Exclusion of candidate genes from a role in cleft lip with or without cleft palate: linkage and association studies

G M Vintiner, K K Lo, S E Holder, R M Winter, S Malcolm

Abstract
Candidate genes and marker loci for cleft lip/palate (CL/P) were tested using linkage analyses and association studies. Eight British families with apparent autosomal dominant inheritance of non-syndromic CL/P participated in the linkage analyses while the association analyses involved 61 unrelated British white people with CL/P and 60 controls.

The report of an association between RARA (17q21) and unrelated Australian persons with CL/P (p = 0.016) was not confirmed in British CL/P persons (p = 0.254, p > 0.1). There was also no evidence of linkage between RARA and the eight CL/P families (Z = -3.211, 0 = 0.001). Linkage was excluded between familial CL/P and F13A1 (map position 6p24-25) with an observed maximum lod score of 0 = 2.052 at 0 = 0.05. No association was found between alleles at VIM (10p13) and the British CL/P subjects (p = 0.110, p > 0.5). Multipoint analysis excluded linkage between familial CL/P and the markers D1S65 and D1S58 which flank the Van der Woude syndrome locus with a maximum lod score of 0 = -4.0. This suggests that the genetic defect underlying VWS is not the same as in non-syndromic CL/P. There was no evidence of linkage between CRTLI (5q15) and the eight CL/P families (Z = -3.466, 0 = 0.05).

Complex segregation analysis in different racial groups with cleft lip with or without cleft palate (CL/P) have suggested that clefting is the result of the action of a major locus with reduced penetrance. In support of the major locus theory is the existence of families with an apparently autosomal dominant pattern of inheritance of CL/P.

In an attempt to identify a putative major gene, an association analysis was carried out by Arding et al. which compared the frequency of polymorphisms at various candidate genes in an American CL/P population and a control population. An association between polymorphisms within transforming growth factor-alpha (TGFA) and unrelated subjects with CL/P was identified. This association has since been confirmed in English and Australian subjects of mainly white north European origin. The presence of the association in three different populations suggests that TGFA contributes to the development of clefting in some people. However, no evidence of linkage was found between TGFA and CL/P in families with an apparently autosomal dominant pattern of inheritance. This suggests that in those families, TGFA is not a gene of major effect.

A second association between unrelated Australian persons with CL/P and the retinoic acid receptor alpha (RARA) locus has recently been reported. An allele at the PstI polymorphism of RARA, map position 17q21, was found more commonly in subjects with CL/P compared with controls (p = 0.016). This suggests that RARA, like TGFA, may contribute to the development of clefting in some subjects. RARA also maps near to a t(5;17)(q15;q23) translocation which has been observed to segregate with affected members in a family with Stickler syndrome. Cleft palate (CP) was among the clinical features observed.

In this study, a combined linkage and association approach has been undertaken to test the reported association between RARA and CL/P in a British population and to test other possible major genes which may be involved in CL/P.

Candidate genes were selected on the basis of map positions close to cytogenetic aberrations causing CL/P, proximity to loci known to cause syndromic CL/P, and from published linkage and association studies. A suggestion of linkage between CL/P and F13A1 (6p24-25) had previously been reported by Eiberg et al. A maximum lod score of Z = -3.66 at 0 = 0.00 for males and 0 = 0.26 for females was reported. This locus was selected as a possible marker for CL/P and was tested in a linkage analysis with the eight CL/P families.

VIM, map position 10p13, is present in the medial cell edge during palate fusion and also maps near to the translocation breakpoint in two subjects with non-syndromic CL/P. The karyotype of one subject was 46,XY,t(2;10)(q33;p13) while the other was 46,XY,t(10;14)(p13;q24). Its potential as a candidate was tested in an association analysis with the unrelated CL/P subjects.

D1S65 and D1S58 were selected as markers for 1q based on the placement of a locus for Van der Woude syndrome (VWS) in the 1q32-42 region. VWS is an autosomal dominant disorder and is characterised by the presence of CL/P, CP, and lip pits. Fusion of lip pits is thought to occur at the same time as fusion of the lip and palate, so the molecular defect underlying this disorder may also be involved.

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in non-syndromic clefting. D1S65 and D1S58 which flank the WVS locus\textsuperscript{15} were used as markers in a multipoint analysis involving the eight CL/P families.

CRTL1, map position 5q15,\textsuperscript{17} is responsible for linking proteoglycan aggregates which are found in cartilage\textsuperscript{19} and maps near to the other translocation breakpoint observed to segregate with affected members in a family with Stickler syndrome.\textsuperscript{11} CRTL1 was tested as candidate for CL/P in a linkage analysis with the eight CL/P families.

Materials

PEDIGREE INFORMATION

Eight white British families with an apparently autosomal dominant pattern of inheritance of CL/P were analysed for linkage. Clinical details of all eight families have been reported previously.\textsuperscript{8} Chromosome analysis was carried out on an affected subject from each of the families. All karyotypes were normal.

ASSOCIATION STUDY INFORMATION

Sixty-one unrelated white British subjects with CL/P participated in the association study with VIM and RARA. Information on the ascertainment of 57 of these subjects is outlined in Holder et al\textsuperscript{18} and their clinical details are outlined in table 1. Sixty unrelated subjects were used as controls; 23 of these consisted of work colleagues while 37 were parents of children with cystic fibrosis. No clefting information was available on this latter group but they were assumed to be normal.

DNA PROBES USED

Information on the DNA probes used is outlined in table 2.

DATA ANALYSIS

Family data were prepared for analysis using LINKSYS and analysed with two point linkage using LIPED. Two different modes of inheritance and three different levels of penetrance were used in the analysis of the CL/P families.

<table>
<thead>
<tr>
<th>Gene/locus</th>
<th>Probe</th>
<th>Polymorphic enzyme</th>
<th>Allele size (kb)</th>
<th>Freq</th>
<th>Ref</th>
</tr>
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<tr>
<td>D1S58</td>
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<td>BglI</td>
<td>5.0</td>
<td>0.49</td>
<td>19</td>
</tr>
<tr>
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<td>TaqI</td>
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<td>0.51</td>
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<tr>
<td>RARA</td>
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<td>PstI</td>
<td>3.6</td>
<td>0.3</td>
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</tr>
</tbody>
</table>

Lod scores were calculated based on an autosomal dominant model of inheritance of CL/P with a gene frequency of 0.001 and 80% penetrance. These values appeared to best reflect the inheritance pattern observed in the eight families with CL/P. Lod scores were also calculated with autosomal dominant inheritance but with a level of penetrance set at 28%. This was obtained by averaging the separate penetrances which were set for males and females in an analysis of American CL/P families by Hecht et al\textsuperscript{23} The second model tested autosomal recessive inheritance with a gene frequency of 0.035 and an average penetrance of 35%. This reflected the model proposed by Chung et al\textsuperscript{24} but the families here are largely uninformative because of their structure. For the microsatellite markers CRTL1 and F13A1, the alleles observed were assigned an equal frequency.

Multipoint linkage analysis was carried out using the LINKMAP section of LINKAGE version 5.04. For the association analyses, $x^2$ analysis was used to assess any difference in allele frequencies between cases and controls.

Methods

Restriction endonuclease digestion was carried out under recommended conditions (Northumbria Biologicals Ltd). The DNA was separated by electrophoresis in 0.8% agarose, denatured in 1·49 mol/l NaCl/0.5 mol/l NaOH solution, then transferred directly to nylon transfer membranes (Hybond-N+, Amersham) in 20 x SSC. Inserts were excised from their vectors and oligolabelled with $^32$P. Probes were hybridised to filters overnight at 65°C then washed in 0·1 x SSC, 0·1% SDS at 65°C. Filters were exposed to x ray film at -70°C for seven days.

MICROSATELLITE ANALYSIS

The following sequences were used for PCR amplification. CRTL1.\textsuperscript{25} A 222 to 240 bp fragment was observed with the CRTL1 primers: 5'-CCG GTG TCA GCA TCT TC 3' forward primer, 5'-GCC TTC GTG CAT GAC AGA GCT CA 3' reverse primer. Conditions for amplification were: one minute at 94°C denaturation, one minute at 65°C annealing, one minute at 72°C extension for 30 cycles. F13A1.\textsuperscript{26} A 195 bp fragment was observed with the F13A1 primers: 5'-GAG GTT GCA CTC CAG CCT TT 3' forward primer, 5'-ATGCCA TGC AGA TTA GAA 3' reverse primer. Conditions for amplification were: one minute at 94°C denaturation, two minutes at 55°C annealing, two minutes at 72°C extension for 27 cycles.

PCR amplification with $^32$P incorporated in the reaction (1 μCi per sample) was performed on a Techne PHC-2 amplifier. The amplified fragments were loaded onto a 6% denaturing polyacrylamide gel and electrophoresed at 50 W for two hours. The gel was exposed overnight to x ray film at -70°C. Fragments were sized against a sequenced M13 vector.
Exclusion of candidate genes from a role in cleft lip with or without cleft palate (Sequenase kit Version 2.0, United States Biochemical).

**Results**

**CANDIDATE LOCUS RARA**

The results of the association analysis between RARA and 61 unrelated British CL/P subjects are presented in tables 3 and 4. In table 3, the allele and genotype frequencies for the *PsrI* RFLP at the RARA locus in the CL/P and control populations are presented. The RFLP distribution in cases and controls and results of the \( \chi^2 \) analysis are given in table 4. There was no significant association with either allele at the RARA locus and the presence or absence of clefting (\( \chi^2 = 0.954, p > 0.1 \)).

A two point linkage analysis between RARA and the eight CL/P families was carried out. In table 5, the lod scores resulting from an analysis testing an autosomal dominant pattern of inheritance of CL/P with penetrance set at 80% were presented. Linkage was excluded between the two with a lod of \( Z = -3.211 \) at \( \theta = 0.001 \) and \( Z = -1.938 \) at \( \theta = 0.05 \). The other models also failed to show evidence of linkage, although they were less informative (table 5).

**CANDIDATE LOCUS VIM**

An association analysis was carried out between polymorphisms at the vimentin locus and the presence or absence of CL/P. The allele and genotype frequencies for the *BclI* polymorphism at the vimentin locus in the CL/P and control populations are presented in table 6. The distribution of the two alleles between cases and controls and results of the \( \chi^2 \) analysis are presented in table 8. There was no significant association with either allele at the vimentin locus and the presence or absence of clefting (\( \chi^2 = 0.110, p > 0.5 \)).

**MARKER LOCUS F13A1**

F13A1 was used in a two point linkage analysis with the eight families with CL/P. Crossovers were observed between affected subjects and F13A1 in families CD and HN. Family PL was uninformative. The lod scores for all three models are presented in table 6. Assuming an autosomal dominant pattern of inheritance of CL/P and penetrance of 80%, linkage between F13A1 and CL/P was excluded with a lod score of \( Z = -2.052 \) at \( \theta = 0.05 \).

**MARKER LOCI D1S65 AND D1S58**

Multipoint analysis was carried out using loci D1S65 and D1S58 which flank the Van der Woude locus and the eight CL/P families. The distance between D1S65 and D1S58 was estimated to be 15 cM. This was converted to a recombination fraction of \( \theta = 0.13 \) using the Haldane mapping function. Crossovers between the two marker loci were observed in families HN, SH, SN, and TY, while between the putative disease locus and affected subjects, crossovers were observed in families BR and SH.

The results of the multipoint analysis, testing an autosomal dominant pattern of inheritance with 80% penetrance, are outlined in the figure. There was no evidence of linkage between CL/P and the chromosome 1 loci with a maximum multipoint lod score of \( Z = -4.0 \). A lod score of \( Z = -2.0 \), which is indicative of no linkage, was found 17 cM from D1S65 and 12 cM from D1S58. Linkage was also
excluded using reduced penetrance (28%) and autosomal recessive inheritance.

**Table 9** Lod scores for two point linkage of CL/P and CRTL1. Total lod scores with (1) autosomal dominant pattern of inheritance of CL/P, 80% penetrance; (2) autosomal dominant pattern of inheritance of CL/P, penetrance 28% (3) autosomal recessive pattern of inheritance of CL/P, penetrance 35%.

<table>
<thead>
<tr>
<th>Family</th>
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<th>0.000</th>
<th>0.001</th>
<th>0.05</th>
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<th>0.3</th>
<th>0.4</th>
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<tbody>
<tr>
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<td>-0.561</td>
<td>-0.314</td>
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<td>-0.040</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Total (1)</td>
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<td>Total (2)</td>
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<td>-0.762</td>
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<tr>
<td>Total (3)</td>
<td>-0.051</td>
<td>-0.050</td>
<td>-0.017</td>
<td>0.002</td>
<td>0.017</td>
<td>0.012</td>
<td>0.004</td>
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</table>

**Discussion**

Candidates for the role of a major gene in non-syndromic CL/P were selected using several criteria and were tested through a combination of linkage analyses and association studies. Those loci used in a linkage approach included the markers D1S65 and D1S58, CRTL1, F13A1, and RARA. It was hoped that a significant lod score generated by any of these loci would either indicate direct involvement in familial clefting or suggest a chromosome region. Using an association approach, the loci VIM and RARA were tested for their involvement in a group of unrelated CL/P subjects. Contrary to the findings reported by Chenex-Vrench et al., there was no significant association between British CL/P subjects and RARA polymorphisms ($\chi^2 = 0.954, p = 0.1$). In the association analysis carried out by Chenex-Vrench et al., the frequency of alleles among Australian CL/P and control subjects at other candidate loci was also tested. Of the 61 British CL/P subjects, all white, who participated in the association analyses presented as part of this work, a study involving 57 of them had previously shown an association with TGFA. As both the Australian and British populations had shown associations with TGFA, it seemed likely that the two study groups contained persons of similar racial origin and this would be reflected in a RARA association. The apparent lack of association seen with the British CL/P subjects may suggest that the association between RARA and Australian subjects with CL/P is the result of a type I error. Replication of this work in other populations may resolve this.

Several reports have suggested the short arm of chromosome 6 as being a possible candidate region for CL/P. Eiberg et al. carried out a linkage analysis which suggested linkage between F13A1 and the CL/P phenotype. There have also been two reports of subjects with syndromic clefting and chromosome aberrations involving 6p23, 29, 30.

The possibility of a CL/P locus within the 6p24–25 region was not confirmed in the linkage analyses carried out with F13A1 in the British families with any of the models of inheritance tested. There is also some evidence against a gene for CL/P within the human leucocyte antigen (HLA) region, map position 6p21.3. In a sib pair analysis, the segregation of HLA haplotypes in eight families with CL/P did not differ significantly from random mendelian expectation, ruling out linkage between HLA and CL/P in those families.

It is possible that a gene for non-syndromic CL/P lies distal to 6p21.3 and proximal to 6p24–25. Of the two subjects with chromosome 6p23 aberrations, one involved a t(6;9)(p23;q22.3) translocation, while the other was a 6p23 terminal deletion. Both these subjects had many other clinical manifestations apart from CL/P. It is possible that the phenotype observed in the translocation subject is the result of a gene which affects other tissues in addition to the lip and palate. The syndromic clefting associated with the 6p23 terminal deletion could result from the loss of a number of genes. This region of 6p may not be a strong candidate for a major locus for non-syndromic clefting.

Vimentin is a protein belonging to the class of intermediate filaments of the cell and its synthesis occurs in tissue of mesenchymal origin. It was selected as a possible candidate for CL/P based on its presence in the medial cell edge during palate fusion and its proximity to a translocation breakpoint in two subjects with non-syndromic CL/P. The results of the association study, however, suggest that vimentin does not contribute to the clefting phenotype in the unrelated British subjects analysed. There was no significant difference between the allele frequencies at VIM in subjects with

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CL/P compared with the control population ($\chi^2 = 0.110, p > 0.5$).

The markers D1S65 and D1S58 map to 1q31-32 and appear to flank the VWS locus.\(^7\) These markers were used in a multipoint analysis with the eight CL/P pedigrees. The presence of crossovers between the two loci in four of the families reflects the estimated 15 cM which separate these markers.\(^7\)

In all three linkage models tested, there was no evidence of linkage between D1S65 and D1S58 and the CL/P phenotype, suggesting that the molecular defect underlying VWS is not the same as in non-syndromic clefting. These results are in agreement with Hecht et al.\(^3\) who reported a lack of linkage between CL/P and renin, to which VWS has been linked.\(^3\) One family within which cleft palate (CP) was segregating was also analysed and found not to be linked to the interval thought to contain the VWS gene.

Two other candidate genes for VWS had been considered by Murray et al.\(^5\) The first was lamin B2 (LAMB2) which is a component of the laminin molecule found in basement membranes, while the second is DAF, a complement-regulating factor (DAF) which is involved in programmed cell death. Both of these were excluded as candidates for VWS owing to the occurrence of crossovers between affected subjects. LAMB2 has been mapped 8 cM proximal to D1S65 while DAF is located 3 cM distal to D1S58.\(^7\) From the results of the multipoint analysis presented in this study (figure), there was no evidence of linkage between these two other candidate loci and CL/P.

The polymorphic CA repeat identified within the CRTL1 gene\(^6\) proved useful in a linkage analysis with the eight CL/P families. The finding of crossovers between affected subjects in five out of eight families suggests that this locus is not involved in the CL/P phenotype observed in these families. However, although a strongly negative lod score was generated with an autosomal dominant pattern of inheritance and 80% penetrance ($Z = -3.456$ at $\theta = 0.05$), family SN alone generated a positive lod score of $Z = 0.823$ at $\theta = 0.0$. CRTL1 could be a gene of major effect in this family although the lod score does not reach significance.

The ability to test candidate genes or regions of chromosomes in a linkage analysis with CL/P is hampered by the scarcity of large multigeneration families. The eight families studied provided a unique opportunity to test various genes and markers under the hypothesis of a single major gene for CL/P. As a result of this work it is possible to conclude that there was no evidence of linkage between chromosome region 1q32, CRTL1, chromosome region 6p24-25, and RARA and a single major clefting gene in the majority of families studied. However, caution should be exercised in interpreting the results of negative linkage studies using apparently 'dominant' pedigrees segregating for CL/P, in view of recent studies which suggest CL/P may be caused by multiple interacting loci\(^3\)\(^-\)\(^4\) and the difficulties in showing linkage with one locus if this were the case.\(^3\)

From the population based association approach, there was no evidence of association with CL/P and VIM or RARA.

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