The application of molecular genetics to detection of craniofacial abnormality

GUDRUN MOORE1, ALASDAIR IVENS1, JOANNA CHAMBERS1, ARNI BJORNSSON2, ALFRED ARNASON3, OLAFUR JENSSON3 and ROBERT WILLIAMSON1

1Department of Biochemistry and Molecular Genetics, St Mary's Hospital Medical School, University of London, London W2 1PG, UK, 2Department of Plastic Surgery and 3Genetics Division of the Blood Bank, National University Hospital, Reykjavik, Iceland

Summary

Congenital malformations such as secondary cleft palate can be exclusively monogenic or polygenic, but most cases have a multifactorial origin involving both environmental and genetic factors, making genetic analysis difficult. The new techniques of molecular genetics have allowed the successful chromosomal localization of mutant genes in disorders that show a simple Mendelian segregation, whether autosomal dominant (e.g. Huntington's disease), autosomal recessive (cystic fibrosis) or X-linked (Duchenne muscular dystrophy). Recently, a large Icelandic family (over 280 members) with X-linked secondary cleft palate and ankyloglossia (tongue-tied) has been used as a model to localize the mutant gene associated with this craniofacial clefting. The gene has been subchromosomally localized to Xq13–q21.1, using anonymous probe DXYS1; a LOD score of 3.07 was obtained.

We are preparing cosmid libraries from DNA from mouse cell lines containing only the relevant part of the human X chromosome, introduced by chromosome-mediated gene transfer. Cosmids that contain human X-chromosome sequences will be isolated and analysed for overlapping sequences and RFLPs (restriction fragment length polymorphisms) and the regions further defined by pulsed-field gel electrophoresis and the identification of coding sequences. This should give data on the location and structure of a gene involved in the craniofacial development of the human palatine shelves. This gene, and its protein product, will identify one component of the pathway that causes nonfusion of the palate. In the long term, the understanding of the expression of this sex-linked gene for secondary cleft palate and ankyloglossia will provide a model for the molecular identification of other genes regulating processes in craniofacial development whose expression is hidden in phenotypic, polygenic complexity.

Key words: molecular genetics, cleft palate, DNA, X-chromosome, Y-linked disorders, ankyloglossia, craniofacial malformation.

Introduction

The majority of congenital craniofacial malformations occur during the 5–12 weeks of development. The earlier part of this period approximates to the time in which teratogens can harm the developing fetus, spanning the embryonic period from 3 to 9 weeks postconception (Moore, 1982). The most common congenital abnormality of the face is cleft lip (CL), a condition which involves nonfusion of the upper lip and the anterior part of the maxilla during weeks 5–7 and occurs at an incidence of approximately 1 in 1000 births (Thompson & Thompson, 1986). Although CL is frequently associated with cleft palate (CP), CL and CP are different both temporally and with respect to developmental lineage.

CP alone results from failure of the mesenchymal masses of the palatine processes to fuse during weeks 7–12. Generally, combined cleft lip and palate (CL+P) and CL alone are more frequent in males, whereas for isolated CP the reverse is true (Fogh-
X-linked cleft secondary palate and ankyloglossia in an Icelandic family

A

B

Legend:
- ◯ Not investigated
- ■ Cleft secondary palate
- ◯ + Ankyloglossia
- ◯ Investigated
- ◯ Ankyloglossia
- ◯ Obligatory carrier
- ◯ High vaulted palate
- ◯ Cleft secondary palate only
The incidence of CL ± CP is higher than that of CP, varying from 2.1-1000 in Japan to 0.4-1000 in Nigeria (Leck, 1984), with the geographical variation being less important than ethnic differences. However, CP alone has an average incidence of 0.7-1000 and shows little variation in different racial groups. This may mean that CP alone will not fit the purely multifactorial model. Such a model includes both polygenic origin and undefined environmental factors that will increase the variation in incidence both geographically and to some extent racially. Previous studies have shown that CP may include both polygenic and monogenic types (Bixler, Fogh-Anderson & Connolly, 1971; Bear, 1976). To date, there have been only three pedigrees reported in which CP is clearly inherited as a single-gene X-linked disorder (Lowry, 1970; Rushton, 1979; Bjornsson & Arnason, 1986). Therefore, 1976). Multifactorial inheritance is more difficult to analyse than other types of inheritance, but is thought to account for much of the normal variation in families, as well as for many common disorders, including congenital malformations. The genetic contribution to multifactorial inheritance has traditionally been analysed by comparing the ratio of the concordance rate of the condition in monozygotic and dizygotic twin pairs with the normal rate of the condition in the general population.

It appears that many congenital malformations result from a failure in a specific developmental process so that subsequent normal stages cannot be reached. The normal rate of development can be thought of as a continuous distribution, resulting from many environmental and genetic factors. If the normal rate of development is perturbed, a serious malformation may result, dividing the continuous variable distribution into a normal and abnormal class separated by a threshold. Several human congenital malformations show family patterns that fit this multifactorial-threshold model, CL ± CP and CP being two of them. Analysis is made by collection of information on the frequency of the malformation in the general population and in different categories of relatives. This allows the empirical risk of the malformation to be estimated in subsequent pregnancies. This risk is based solely on past occurrences and does not rely on knowledge of the genetic and environmental factors that give rise to the malformation (Table 1 gives recurrence risks).

Table 1. Recurrence risks (%) for cleft lip ± cleft palate

<table>
<thead>
<tr>
<th>Number of sibs affected</th>
<th>One sib and one second-degree relative</th>
<th>One sib and one third-degree relative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Neither parent affected</td>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>One parent affected</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Both parents affected</td>
<td>34</td>
<td>40</td>
</tr>
</tbody>
</table>

Data from Bonaiti-Pellie & Smith, 1974.

Multifactorial traits are defined as those that are determined by a combination of factors, genetic and nongenetic, each with a minor but additive effect (such as blood pressure) (Thompson & Thompson, 1986). It is important to distinguish between multifactorial and polygenic inheritance; the latter term should be used in a more restricted sense with reference to conditions determined exclusively by a large number of genes, each with a small effect, acting additively, such as hair colour (Fraser, 1976).
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Fig. 3. TaqI-digested DNA samples from the pedigree are shown hybridized to the anonymous DXYSl marker. Section of the Icelandic family showing the X-linked inheritance of CP and A.

Methods

DNA extraction

Genomic DNA was prepared from 10 ml of whole blood collected in EDTA (Kunkel et al. 1977).

Restriction enzyme analysis

The analysis of inheritance of RFLPs was carried out using standard techniques. 4 μg DNA was digested with the restriction enzyme revealing the polymorphism for each X-chromosome probe. The digested DNA was fractionated by electrophoresis on 1 % agarose gels and transferred to Hybond-N™ membranes (Amersham International) (Southern, 1975). The probes used were labelled to a specific activity of 1x10^9 disints min⁻¹ μg⁻¹ by synthesis using random oligonucleotide primers (Feinberg & Vogelstein, 1983). The filters were washed down to a final salt concentration of 0.1xSSC (SSC is 0.15 M NaCl, 0.15 M sodium citrate) in the presence of 0.1 % SDS at 65°C. Autoradiography was for 24 h at -70°C with an intensifying screen. For the linked probe pDP34 in this figure, three bands are seen. The 13 kb band is Y-chromosome specific and can be seen in all males. The polymorphism for this probe gives two bands at 12 and 11 kb. The 12 kb band segregates with the CP+A mutation, i.e. all affected males and female carriers must show the 12 kb band.

using rats and different mouse strains, and epidemiological studies followed in man. The nucleotide analogue FUDR has been found to inhibit acid mucopolysaccharide synthesis and to decrease palatine shelf force inducing CP in rat fetuses (Ferguson, 1978). Mutant genes in mice have been associated with CP cho, which inhibits jaw growth (Seegmiller & Fraser, 1977) and ur, which reduces palatine shelf width (Fitch, 1957).

In man, associations of CP and CL ± CP with HLA typings have been excluded (Van Dyke, Goldman, Spielman & Zmijewski, 1983; Van Dyke, Goldman, Spielman, Zmijewski & Oka, 1980) despite evidence of associations of the mouse MHC regions H-2 and H-3 with glucocorticoid- and phentoin-induced CP (Gasser, Mele, Lees & Goldman, 1981a; Gasser, Mele & Goldman, 1981b). It is still not clear exactly how the genes involved in the failure of the palate to fuse, and potential thresholds to teratogenic agents, combine; large parts of the complex biochemical mechanisms are still only postulated at the theoretical level. There have been few definitive findings which hold up in more than a single model system, and any genetic or environmental factor that appears critical in one case can be excluded in another.

The analysis of single gene mutations using RFLPs for linkage studies has had considerable success in determining the chromosomal location of several common inherited disorders. RFLPs are specific enzyme sites that are polymorphic and segregate in Mendelian fashion. These sites can segregate in a linked or unlinked manner depending on their proximity to the disease locus. Both in cases where the affected chromosome is known, as for Duchenne muscular dystrophy (Davies et al. 1983) and chronic granulomatous disease (Royer-Pokura et al. 1986), and when neither the autosome nor the biochemical defect is identified, as for Huntington's disease (Gusella et al. 1983) and cystic fibrosis (Knowlton et al. 1985; Wainwright et al. 1985; White et al. 1985), linkage to within five centimorgans has been achieved. However, the analysis of polygenic disorders by linkage studies with RFLP markers is at present more complex both practically and theoretically.

One approach to dissecting the aetiology of disorders with complex combinations of genetic and environmental factors is to use as a model a family in which the phenotype is due to a single gene defect, but displays the same features as more common multifactorial and sporadic cases. Such a 'model' for midline congenital defects has been found in a large Icelandic family (over 280 individuals) (Fig. 1) showing Mendelian inheritance of X-linked secondary cleft palate and ankyloglossia ('tongue-tied') (CP+A) (Fig. 2). Both the large size of this pedigree and the availability of many well-localized X-chromosome probes has made it possible to localize this defect subchromosomally. The CP+A locus has been found to be closely linked to one anonymous DNA
Fig. 2. Photographs of the disorder in affected male children showing the variation in the severity of the cleft palate and ankyloglossia. (A) Partial clefting of the secondary palate; (B) complete clefting of the secondary palate; (C) ankyloglossia or tongue-tied.
Fig. 4. Diagramatic description of the X chromosome showing both the in situ localization of the X probes and their maximum LOD scores in the linkage analysis of the CP+A family.

**Linkage and segregation analysis**

Combined LOD scores were calculated using the computer program package LINKAGE as described by Lathrop (1984). LOD = decimal logarithm of odds ratio, likelihood of observed recombination frequency to likelihood at 50% recombination (completely unlinked). The standard significant cut off points for positive linkage and exclusion are +3 and −2 respectively. Multipoint linkage analysis between informative markers was carried out as described in Farrall, Scambler, North & Williamson (1986). Fig. 4 gives the maximum LOD scores at recombination fractions from 0 to 0.5 for all the informative probes. These data locate CP+A to Xq13–Xq21.1.

The exclusion map on Fig. 4 is composed of 14 probes that did not show linkage to CP+A. Minimal multipoint linkage was feasible between these probes due to their low information content within this family. However from these data large sections of Xq and Xp have been excluded.
probe DXYS1 (probe name pDP34), which maps to Xq13–21.1 (Moore et al. 1987) (Fig. 3).

Once localization on the X chromosome has been more accurately achieved with finer mapping, cosmid libraries can be used to jump or walk from specific restriction enzyme sites to the physical location of the gene. Subsequent cloning and sequencing of the normal gene and its mutation and analysis of its functional expression using cDNA libraries and Northern blots will allow one part of the pathway that leads to cleft palate to be clarified. Autosomal sequences may either be homologous, or specify other parts of the same pathway, or at least give clues to potential candidate genes involved in neural crest cell and craniofacial development.

Discussion

Localization of the mutation causing cleft palate in this family to Xq13–21.1 is a first step in understanding the genetic component of congenital neural crest defects. This region of the X chromosome contains an XY homologous region (Page, Harper, Love & Botstein, 1982). As the limits of genetic mapping in this family are approached, the techniques of cosmid walking and jumping and pulsed-field gel electrophoresis (Schwartz & Cantor, 1984; Poustka & Lehrach, 1986) will allow the gap between the genetic and physical map to be bridged.

The isolation of the gene and studies of its spatial and temporal expression during development of the secondary palate should lead us to a greater understanding of mechanisms which may lead to the failure of closure of the palate. This mechanism can be compared with those which may cause sporadic cases in humans, or in animal models. There are also families known in which an X-linked form of spina bifida and anencephaly occur (Burn & Gibbens, 1979; Fitch, 1957). Any homology in linkage between these families and the CP+A Icelandic family would be of interest.

The cloning of this mutation can now be used as a starting point for the analysis of other developmental abnormalities. For example, this gene may be a member of a multigene family involved in the embryonic development of other tissues. Bodmer (1983) has suggested that genes with related functions whose coordinated expression gives rise to complex phenotypes may be clustered on one chromosomal region as supergenes. In mouse, five related homeotic genes are known to be clustered in a supergene family at a single genetic locus. Such structure–function relationships may allow direct entry into complex genetic systems involved in pattern formation and ontogeny. Analysis of CP+A as a sex-linked single gene provides a model for other genes regulating processes in embryonic development.

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References


