic force to cause shelf elevation [9]. However, contraction of elastic fibers and/or skeletal muscle fibers, and an increase in vascularity of the developing palate have also been proposed as alternative mechanisms underpinning shelf elevation. Palatal fusion itself appears to be driven by several cell adhesion molecules, including nectin 1, desmosomes and type IX collagen, and growth factors, such as TGFα/EGFR and TGF-β3 [8,9].

The search for candidate genes
A variety of genetic approaches have been used to identify candidate genes and loci responsible for clefting [4]. Compiled in Table 1 is a list of candidate genes derived from linkage and association studies, studies of the roles these genes play in animal development and the phenotypes they generate when disrupted in mouse knockouts [1,5]. Genome-wide linkage scans have also provided some important clues. To date, 13 genome-wide scans
for nonsyndromic CL/P have been performed, and a meta-analysis (see Glossary) of these individual scans revealed significant heterogeneity LOD scores (see Glossary) on chromosomes 1p, 6p, 6q, 14q and 15q, and a particularly strong signal on 9q [10].

The past two years in particular have witnessed several exciting new advances in the mapping of genes for clefting. The latest data from mouse and human studies have helped identify several genes known to underlie Mendelian syndromic forms of CL/P as also playing a role in the etiology of isolated clefts. These include IRF6 [11**, MSX1 [12], PVRL1 [13], TBX22 [14] and FGFR1 [15] (Table 1).

**Clues from Mendelian forms of clefts**

Mendelian forms of clefting with phenotypes closely mimicking those of isolated clefts can greatly facilitate the mapping of genes underlying the isolated forms [2]. The autosomal dominant Van der Woude syndrome (VWS) is the best model studied to date. In addition to clefts, pits in the lower lip and hypodontia are the only additional features in VWS patients. Recently, mutations in the interferon regulatory factor 6 (IRF6) gene were reported to underlie VWS [16], and, subsequently, variants in IRF6 were found to be significantly associated with nonsyndromic clefting as well [11**,17]. In the mouse, Irf6 transcripts are highly expressed in the palatal medial edge epithelium (MEE) immediately before and during fusion of the palatal shelves [16] (Figure 1). It has been speculated that mutations in IRF6 might repress the TGF-β signaling pathway in a manner analogous to IRF1-mediated repression, leading to increased epithelial apoptosis before the bilateral processes have managed to fuse [8].

Knocking out a second gene, Msx1 (msf homeobox homolog 1), in mice results in clefting [18]. The Msx proteins are known to play key roles in epithelial–mesenchymal tissue interactions during craniofacial development [19]. In humans, MSX1 is deleted in cases of a 4p deletion syndrome that is frequently associated with clefting [20]. Moreover, a nonsense mutation in exon 1 of MSX1 caused tooth agenesis and various combinations of clefts in a Dutch family [21]. In a follow-up study of 1000 unrelated individuals with CL/P, complete sequencing of the gene showed that mutations in MSX1 alone could account for 2% of isolated CL/P [12,22].

A third gene, FGFR1 (fibroblast growth factor receptor-1), encodes a transmembrane receptor tyrosine kinase that transduces signals from secreted FGFs [23]. Loss-of-function mutations in FGFR1 cause the autosomal dominant form of Kallmann syndrome (KAL2), which is characterized by hypogonadism and anosmia, and clefting in around 5–10% of the cases [15]. The variable expression of FGFR1 variants results in some affected individuals presenting with isolated CL/P alone (JC Murray, unpublished).

Mutations in TP63 are implicated in five distinct human developmental disorders, characterized by limb abnormalities, ectodermal dysplasia and orofacial clefts [24]. Interestingly, the distribution of mutations over the different p63 protein domains shows a clear pattern of genotype–phenotype correlation. Other notable examples of clefting syndromes that might include phenocopies of isolated clefts are X-linked cleft palate with ankyloglossia, caused by mutations in TRX2 [14,25], cleft lip and palate-ectodermal dysplasia syndrome (PVRL1) [13,26], and lymphedema-distichiasis syndrome (FOXC2) [27,28]. Other genes underlying additional clefting syndromes that are also excellent candidates for investigating the causes of isolated clefts include FOXE1 in Bamforth-Lazarus syndrome [29] and FLNA in otopalatodigital syndromes types 1 and 2 [30].

**Clues from genomic rearrangements**

Genomic rearrangements can arise when interspersed repeat elements lying in tandem facilitate submicroscopic
deletion and duplication events or translocations and/or inversions between or within chromosomes [31]. Genetic variants that result in a phenotype including clefting and that are found segregating with a genomic rearrangement in multiple members are best represented by the 22q deletion syndrome. Duplications of this same region have been associated with cleft palate [32], suggesting that genome-wide searches using comparative genomic hybridization (CGH; see Glossary), quantitative-PCR (see Glossary) or allele-loss might reveal additional clefting loci.

Recently, two relevant genes or gene clusters with balanced translocations and CL/P have been identified: the first gene at 19q13 [33] and the second gene at 2q32 [34]. The candidate gene transected at 2q32 is SATB2. It

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytogenetic locationa</th>
<th>Gene function</th>
<th>Animal model phenotypeb</th>
<th>Expression data</th>
<th>Linkage/association</th>
<th>Known syndrome</th>
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<td>1p36</td>
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<td>+/−</td>
<td>+</td>
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<td>2p13</td>
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<tr>
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<td>TF</td>
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<td>+</td>
<td>−</td>
<td></td>
</tr>
<tr>
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<td>17q21</td>
<td>CS</td>
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<td>+</td>
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<td>TF</td>
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<td>−</td>
<td>+</td>
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<tr>
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<td>Xq21</td>
<td>TF</td>
<td>NA</td>
<td>+</td>
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</tbody>
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Abbreviations: CAM, cell adhesion molecule; CS, cell signaling; EEC, ectrodactyly ectodermal dysplasia; GF, growth factor; GFR, growth factor receptor; NA, not available; TF, transcription factor; −, negative; +, positive; +/−, weak.

Table 1 is based in part on data compiled from references [1,6,8].

b From the Mouse Genome Informatics database (MGI; http://www.informatics.jax.org/).

Figure 1

Ir6 expression in the E14.5 prefusion mouse palate. The figure depicts an in situ frontal section through the posterior palate. Nuclei were stained with DAPI for background staining and the silver grains were pseudo-colored red in the merged image. (Photograph kindly provided by Alexandra Knight and Professor Michael J Dixon).
is highly expressed in both the lip and the palate, making it an excellent candidate for isolated CL/P. Expression analyses in the mouse secondary palate reveal that the strongest expression of Satb2 occurs before palatal shelf fusion (E13.5), with a dramatic down-regulation after the shelves have fused (E14.5) (Figure 2). Additional examples of clefts arising from displaced genomic material are the Dancer mutation [35] and clefts in the 22q and 1p36 deletion syndromes [36,37].

The transforming growth factors
Transforming growth factors are one of the most extensively studied gene families in relation to clefting. One member of this superfamily, transforming growth factor α (TGF-α), binds to the epidermal growth factor receptor (EGFR) and elicits responses similar to but more potent than EGF. The expression pattern of TGF-α in palatal tissues, especially in the midline seam and subjacent mesenchyme of the palatal shelves at the time of shelf fusion, supports a role for TGFA in clefting. Although inconclusive, data from association studies indicate that either TGFA itself or markers in its vicinity might play an important role in clefting [38].

Studies of expression patterns have shown that, although each Tgfb is temporally and spatially expressed in the developing palate, only the Tgfb3−/− knockout inhibited normal palatal shelf fusion in mice [39]. Moreover, the mechanism by which Tgf-β3 affects palatal shelf fusion appears to be targeted and specific: the MEE in Tgfb3−/− mice fails to stop cellular proliferation [40], displays reduced apoptosis [41], fails to alter its morphology and adheres less well [42], fails to degrade the basement membrane and fails to undergo EMT [43]. Furthermore, exogenous TGF-β3 can induce palatal fusion in the chicken through a process that requires physical contact of the MEE and formation of the midline seam [39]. Thus, Tgfβ3 signaling is unequivocally a key pathway in palate development in the mouse. It also appears to be involved in palatal development in humans, because association studies have provided some corroborative data [38].

Animal models and expression data
Molecular studies in the mouse and chick have been pivotal in the identification of genes that regulate the dynamic cellular changes in the MEE. Although chick palatal shelves grow towards one another above the tongue and make contact, they do not actually fuse. The chick, therefore, has the advantage of mirroring the pathology seen in cleft palate, whereas the mouse provides an excellent model to study palatal shelf fusion. Indeed, studies in these animal models have helped to identify a battery of genes essential for palatal formation: Tgfβ3 [44]; Bmps [45]; Tbx22 [46]; Fgfs [47]; Pdgsf [48]; Rhoa [49]; the gene encoding PtdIns-3 kinase [43]; Gabrb3 [50]; Gad1 [51,52]; Cspg [53]; and Mmps and Timp2 [54].

Animal models with clefts arising spontaneously or as a result of mutagenesis experiments provide another exciting avenue for gene mapping [55]. The mouse is an excellent model for studying human clefting because the development of craniofacial structures in these two species is remarkably similar. Whereas cleft palate is a common phenotype in the mouse, cleft lip is rare. To date, four mutations have been reported with cleft lip and palate phenotypes in mice. These include two spontaneous mutations called Twirler and Dancer, a transgene insertion-induced deletion mutation called Legless, and a radiation-induced mutation called Brachyphalangy [56]. Both the Dancer and Twirler mutations are almost fully penetrant for CL/P in homozygotes. Furthermore, Dancer was shown to arise from a translocation of the p23 gene.
sequence into the *Tbx10* locus, resulting in ectopic expression of *Tbx10* under the influence of the p23 promoter [35].

In addition to these mutant strains, cleft lip also occurs spontaneously in around 5–30% of embryos and neonates in a well-studied family of inbred mouse strains (the ‘A’ strains) [56]. A genome-wide screen for cleft susceptibility loci in the *A/WySn* strain identified two epistatically interacting loci, *clf1* and *clf2*, that contribute to the cleft lip phenotype [57]. The *clf1* locus contains two *Wnt* genes, *Wnt3* and *Wnt9b*, suggesting a potential role for the *Wnt* signaling pathway in orofacial development [58].

As to expression analysis, the strongest candidate genes are likely to be those whose normal expressions encompass the critical time and tissue for lip and palate development. Three global approaches are currently available for gene expression analysis in craniofacial structures: (i) the ongoing studies of the Craniofacial and Oral Gene Expression Network (COGENE), which provides public web access to genome-wide expression analysis data of craniofacial tissues isolated from human embryos (http://humgen.wustl.edu/COGENE/); (ii) Optical Projection Tomography (OPT), which enables the visualization of the relative expression of genes both temporally and spatially [59]; and finally, (iii) the mouse N-ethyl-N-nitrosourea (ENU) mutagenesis projects [60], which in addition to helping identify potential candidates for craniofacial development also serve as a means of verifying whether the expression patterns of existing candidate genes are consistent with hypotheses about function. A recent study [61] of the effects of ENU mutagenesis on the offspring of male mice suggested that genes related to isolated cleft palate might be recessive in phenotype, whereas point mutations appeared to be more relevant to the pathogenesis of cleft lip and palate. See Box 1 for additional resources on the internet.

**A role for environmental risk factors**

Birth defects are likely to recur in families not only because of shared genetic factors but also as a result of

**Box 1 Additional resources on the internet.**

| Center for Craniofacial Development and Disorders (CCDD) | http://www.hopkinsmedicine.org/craniofacial/Home/Index.cfm |
| OMIM is a curated database of human genes and genetic disorders. It enables rapid and direct linking between disease, gene sequence and chromosomal locus. |
| Craniofacial and Oral Gene Expression Network (COGENE) | http://humgen.wustl.edu/COGENE/ |
| COGENE represents a consortium of investigators involved in describing human gene expression changes that occur during early stages of development, with particular emphasis on craniofacial development. |
| Developmental Genome Anatomy Project (DGAP) | http://www.bwhpathology.org/dgap/ |
| DGAP looks for apparently balanced chromosomal rearrangements in patients with multiple congenital anomalies, and uses this information to map and identify genes that are disrupted or dysregulated at critical stages of human development. |
| Entrez Gene provides a unified query interface for gene-oriented searches. It provides information on official nomenclature, aliases, sequence accessions, phenotypes, homology, map locations, and related websites. |
| Mouse Genome Informatics (MGI) | http://www.informatics.jax.org/ |
| MGI provides integrated access to data on the genetics, genomics and biology of the laboratory mouse. |
| Murray laboratory website | http://genetics.uiowa.edu/ |
| This is JCM’s laboratory website. The web pages provide information on review protocols currently used in the lab, access to both published and unpublished data regarding genes and ongoing studies. Also included are extensive descriptions of each major project currently underway and options for obtaining additional information about them. |
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shared environmental factors [62]. Cigarette smoking during pregnancy, with the attendant hypoxia, is associated with several adverse reproductive outcomes. The most recent meta-analysis on the effects of smoking indicates a moderately increased risk of orofacial clefts [63]. Specifically, estimates of relative risks were 1.34 (95% confidence interval (see Glossary) (CI); 1.25–1.44) for CL/P and 1.22 (95% CI; 1.10–1.35) for CPO.

Maternal nutrition during pregnancy also appears to play an important role. For example, low dietary intake of B-complex vitamins, in addition to exposure to deficient or excessive amounts of vitamin A, have been linked to increased risks of clefts [64,65]. Increased risks from exposures can suggest metabolic pathways whose disruption might trigger the development of clefts. Several studies have shown that folic acid and other B-complex vitamins might have a beneficial effect on reducing the risk of orofacial clefts [66–69].

The role of cholesterol-lowering drugs (e.g. statins) in prenatal development has been recently discussed [70]. Statins that reach the embryo through maternal intake of the drug might inhibit cholesterol biosynthesis and, consequently, affect the sterol-dependent Hedgehog family of morphogens, which is critical for the proper development of a range of structures, including the face. In a study of the adverse effects of gestational exposure to statins, two cases had cleft lip and two others had cleft palate among 31 adverse birth outcomes [71]. Other drugs, such as corticoids, have also received some attention, although the effects are modest in size [72]

Of particular importance to a complex trait such as clefts is the study of the likely impact of both genetic and environmental factors. Several studies have investigated interactions of a range of common environmental factors, such as cigarette smoking, alcohol intake, multivitamin/folic acid supplementation and the use of medication, with variant alleles in several genes that include TGFα, TGFβ3, MSXI, BCL3, RARA, MTHFR, CYP1A1, NAT1, NAT2, GSTT1 and EPHX1. These have been reviewed elsewhere [1,73,74].

In assessing disease risk, most previous studies have typically focused on the affected child as the unit of analysis. Recent works in clefts, however, have started to focus on parental contributions too, particularly for the analysis. Recent works in clefts, however, have started to focus on parental contributions too, particularly for the analysis. Recent works in clefts, however, have started to focus on parental contributions too, particularly for the analysis. Recent works in clefts, however, have started to focus on parental contributions too, particularly for the analysis. Recent works in clefts, however, have started to focus on parental contributions too, particularly for the analysis. Recent works in clefts, however, have started to focus on parental contributions too, particularly for the analysis. Recent works in clefts, however, have started to focus on parental contributions too, particularly for the analysis. 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Conclusions

Great strides have been made in recent years in our understanding of how orofacial clefts arise at a molecular level. Contributions from the single genes IRF6, MSXI and FGFR1 now seem to explain approximately 15% of isolated clefts. Cigarette smoking and, possibly, disruptions in the folate biosynthetic pathway represent potential environmental risks. Given the now large sample sets available for study, linkage scans will hopefully have sufficient power to identify gene–environment interactions. Coupled with new discoveries in gene expression and animal models, researchers are finally starting to unravel the causes of orofacial clefts and, hopefully, new opportunities for improvements in diagnosis and treatment of this complex genetic can soon be made available to cleft patients and their families.

Update

A genome-wide scan for loci involved in CPO was recently conducted in a group of Finnish multiplex families [79]. Finland has one of the highest rates of isolated CPO among Caucasian populations, and even more intriguing is the higher observed prevalence of CPO compared to CL/P. Finland is therefore especially attractive for the study of isolated cleft palate. This study reported suggestive linkage at 1p34, 2p24-p25, and 12q21. The authors also screened nine unrelated affected individuals for mutations in IRF6, but no mutation was found.

Lately, Loeys et al. [80] reported that mutations in TGFBR1 or TGFBR2 were the cause behind a novel syndrome that is characterized by altered cardiovascular, neurocognitive, skeletal and craniofacial development. Tissues from affected individuals showed increased TGF-β signaling, reflected by nuclear enrichment of phosphorylated Smad2. In a related paper, Cui and co-workers [81] demonstrated that over-expression of Smad2 could rescue the cleft palate phenotype in Tgf-β−/−mutant mice. These reports provide further evidence that aberrant TGF-β signaling plays a prominent role in the pathogenesis of many common human malformations, including cleft palate.

Data from a recent study on Bmp-signaling in lip and palate fusion in mice uncovered a Bmp4–Bmpr1a genetic pathway involved in lip fusion, and revealed distinct roles of Bmp-signaling in lip and palate development [82]. Whereas Bmpr1a mutants had fully penetrant bilateral CL/P with tooth agenesis, most likely as a result of defective proliferation, Bmp4 mutants had isolated cleft lip, possibly caused by premature apoptosis in the medial nasal processes. This suggests that Bmp-signaling plays distinct roles in lip fusion and secondary palate development. Interestingly, signaling through Bmpr1a appeared to affect the expression of transcriptional regulators such as Barx1 and Pax9, but not of Msx1, Tbx22 or Osr2.
As to studies of gene–environment interactions in relation to clefts, a recent meta-analysis examined the association between maternal cigarette smoking and infant’s genotype at the TaqI site in TGFA [83]. Although maternal smoking was a consistent risk factor for both CL/P and CPO across all studies, the modest effects of interaction seemed to be restricted to cleft palate only.

Acknowledgements

We thank Dr Temis M Felix for reviewing this manuscript and apologize to all those authors whose work could not be cited because of lack of space. AJ is supported by a postdoctoral fellowship from the Research Council of Norway (NFR) and JCM by National Institutes of Health (NIH) grants DE08559 and DE16125. We would especially like to thank the many families and students who have contributed to our research over the years, and our colleagues Kaare Christensen, Mike Dixon, David Fitzpatrick, Andrew Lidral, Mary Marazita, Brian Shutte and Rolf Terje Lie for many helpful discussions.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
• • of outstanding interest


Using the IRF6 gene, in which haploinsufficiency (see Glossary) results in the autosomal dominant Van der Woude’s syndrome, Zucchero et al. studied 36 single nucleotide polymorphisms from ten world-wide populations to demonstrate very strong transmission distortion of a particular risk allele of IRF6 in isolated clefts. Allelic variants of IRF6 had an attributable risk of approximately 12% for cleft lip and palate. This report represents the first time such a strong single-gene effect has been demonstrated in isolated clefting.


34. FitzPatrick DR, Carr IM, McLaren L, Leek JP, Wighton PM, William K, Gauptier N, McGill N, Hayward C, Firth H et al.: Identification of SATB2 as the cleft palate gene on 2q32-q33. Hum Mol Genet 2003, 12:2491-2501. Fitzpatrick et al. used breakpoint mapping techniques (see Glossary), coupled with expression analyses, to identify the SATB2 gene located at a breakpoint in two different families with CLP. SATB2 encodes a 733 amino acid DNA-binding protein of remarkable conservation between human and mouse. Although mutations were not identified in an initial screen of isolated cleft palate cases, the gene itself and its regulatory elements still remain strong candidates for isolated clefts of the lip and/or palate.


77. Both fetal and maternal alleles might influence the outcome of pregnancy, particularly for genes that are involved in the metabolism of essential nutrients or the detoxification of harmful chemicals. A special strength of this study was that it assessed effects of the mother’s alleles separately from those of the fetal alleles at two MTHFR variants (C677T and A1298C) in a case–parent triad setting. There was no indication of an increased risk with the child’s genotypes at either C677T or A1298C, but mothers with either CT or TT at C677T appeared to lower the risk of CL/P in their children. In CPD, a dominant pattern of increased risk was observed with the child’s C677T genotypes. A meta-analysis in the cleft palate category showed that, except for 677T/1298A, none of the other haplo-types were transmitted significantly in excess or in deficiency.


