Abstract The malformation of nonsyndromic cleft lip with or without cleft palate (CL/P) is a common congenital disease that affects approximately 1/1000 newborns in Caucasian populations. Genetic studies indicate that CL/P has the characteristics of a complex genetic trait. Linkage analysis and mouse-model knockout studies have suggested several candidate genes mapping in different chromosome regions for CL/P malformation. On these grounds, we have investigated, by linkage disequilibrium (LD) and parametric and nonparametric linkage analyses, five different candidate genes, including those for the β3 subunit of the γ-aminobutyric acid receptor (GABRB3), glutamic acid decarboxylase 1 (GAD1), retinoic acid receptor α (RARA), transforming growth factor β3 (TGFB3), and msh (Drosophila) homeobox homolog 1 (MSX1). Interestingly, a significant LD between GABRB3 and CL/P was obtained (P-value=0.008 in the allele-wise analysis for multiallelic markers), suggesting that the GABRB3 gene is involved in this congenital disease. This new finding in humans is in agreement with previously reported data obtained with the murine model. Indeed, mouse studies indicate a role for γ-aminobutyric acid (GABA) and its receptor in normal palate development. Exclusion of the GAD1 gene, which encodes the GABA-producing enzyme, in CL/P pathogenesis was obtained in our study. Moreover, we were unable to confirm the involvement of the MSX1 gene in nonsyndromic CL/P. Moderate evidence of LD between marker alleles and CL/P was found at the RARA and TGFB3 loci suggesting a minor role for these genes in our family set of nonsyndromic CL/P.

Introduction

Nonsyndromic cleft lip with or without cleft palate (CL/P; MIM 119530) is a common birth defect with characteristics of a genetically complex trait. Epidemiological studies and segregation analyses have established the importance of genetic factors in clefting. However, no definitive evidence of any single genetic mechanism has been found. Since a major gene effect or an oligogenic model seems to fit better the observed data, many studies in the last decade have been carried out in an effort to identify the clefting genes. Several candidate genes and loci mapping in various chromosome regions have been claimed to be involved in cleft determination, by both association and linkage approaches. Conflicting results were frequently obtained when different samples were tested to replicate previous findings (reviewed by Shutte and Murray 1999). The difficulties encountered are mainly related to incomplete penetrance, genetic heterogeneity, limited sample size or the relative importance assumed by inves-
tigated risk factors in different populations. Linkage analyses have focused on chromosome regions 6p23, 19q13, 2p13, and 4q31 (L. Scapoli et al. 1997; Stein et al. 1995; Pezzetti et al. 1998; Beiragh et al. 1994). Association studies have been used to examine candidate genes in CL/P, such as those for retinoic acid receptor α (RARA), transforming growth factor α (TGFA), msh (Drosophila) homeobox homolog 1 (MSX1), transforming growth factor β3 (TGFβ3), and 5,10-methylenetetrahydrofolate reductase (MTHFR) (Chenevix-Trench et al. 1992; Ardinger et al. 1989; Lidral et al. 1998; Maestri et al. 1997; Martinelli et al. 2001).

The advent of gene targeting has led to the identification of several additional candidate cleft genes. Indeed, some dominant negative mice have an orofacial cleft as a part of their phenotype. Interestingly, knockout of endothelin-1 (Edn1), which is homologous to EDN1 mapping to the chromosomal region 6p23 in humans, shows craniofacial abnormalities including cleft palate (Kurihara et al. 1994). Moreover, mice deficient in the endothelin converting enzyme-1 (Ece1) or in endothelin-A receptor (Edra) genes exhibit virtually identical abnormalities to those of Edn1-deficient mice (Yanagisawa et al. 1998; Clouthier et al. 1998). In a recent study, we analyzed EDN1 related genes for linkage in a selected sample composed of 6p23-unlinked multiplex CL/P families (Pezzetti et al. 2000). Despite suggestive mouse data, the results from our study excluded the possibility that the EDN1 pathway (ECE1, EDNRA, and EDNRB genes) plays a major role in the etiology of nonsyndromic familial CL/P in humans. Data indicating EDN1 exclusion in human clefting was also obtained in a mutation analysis of the gene (Schultz et al. 1999).

Other pathways seem to be implicated in the normal development of the mouse palate. Genetic observations have suggested a role for the neurotransmitter γ-aminobutyric acid (GABA). Indeed, knockout of the GABA-producing enzyme glutamic acid decarboxylase (Gad1) gene or the β3 subunit of the GABA_A receptor (Gabrb3) gene results in clefting of the secondary palate (Asada et al. 1997; Homanics et al. 1997). Phenotypes that include oral cleft have also been obtained in studies involving the Tgfβ3 and Msx1 genes (Kaartinen et al. 1995; Satokata and Maas 1994). Some of the candidate genes from mouse model have been investigated in humans. Recently, linkage disequilibrium (LD) was found for TGFβ3 and MSX1, and thus an etiologic role for those genes was proposed (Maestri et al. 1997; Lidral et al. 1998). Van den Boogaard et al. (2000) identified a stop codon in the MSX1 gene in a three-generation Dutch family with tooth agenesis and various combinations of cleft lip and cleft palate. This finding provided further evidence for the involvement of this gene in orofacial clefting. However, its involvement in the nonsyndromic form of cleft remains to be demonstrated. On these grounds, the aim of the present work has been the analyses of five CL/P candidate genes, TGFβ3, MSX1, RARA, GABRB3 and GAD1, to verify their putative involvement in this frequent human malformation.

### Materials and methods

**Families**

The pedigree collection of 38 families has been extensively described in a previous report (L. Scapoli et al. 1997). In brief, all families were collected from regions of northeastern Italy and ascertained through the Archive for Cleft Lip and Palate Cases of the Clinic of Oral-Maxillofacial Surgery, General Hospital, Vicenza, Italy. They comprised subjects that presented CL/P as the only familial disease. All patients were nonsyndromic, and the use of clefting drugs in pregnancy was excluded. After informed consent was obtained, blood samples were drawn from 268 individuals; 78 were CL/P-affected. DNA was extracted following the method of Higuchi (1989), as before (Carinci et al. 1995).

**Markers**

A microsatellite marker was selected for each CL/P candidate gene. Intragenic markers, named MSX1 CA and GABRB3 CA, were used for the MSX1 and GABRB3 genes, respectively (Pezzetti et al. 1997; Mutirangura et al. 1992). D14S61 was selected for TGFβ3 because the marker and gene were on the same bacterial artificial chromosome clone (RPC11–270M14; GenBank AF107885). D2S335 was selected because it lies in close proximity of the GAD1 gene (<2 cM; Gene Map at NCBI web site, Deloukas et al. 1998). The dinucleotide repeat THR1A, at the thyroid hormone receptor locus, was chosen because it is tightly linked to RARA gene, whereas LD was previously observed between this marker and CL/P (Maestri et al. 1997).

Polymerase chain reactions (PCRs) were carried out with fluorescent-dye-labeled primer pairs and 50 ng DNA template in a 12.5-μl final volume. Samples were processed through 35 cycles, at temperatures and times indexed for the various sets of primers. The products, which were diluted and mixed with a size standard, were electrophoretically analyzed in a 377 ABI PRISM sequencer apparatus for approximately 2 h in a 4.25% polyacrylamide gel. Multiplex electrophoresis was carried out by co-loading different dye-labeled PCR products in the same lane. A control DNA of known genotype was included in each PCR and electrophoretic analysis. The electrophoretic results were processed by GENESCAN software, and allele assignment was carried out by using Genotyper software, version 1.1.

**Linkage analysis**

The estimation of markers allele frequencies was carried out from our pedigree data by using the ILINK program of the LINKAGE package (version 5.1; Terwillinger and Ott 1984). Linkage analyses were carried out by parametric and nonparametric approaches. Two point LOD scores were calculated between each marker and the disease by using the MLINK program of the LINKAGE package and by adopting a dominant mode of inheritance with reduced penetrance. The disease allele frequency was 0.0035, and penetrance was 0.12 for male and 0.06 for female (C. Scapoli et al. 1999). To verify the hypothesis of genetic heterogeneity, the results obtained from the linkage analysis were further analyzed by using the HOMOG computer program (Ott 1991). Nonparametric linkage (NPL) was calculated on the basis of the observed and the expected identical-by-descent allele-sharing among affected relative pairs by the computer software package GENEHUNTER (Kruglyak et al. 1996). Families were weighted equally, and the score function “all” was used.

**LD analysis**

The transmission disequilibrium test (TDT) was performed to test for LD in familial data (Spielman et al. 1993). The extended TDT (ETDT) package, introduced for analyses of markers with multiple