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ORIGINAL ARTICLE

No evidence of *HAND2* involvement in nonsyndromic cleft lip with or without cleft palate

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Abstract Craniofacial morphogenesis is determined by multistep processes involving signalling molecules and transcription factors, which are organised into highly coordinated pathways. Derailment from this intricate network can lead to congenital malformations. Cells migrate from neural crests to populate different structures, such as branchial arches, involved in embryonal orofacial development. The EDN1 pathway is involved in branchial arch development. Gene knockout and knockdown experiments on EDN1 or its downstream effector dHAND resulted in mice that were characterised by craniofacial defects and cleft palate. Our aim was to evaluate whether the transcription factor *HAND2* could be implicated in non-syndromic cleft lip with or without cleft palate (CL/P) aetiology. A sample study composed of 39 multiplex Italian pedigrees was enrolled to test linkage between two microsatellite flanking *HAND2* locus and CL/P. No evidence of linkage between *HAND2* and CL/P was obtained. Indeed, formal levels of exclusion were obtained with different inheritance models. Investigation results did not support a role of *HAND2* in CL/P aetiology. Nevertheless a minor contribute of the gene in clefting could not be ruled out.

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Introduction

A variety of specific transcription factors act synergistically with perfectly orchestrated timing to give birth to the craniofacial morphogenesis. It is, therefore, conceivable that a failure in this delicate web could determine developmental alterations, such as cleft lip with or without cleft palate (CL/P). Certain transcription factors that are possibly involved in CL/P aetiology have been the subject of different investigations, considering the wide geographical distribution of this congenital malformation, with an average birth prevalence of 1/700.

Association has been reported between *IRF6* (interferon regulatory factor-6) and CL/P and confirmed by different authors in different populations worldwide [1–3]. *TP63* gene, that encodes a member of the p53 family of transcription factors, has been investigated on the basis of its involvement in ankyloblepharon-ectodermal dysplasia-clefting (AEC), a rare syndrome which is characterised by congenital ectodermal dysplasia and cleft lip and/or cleft palate [4, 5]. Demonstration of the crucial role of both p63 and *IRF6* in palate development has been recently provided in a murine model, showing cooperation between the two transcription factors [6]. Leoyklang [7] supported a role for this gene in non-syndromic CL/P, establishing three novel non-synonymous changes (S90L, R313G, and D564H) in addition to 21 variant sites. The *MSX1* gene has been investigated because of its involvement in tooth agenesis with various combinations of cleft lip and cleft palate [8]. The role of this encoded protein, which functions as a transcriptional repressor during embryogenesis, was confirmed

in humans by Otero in a Colombian CL/P familiar sample study [9]. Van den Boogaard reported a significantly increased CL/P risk for offspring carrying a specific allelic variant of *MSX1* and whose parents smoked during the periconceptional period [10].

Neural crest cells play a key role in craniofacial development, migrating to populate diverse structures, including branchial arches. The endothelin family of secreted polypeptides is a determinant in regulating branchial arch development, as well as the basic helix-loop-helix transcription factor dHand, which is expressed in the mesenchyme of branchial arches [11, 12]. In fact, Yanagisawa et al. [13] demonstrated that mice lacking the dHand branchial arch enhancer died perinatally and exhibited a spectrum of craniofacial defects that included cleft palate. In addition, data produced by Xiong [14] showed that *HAND2* is an essential component for palatogenesis when expressed at both epithelial and mesenchymal levels. Interestingly, *HAND2* acts downstream to the Edn1 signalling cascade, a pathway that seems crucial for craniofacial development in mice [15]. The aim of the present study was to investigate a possible involvement of *HAND2* in the aetiology of CL/P. The present study represents a first investigation regarding the role of this gene in humans.

Materials and methods

Sample study

The pedigree collection, which was made up of 40 multiplex families, was extensively described in a previous paper [16]. In brief, all the families were from regions of north-eastern Italy, and included subjects who presented CL/P as the only familiar disease. All the patients were nonsyndromic and the use of clefting drugs in pregnancy was excluded. The pedigrees were made up of a total of 420 individuals, 100 of whom were affected by CL/P. After obtaining informed consent, blood samples were drawn from 284 individuals, 84 of whom were affected. DNA was prepared from peripheral blood cells [17] and then used as template for standard PCR.

Markers and DNA typing

Two microsatellite markers were chosen to study the linkage between CL/P and the *HAND2* gene. The markers flank the *HAND2* locus with an interlocus distance of 196 kb and a genetic distance of 0.72 cM. D4S2991 and D4S621 oligonucleotide primer pairs were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and custom synthesized (www.biomers.net). The forward primer for both markers was labelled in the 5′ position with 6-FAM fluorescent dye. Duplex PCR were performed on the genomic DNA samples in a 12.5 μl final volume, obtaining amplimers differing between markers of at least 30 bp. PCR products were separated by capillary electrophoresis on an ABI 310 Genetic Analyzer following the manufacturer's protocol (Applied Biosystems, Monza, Italy). Genotypes were collected using GeneMapper 4.0 software (Applied Biosystems, Monza, Italy). A control sample of known genotype was included for each PCR and electrophoretic analysis.

Linkage analyses

Linkage between the markers and CL/P was tested with three different methods: (a) a parametric LOD score analysis, which requires inheritance modes to be specified; (b) a nonparametric linkage (NPL) analysis, which requires marker allele frequencies specification; and (c) a non-parametric transmission disequilibrium test (TDT), in which no assumptions about either disease-gene model or marker allele frequencies are required.

Multipoint LOD scores were calculated with the LINKAGE package using different genetic models. A complex segregation analysis was performed on our family set [18] Based on these analyses, we used a dominant mode of inheritance with a disease allele frequency of 0.0035 or a recessive model with a disease allele frequency of 0.187 for the LOD score calculations. The penetrance values were set to 0.12 for males and 0.06 for females in both models; moreover, other calculations were carried out with penetrances, which were alternatively set at 0.9, 0.6, 0.3 and 0.001; the latter value implemented the affected-only method [19]. Marker allele frequency was obtained from unrelated pedigree founders. LOD score calculations under the hypothesis of genetic heterogeneity and NPL analyses were performed using the GENEHUNTER computer program [20]. Power calculations were performed using only the dominant model with parameter values obtained by segregation analysis. 1,000 replicates of the sample were simulated using the SLINK program, for a marker at recombination fraction $\theta=0$. The replicates were analysed using the MSIM and HELODHET programs [21, 22]. Power to detect linkage, i.e. LOD score>3, was good; in fact, in the analysis, all the replicates exceeded this value as far as locus homogeneity was concerned. However, the power decreased significantly when heterogeneity was present. With a 50% proportion of unlinked families, the replicates that gave LOD scores of >3 were 53% and LOD scores of >2 were 76%.

TDT compares the alleles transmitted from heterozygous parents of affected individuals to those alleles which are not transmitted, and allows the detection of linkage disequilib-

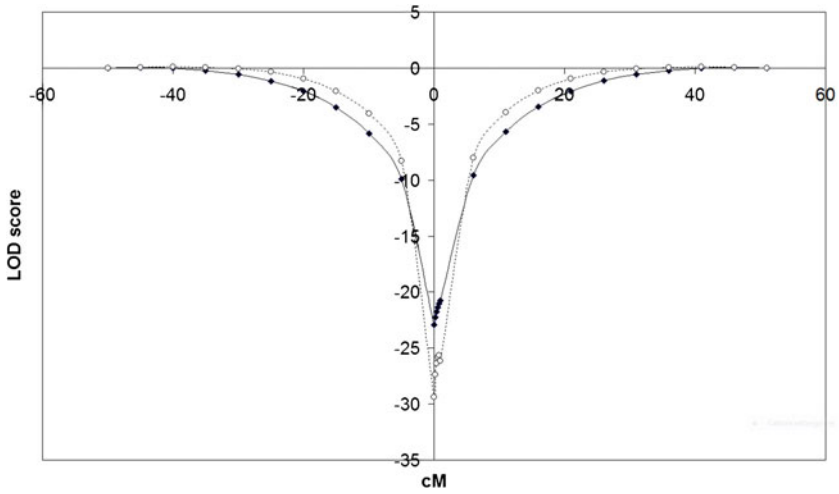

Fig. 1 Multipoint LOD score analysis. LOD scores were calculated under a dominant model of inheritance (*continuous line*) and under a recessive model (*dotted line*)

rium [23]. A likelihood ratio method implemented in the ETDT program, was used to test for linkage disequilibrium with multiallelic markers [24]. In fact, the program calculates the log likelihood under the null hypothesis (L0) and under an alternative hypothesis that transmission probabilities may deviate from 50% in an allele specific (L1) or genotype specific (L2) manner. Twice the log-likelihood ratios are χ^2 statistics, which have a degree of freedom that is equal to the number of alleles, or the number of genotypes observed in the genotype-wise analysis. Firstly, TDT was performed including every nuclear family to maximize the power of linkage, then including only unrelated families for a more accurate allelic association test.

Results

Two microsatellite markers flanking the *HAND2* gene were typed to test linkage between the gene and CL/P. The number of observed alleles was 13 at D4S2991 and 7 at D4S1595, while the calculated expected heterozygosity from all unrelated family founders were 0.78 and 0.62, respectively. The intermarker genetic distance was 1.1 cM (LOD score 21.66), in agreement with published genetic maps.

No evidence of linkage was detected between markers and CL/P under both dominant and recessive inheritance modes (Fig. 1). In fact, LOD scores lower than −2 were obtained in a 30-cM region surrounding *HAND2*. Similar results were obtained using different penetrance values, or by maximizing the LOD scores under the hypothesis of genetic heterogeneity. The nonparametric linkage method rejected the linkage hypothesis (P=0.72).

There were 61 heterozygous parents used for TDT analysis for D4S2991 and 92 for D4S1595. Likelihood ratio approach did not support linkage disequilibrium between the markers and CL/P alleles with either the allele-wise model (P=0.29; P=0.39, respectively), or the genotype-wise model (P=0.34; P=0.09, respectively). No significant transmission distortion was found for any marker allele with the McNemar's test (Table 1).

TDT was repeated in order to include only one affected subject and his/her parents for each pedigree

Table 1 TDT for observed marker alleles

Marker	Allele	T[a]	NT[b]	Chi-squared[c]	*p* value
D4S1595	1	2	0		
	2	8	6	0.29	0.59
	3	1	1		
	4	22	25	0.19	0.66
	5	28	26	0.07	0.79
	6	0	2		
	7	0	1		
D4S2991	1	3	4		
	2	25	28	0.17	0.68
	3	5	7	0.33	0.56
	4	8	3	2.27	0.13
	5	10	6	1.00	0.32
	6	0	3		
	7	5	6	0.09	0.76
	8	1	3		
	9	16	11	0.93	0.34
	10	6	11	1.47	0.23
	11	7	7	0.00	1.00
	12	5	3		
	13	1	0		

[a]Transmission count

[b]Non-transmission count

[c]Test performed when allele counts more than 10

and then data was stratified by parental sex. In either case, no significant deviation from the null hypothesis was obtained, thus excluding significant allelic association and imprinting.

Discussion

Orofacial clefting is caused by alterations in the complex morphogenetic process controlled by a highly coordinated genetic network at an embryological level. Embryologists have extensively studied the fate of cranial neural crest (CNC) cells in order to understand the basis of human congenital syndromes, characterised by the abnormal development of face and other branchial arch derived structures. Among these, the Pierre Robin sequence which presents clefting as a feature [25]. The basic helix-loop-helix transcription factor dHAND is required for craniofacial development and is expressed in the CNC-derived mesenchyme of the first and second branchial arches [12]. It acts as a downstream effector for the endothelin-1 pathway, is an intrinsic regulator in the epithelium and is required for palate development, as Xiong demonstrated in a mouse model [14]. The hypothesis that *HAND2* could be involved in the aetiology of non-syndromic CL/P induced our group to test a sample of multiplex CL/P pedigrees for genetic linkage between the gene and the malformation. A couple of microsatellite markers flanking the gene were selected to maximize the chance to obtain information from each family. No evidence of linkage was found; indeed, LOD scores values providing formal exclusion of linkage were obtained. Wrong parameters specification could cause false-negative results; however, different inheritance modes and penetrance values provided similar results. Alternative statistical methods, model-free or non-parametric, thus more suited for a complex disease like CL/P, did not support either the involvement of *HAND2* in the malformation. However, it should be noted that these methods suffer of relatively lower power with respect to the parametric method. Overall, the different approaches provided homogeneous results that did not support a major role for *HAND2* in CL/P aetiology. Nevertheless, it is not excluded that *HAND2* mutation could contribute to CL/P in a small fraction of cases or that polymorphisms of the gene could act as modifiers.

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Conflict of interest The authors declare that they have no conflict of interest.

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