LETTER TO JMG

A genome-wide scan of non-syndromic cleft palate only (CPO) in Finnish multiplex families

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J Med Genet 2005;42:177–184. doi: 10.1136/jmg.2004.019646

Oral clefts are the most common congenital malformations worldwide. Cleft palate can be non-syndromic (MIM 119540) or it can appear as a part of a syndrome or recurrence pattern. Non-syndromic cleft palate and non-syndromic cleft lip with or without cleft palate (CL/P) are considered to be separate entities, on the basis of different embryonic timing and epidemiology. However, in some syndromes, both of these cleft types segregate in the same pedigree, suggesting that they might share a common genetic background. Oral clefts manifest in over 300 different syndromes, and in some of these syndromes the gene defect is already known (Online Mendelian Inheritance in Man database 2004, <http://www.ncbi.nlm.nih.gov/Omim/>). Identified mutations in cleft syndromes have shown that functionally and structurally very distinct types of genes have an effect on palatogenesis. Recently, a mutation in interferon regulatory factor 6 (IRF6) was found to cause van der Woude syndrome (VWS) (MIM 119300),¹ which is one of the most common cleft syndromes.

Mutations in very different type of genes can lead to cleft palate in mice. These genes encode growth factors, receptors, transcription regulators, and enzymes for signalling molecule synthesis. Cleft palate, in addition to other congenital malformations, is found in ~70 knock out mice strains (The Transgenic/Targeted Mutation Database, <http://tbase.jax.org/>). Other anomalies occur frequently, and therefore no exact model for non-syndromic cleft palate exists. Usually the penetrance is not complete, but *Msx-1* knock outs result in 100% cleft palate.²

It has been suggested that, in humans, ~50% of cases of cleft palate are non-syndromic.³ The etiology and pathogenesis of non-syndromic cleft palate—and also of all other clefts—are poorly understood. Extrinsic factors, such as maternal smoking⁴ with a particular allele in *TGF α* locus,^{5–7} maternal alcohol consumption,^{8,9} maternal intake of drugs during the first trimester,¹⁰ and advanced paternal age,¹⁰ have been shown to increase the risk of cleft palate only (CPO) in some studies, but conflicting results have also been published. Sparse intake of folic acid during the first trimester has been suggested to increase the risk for cleft palate.^{11,12} The increased recurrence risk for siblings and the even higher risk for monozygotic twins suggest a genetic impact on cleft formation.^{3,13,14} Some families have shown a dominant mode of inheritance,¹⁵ but oligogenic models with reduced penetrance and influence of extrinsic factors have also been proposed.^{16,17} The risk of recurrence is ~2% if one child already has a CPO, ~6% if one parent has it, and ~15% if one child and one parent have it.¹⁸ At the moment, non-syndromic cleft palate is considered to be a multifactorial disease with a strong genetic background combined with extrinsic factors.^{3,19–21}

The incidence of non-syndromic CPO is 1/1000 live births in Finland, and this is one of the highest rates among white people.²² High incidences are seen in regions near Oulu and in central Finland.²² These regional differences become even more striking when the birthplaces of grandparents of

Key points

- Non-syndromic cleft palate is one of the most common congenital malformations worldwide.
- We performed a genome-wide scan of nine Finnish multiplex families affected with non-syndromic cleft palate.
- Chromosomal region 1p34 and the entire chromosomes 2 and 4 were screened in an additional 15 families and with a denser marker map.
- No mutations were detected in *IRF6* when screening nine affected individuals from separate families.
- We found suggestive linkage in 1p34 ($z=1.52$), 2p24–p25 ($z=2.29$), and 12q21 ($z=1.80$).

proband is analysed; the Oulu region is heavily over-represented.²² Usually, the incidence of cleft lip and palate (CL/P) is higher than the incidence of CPO,²³ but in Finland the ratio is the opposite.²²

Several association and linkage studies on CPO have been carried out. An association between *TGF α 2* and CPO has been found in three studies.^{5,6,24} Later, Lidral *et al* found no association between CPO and *TGF β 1*, *TGF β 2*, *TGF β 3*, or *MSX1*.²⁵ An association between the risk of CPO and variation at the *TGF β 3* locus was found in a Danish population.²⁶ Evidence of linkage disequilibrium between CPO and *MSX1* has been suggested.²⁶ Recently, Hecht *et al* found evidence for a CPO locus on chromosome 4 near *MSX1*.²⁷ *MSX1* is of special interest because a mutation leading to a preterm stop codon was found to cosegregate with CL/P in a large pedigree.²⁸ Linkage between CPO and VWS region 1q32–q41 has been excluded.²⁹ Deletions in 4p16–14 and 4q31–35 were found to be highly significantly associated with cleft palate.³⁰ Duplications in bands 3p24–23, 3p26, 3q23–25, 7q22–32, 8q21, 10p15–11, 14q11–21, 16p12–13, and 22q12–13 were also found to be significantly associated with cleft palate.³¹ Two patients with cytogenetic rearrangements involving the same region of chromosome 2q32 were reported to have CPO with mild facial dysmorphism and learning disabilities.³²

A genome-wide scan has not previously been performed in families with CPO, although several genome scans for CL/P have been published.^{33–38} Here, we present the results of the first genome-wide scan in Finnish multiplex families with non-syndromic CPO.

METHODS

Family material

Data were collected from the records of the Cleft Center, Helsinki University Hospital. Patients with cleft palate in

Abbreviations: CL/P, cleft lip and palate; CPO, cleft palate only; NPL, non-parametric linkage; VWS, van der Woude syndrome

Figure 1A Multiplex CPO families, pedigrees 1–8. The genome-wide scan was performed with families 1–9. 1p34 and entire chromosomes 2 and 4 were screened in all 24 families. A cross indicates that a DNA sample was available.

Figure 1B Multiplex CPO families, pedigrees 9–19. The genome-wide scan was performed with families 1–9. 1p34 and entire chromosomes 2 and 4 were screened in all 24 families. A cross indicates that a DNA sample was available.

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Figure 1C Multiplex CPO families, pedigrees 20–24. 1p34 and entire chromosomes 2 and 4 were screened using all 24 families. A cross indicates that a DNA sample was available.

Finland have been treated centrally since 1948 at the Red Cross Hospital for Plastic Surgery, which in 1984 became the Unit of Plastic Surgery of the 1st Department of Surgery, Helsinki University Central Hospital. We searched the medical records of all patients with cleft palate from the years 1967–1996 inclusively. We contacted 250 patients by letter. The patients were chosen on the basis of three criteria: (i) according to the medical record they did not seem to have other malformations or any syndrome; (ii) they had reported at least one similarly affected relative; and (iii) they could be contacted by post.

Nine large multiplex CPO pedigrees were chosen for the first stage of the genome scan. The pedigrees consisted of 29 affected and 60 unaffected subjects, making a total of 89 individuals (fig 1A–C). In addition to these nine families, we scanned the entire chromosomes 2 and 4 and a candidate region in 1p34 in an additional 15 multiplex families (fig 1A–C). In these cases the pedigrees consisted of 63 affected and 112 unaffected subjects, making a total of 175 individuals. The families all came from different regions of Finland. In our study we used the patients' own knowledge about their ancestors and the local church records to build the pedigrees. Ancestors of the patients were traced back three to six generations. Only in one family were the parents of the proband known to be related to each other (pedigree 14). A common ancestor for the probands, previously not known to be related to each other, was found in two pedigrees (pedigrees 7 and 9).

Probands and as many affected relatives as possible were examined by HK to rule out undiagnosed syndromes. The entire nuclear family was asked to meet the examiner if possible. Family members were then examined and any

dysmorphic features noted. A blood sample was taken from all available first degree and also some second degree relatives. The ethical committees of the Social and Health Ministry of Finland, the University Hospital of Helsinki, and the Department of Medical Genetics of Helsinki University approved the study protocols.

In our previous study we excluded linkage between CPO and candidate regions/genes (*MSX1*, *TGFβ3*, 2q32, and 22q11) using these same 24 Finnish multiplex families.³⁹

Genotyping and linkage analysis

DNA was extracted from EDTA preserved blood non-enzymatically.⁴⁰ Genotyping was performed in the Finnish Genome Center (<http://www.genome.helsinki.fi>) and in the Department of Medical Genetics of the Haartman Institute. In the initial scan with the nine multiplex pedigrees (pedigrees 1–9), 442 polymorphic markers from the ABI linkage mapping set were used. Chromosomes 2 and 4 were screened more densely (using 60 and 51 markers in total, respectively) than other chromosomes because they were of special interest on the basis of literature reports; the average distance is ~10 cM except in chromosomes 2 and 4 where it is ~5 cM. In addition, six additional markers (*D1S247*, *D1S513*, *D1S2723*, *D1S380*, *D1S1188*, *D1S2722*) within the region flanked by markers *D1S234* and *D1S2797* in 1p34 were genotyped. The positions of these markers were ascertained from the Marshfield Comprehensive Human Genetic Map (http://research.marshfieldclinic.org/genetics/Map_Markers/maps/indexmap.html). At the second stage, we analysed region 1p34 in an additional 15 pedigrees. Altogether 24 pedigrees were analysed.

PCR assays were performed in 5 μ l reaction volumes containing 20 ng of DNA, 1.7 pmol of each primer, 0.25 mM of dNTP, 1 $\times$ AmpliTaq Gold Buffer, 0.25 mM $MgCl_2$, and 0.2 U AmpliTaq Gold polymerase (Applied Biosystems; www.appliedbiosystems.com). DNA amplification was carried out as follows: 1 $\times$ 95°C for 12 min, 10 $\times$ (94°C for 30 s, 55°C for 30 s, 72°C for 30 s), 20 $\times$ (89°C for 30 s, 55°C for 30 s, 72°C for 30 s), and finally 1 $\times$ 72°C for 10 min. The

alleles were separated electrophoretically on MegaBACE 1000 (Molecular Dynamics) 96 well capillary instruments according to the manufacturer's instructions. The results were analysed and alleles called using Genetic Profiler software from Molecular Dynamics.

The two largest pedigrees (pedigrees 1 and 9) were divided into two for multipoint linkage analysis performed with the Genehunter program.⁴¹ The data were Mendel checked with

Figure 2A Results of multipoint NPL analysis at stage 1 (pedigrees 1–9, chromosomes 1–15). Thicker curves show NPL scores and thinner curves show information content values. Dotted lines are the zero levels for both NPL and information content values.

Figure 2B Results of multipoint NPL analysis at stage 1 (pedigrees 1–9, chromosomes 15–22). Thicker curves show NPL scores and thinner curves show information content values. Dotted lines are the zero levels for both NPL and information content values.

Pedmanager and Pedcheck software and haplotyped with Genehunter2 to check for double recombinants. The lack of a reliable model of inheritance made traditional parametric linkage analysis unsuitable. Instead, we chose to use the non-parametric linkage (NPL) score (z), which calculates whether affected individuals share alleles identical-by-descent more often than expected by chance. All autosomal chromosomes were analysed. The X chromosome was not included in the analysis because there is no evidence supporting X linked inheritance in our families.

The IRF6 gene was screened for mutations using intronic primers flanking all coding exons and the non-coding exons 1 and 2.¹ For amplifying each exon, 15 ng of genomic DNA was used in the following conditions: 1.5 mM $MgCl_2$, 1 μ M of each primer, 0.2 mM of each dNTP, and 0.04 U/ μ l of AmpliTaq DNA polymerase (Applied Biosystems) or Hot Star Taq (Qiagen; www.qiageninstruments.com) using the suppliers' buffers and recommendations. Primer sequences are available upon request. PCR products were then cleaned from non-incorporated primers and dNTPs using the GFX PCR DNA purification kit (Amersham Biosciences; www4.amershambiosciences.com), further sequenced using the DYEnamic ET Dye terminator kit (Amersham Biosciences), and cleaned of non-incorporated dideoxynucleotides by ethanol precipitation. Sequencing products were injected for 40–80 s at 3 kW and electrophoresed either for 100 min at 9 kW or for 180 min at 6 kW on a MegaBACE 1000 instrument (Molecular Dynamics). Each exon was sequenced in both directions using the same primers as in the PCR amplification step. Sequences were visualised and analysed using Sequence Analyser v 3.0 software (Amersham Biosciences), as well as the Gap4 Staden package.

RESULTS

Stage 1

A genome-wide scan (chromosomes 1–22 with 442 polymorphic markers from the ABI linkage mapping set and with additional markers in chromosomes 1, 2, and 4) of nine multiplex pedigrees detected no significant linkage. The highest NPL score was seen in chromosome 1 ($z = 2.06$, $p = 0.033$, information content 0.58) at a position 50 cM from 1pter. NPL scores reaching values over 1.5 were seen in chromosomes 2 ($z = 1.97$, $p = 0.038$), 6 ($z = 1.58$, $p = 0.062$), 11 ($z = 1.60$, $p = 0.061$), 12 ($z = 1.80$, $p = 0.045$), and 17 ($z = 1.58$, $p = 0.062$). The NPL scores and information content values over entire chromosomes are shown in fig 2A and B.

Stage 2

Analysis of candidate region 1p34 and entire chromosomes 2 and 4 in 24 multiplex families: when using data from all 24 pedigrees, the NPL score in chromosome 1 at position 50.3 cM fell to 1.31 ($p = 0.098$). The highest NPL score in the VWS linked region in 1p34 was 1.52 ($p = 0.069$, inf. cont. 0.86) at position 61 cM (fig 3). When screening the entire chromosomes 2 and 4 in the 24 multiplex pedigrees, the highest NPL scores were 2.29 ($p = 0.016$, inf. cont. 0.67) at position 27.10 cM and 1.56 ($p = 0.064$, inf. cont. 0.67) at position 100 cM, respectively (fig 3). NPL scores over 1.5 ($p < 0.05$) were seen in chromosome 2 at ~5–30 cM. The initial scan of nine families did not find any linkage in chromosome 4, but additional families and a denser marker map raised a narrow and shallow peak of 1.56 ($p = 0.064$) at 100 cM.

Figure 3 Results of multipoint NPL analysis of chromosomes 1p34, 2, and 4 (in pedigrees 1–24). Thicker curves show NPL scores and thinner curves show information content values. Dotted lines are the zero levels for both NPL and information content values.

Exclusion of IRF6 mutations

Altogether, 10 affected and nine healthy individuals belonging to nine families were screened for mutations in the IRF6 gene. All coding exons of the gene (that is, exons 3–8 and the translated part of exon 9) and the two non-coding (5' UTR) exons 1 and 2 were screened by direct sequencing. No mutation/polymorphism was detected in the coding region of the IRF6 gene in the individuals tested. However, a common polymorphism (A/G) located 73 bp before the start codon was detected in exon 2. This polymorphism is referred to as rs861019/ss1266169 in the SNP database (<http://www.ncbi.nlm.nih.gov/SNP/>). Allele frequencies were 0.8/0.2 in the 10 affected individuals and 0.72/0.28 in the nine healthy individuals. This polymorphism does not seem to introduce any new splice site or start codon.

DISCUSSION

Non-syndromic cleft palate can be considered a genetically complex disease. On the basis of previous studies, it seems probable that both extrinsic and intrinsic factors have an

impact on cleft palate formation. No clear Mendelian inheritance pattern can be detected, and locus heterogeneity may be involved. Traditional linkage analysis is not suitable because of the lack of precise parameters.

The results from the genome-wide scan showed no evidence of significant linkage. However, several interesting regions with suggestive linkage were found. In the initial scan with nine pedigrees, the highest NPL score was seen in chromosome 1p in the same region (48–52 cM, $p < 0.05$) as that linked to VWS in a large Finnish pedigree (39–89 cM, $p < 0.0005$).⁴² However, at the second stage, with 15 additional families and six additional markers in 1p34, the NPL scores fell below 1.52 ($p = 0.069$). When they performed genome-wide scans in CL/P, Prescott *et al*³⁴ and Marazita *et al*³⁵ found positive regions in chromosome 1 at 15–25 cM (1p36) and 100 cM, respectively.³⁵ Significant NPL scores were also found in 1p36 in Italian families with non-syndromic orofacial clefts.⁴³ The suggestive NPL scores in our study seemed to be slightly more centromeric compared to the positive scores obtained by Martinelli⁴³ and Prescott *et al*.³⁴ We consider both chromosomal regions 1p34 and 1p36 to be candidate regions in orofacial clefting. We also wanted to rule out the role of the IRF6 gene in our families; no mutations were detected.

When screening all 24 families, suggestive NPL scores (> 1.5 , $p < 0.05$) were seen in chromosome 2 at ~5–30 cM (2p24–p25), with a maximum value of 2.29 ($p = 0.016$). This is the first time that 2p24–p25 has been reported to show any suggestive linkage to CPO. Candidate gene transforming growth factor alpha (TGF α) is located in 2p13 (~71 cM), outside our positive region. The previously reported CPO candidate region, 2q32, did not show any evidence of linkage in our study.³² In chromosome 4, the only positive peak (NPL 1.56, $p = 0.064$) was at 100 cM. Thus, our study could not confirm the role of previously reported candidate regions in chromosome 4 in orofacial clefting.^{27 44 45}

In chromosome 6, NPL scores over 1.5 ($p = 0.062$ – 0.07) were seen at 137–142 cM (6q22). The orofacial cleft (OFC1, MIM 119530) region in 6p24 did not show any evidence of linkage in our families.^{46–49} No linkage or association of orofacial clefts to 6q22 has previously been reported. In chromosome 12 we found a suggestive linkage at 111.0 cM (12q21) ($z = 1.80$, $p < 0.05$). Marazita *et al* found significant associations for two loci in chromosome 12 but at different locations (78 and 166 cM).³⁵ In chromosome 17, the highest NPL ($z = 1.54$, $p = 0.067$) score was at 90 cM and is distal to the candidate gene RARA located in chromosome 17q21.

Although none of the regions reached the level required for significant susceptibility loci, we consider regions 1p34, 2p24–p25, and 12q21 to be candidate regions for further studies on orofacial clefting, especially cleft palate.

ACKNOWLEDGEMENTS

We thank all members of the affected families for their participation. We thank Mrs Riitta Lehtinen and Mrs Sinikka Lindh for technical aid.

ELECTRONIC-DATABASE INFORMATION

The URLs referred to in this study are: Amersham Biosciences, www4.amershambiosciences.com; Applied Biosystems, www.appliedbiosystems.com; Finnish Genome Center, <http://www.genome.helsinki.fi/>; Marshfield Comprehensive Human Genetic Map, http://research.marshfieldclinic.org/genetics/Map_Markers/maps/indexmap.html; Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>; Qiagen, www.qiageninstruments.com; The Transgenic/Targeted Mutation Database, <http://tbase.jax.org/>; and SNP database, <http://www.ncbi.nlm.nih.gov/SNP/>.

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This study was supported by the Sigrid Juselius Foundation, the Academy of Finland, and the Helsinki University Hospital Research Funds.

Conflict of interest: none declared.

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Revised version received 17 May 2004

Accepted for publication 18 June 2004

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