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Application of Genetic Analyses in Studies of Syndromic and Non-syndromic Cleft Lip and Palate

Doctoral Thesis

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Application of Genetic Analyses in Studies of Syndromic and Non-syndromic Cleft Lip and Palate
- Fung Ki Wong

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“If I have seen farther than others, it is by standing upon the shoulders of giants.”

Isaac Newton (1642-1727)

CONTENTS

ABBREVIATIONS

BAC	bacterial artificial chromosome
bp	base pair
CEPH	Centre d'Études du Polymorphisme Humaine
CHLC	Cooperative Human Linkage Center
CLP	non-syndromic cleft lip with or without cleft palate
cM	centiMorgan
DNA	deoxyribonucleic acid
EEC3	ectrodactyly, ectodermal dysplasia, cleft lip/palate syndrome type 3
FIHP	familial isolated hyperparathyroidism
GDB	Genome Database
HGP	Human Genome Project
HPE2	holoprosencephaly type 2
HPT	hyperparathyroidism
HPT-JT	hyperparathyroidism-jaw tumor syndrome
IBD	identical by descent
IBS	identical by state
kb	kilobase
LDB	genetic location database
LOD	decimal logarithm of the odds ratio
LOH	loss of heterozygosity
Mb	megabase
MEN 1	multiple endocrine neoplasia type 1
MF/T	multifactorial threshold
MSX1	homologue of drosophila muscle segment homeobox 1
NPL	non-parametric linkage
OFC1	orofacial cleft 1
OMIM	on-line Mendelian inheritance in man
p	the short arm of a chromosome
PAC	P1-derived artificial chromosome
PAX9	paired box gene 9
PCR	polymerase chain reaction
PPS	popliteal pterygium syndrome
PTH	parathyroid hormone
q	the long arm of a chromosome
RARA	retinoic acid receptor alpha
RFLP	restriction fragment length polymorphism
SHH	homologue of drosophila sonic hedgehog
SIX3	homologue of drosophila sine oculis homeobox 3
SNP	single nucleotide polymorphism
STRP	short tandem repeat polymorphism
TDT	transmission disequilibrium test
TGFA	transforming growth factor alpha
TGFB3	transforming growth factor beta 3
VNTR	variable number of tandem repeats
VWS	van der Woude syndrome
Z	LOD score
θ	theta, recombination fraction

ABSTRACT

Application of Genetic Analyses in Studies of Syndromic and Non-syndromic Cleft Lip and Palate

Fung Ki Wong

Linkage analysis is one of the first and essential steps in the localization of human disease genes. In this study, the method was first applied in mapping of the disease in two families with isolated hyperparathyroidism (FIHP) to the hyperparathyroidism-jaw tumor (HPT-JT) syndrome locus at 1q21-q32. The results suggest that a subset of FIHP families are a variant of HPT-JT. Loss of heterozygosity at 1q21-q32 was found in tumors from a FIHP and a HPT-JT family, implying that the gene involved is a tumor suppressor gene (Paper I).

Genetic analyses were applied to the studies of syndromic and non-syndromic cleft lip and palate. Van der Woude syndrome (VWS), the most common form of syndromic cleft, has been mapped to 1q32-q41. Two Swedish and three Finnish families were mapped to 1q32-q41 and the critical region was tentatively refined to 130 kb by observing recombination and shared haplotypes in the families. Microdeletion of the VWS region was unlikely. Two Finnish families were unlinked to the VWS region in 1q thus providing the first evidence of genetic heterogeneity in VWS. All families were tested for the possible modifying effect on the cleft palate phenotype from a locus in 17p11.2-p11.1. Linkage was clearly excluded, meaning that the 17p locus is not a major modifying gene (Papers II, III).

A Swedish boy and his mother who both have cleft lip and palate were examined for additional clinical features. The presence of lip pits and a distinct toe malformation led to the diagnosis of popliteal pterygium syndrome (PPS). Our analysis showed that microdeletion of the 1q VWS region was unlikely but linkage to the region could not be excluded (Paper IV).

Non-syndromic cleft lip with or without cleft palate (CLP) has been tentatively mapped to candidate loci in chromosome 4, chromosomes 2, 6 and 19 with heterogeneity. Linkage to these regions as well as the 1q VWS locus was tested in 19 Swedish multi-generation CLP families. Our results showed that the group of families was unlinked to any of these regions and heterogeneity was not evidenced. This might imply a new locus or loci in the Swedish CLP families, although linkage to individual loci could be hidden by insignificant heterogeneity in the family group. Results from the non-parametric linkage analysis were insignificant. Although linkage could not be confirmed with the limited sizes of these families, data from individual families will form the basis for future studies of candidate genes derived from these loci (Paper V).

In summary, genetic linkage analyses have been applied to elucidate the genetic basis of syndromic and non-syndromic cleft lip and palate, generating important clinical and genetic data. These results will be incorporated into the ongoing gene mapping efforts of these diseases. Identification of the genes involved in cleft lip and palate is essential for the diagnosis, prediction and in the future treatment of these congenital malformations.

Keywords: Linkage analysis, cleft lip, cleft palate, van der Woude syndrome, popliteal pterygium syndrome, hyperthyroidism, candidate gene

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LIST OF PAPERS

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The published articles are reprinted with the permission of the respective publishers.

I. Teh BT, Farnebo F, Twigg S, Höög A, Kytölä S, Korpi-Hyovalti E, **Wong FK**, Nordenström J, Grimelius L, Sandelin K, Robinson B, Farnebo LO, Larsson C.
Familial isolated hyperparathyroidism maps to the hyperparathyroidism-jaw tumor locus in 1q21-q32 in a subset of families.
J Clin Endocrinol Metab 1998;83:2114-2120.

II. **Wong FK**, Karsten A, Larson O, Huggare J, Hagberg C, Larsson C, Teh BT, Linder-Aronson S.
Clinical and genetic studies of van der Woude syndrome in Sweden.
Acta Odontol Scand 1999;57:72-76.

III. **Wong FK**, Koillinen H, Rautio J, Teh BT, Karsten A, Larson O, Linder-Aronson S, Huggare J, Larsson C, Kere J.
Genetic heterogeneity and exclusion of a modifying locus at 17p11.2-p11.1 in Finnish families with van der Woude syndrome.
(submitted).

IV. **Wong FK**, Gustafsson B.
Popliteal pterygium syndrome in a Swedish family - clinical findings and genetic analysis with the van der Woude syndrome locus at 1q32-41.
Acta Odontol Scand 2000;58 (in press).

V. **Wong FK**, Hagberg C, Karsten A, Larson O, Gustavsson M, Huggare J, Larsson C, Teh BT, Linder-Aronson S.
Linkage analysis of candidate regions in Swedish non-syndromic cleft lip with or without cleft palate families.
Cleft Palate Craniofac J 2000;37 (in press).

INTRODUCTION

General Introduction

Our interest in mapping human traits dates back long before the description of the double helix by Watson and Crick in 1953 and the recognition of the number of human chromosomes in 1956 (Ford and Hamerton 1956, Tjio and Levan 1956). Colorblindness was mapped to the X chromosome by its inheritance pattern (Wilson 1911), which was also the first locus being mapped to a specific chromosome in mammals. In fact, genetic linkage analysis was reported in 1865 by Gregor Mendel on the inheritance of traits in the pea plant. The results from his careful work laid down the foundations for the genetic studies of today.

"All human disease is genetic," stated the Nobel laureate Paul Berg, in a cancer symposium. Although this statement seemed slightly exaggerated, it is becoming increasingly evident that many human afflictions, from cancer to psychological disorders and susceptibility to infection, bear genetic origins. Before genetic mapping information was available, the identification of human disease genes depended on the knowledge of the gene product, a method known as functional cloning or forward genetics. However, the success of pure functional cloning has been very limited to only a handful of human diseases in which the biochemical pathways were known or the gene products could be purified.

Today the identification of human disease genes rarely relies on the function, but on their locations in the human genome, an approach commonly known as positional cloning or reverse genetics. An outline of this approach is shown in Figure 1. To facilitate positional cloning, the disease phenotype must be defined as precisely as possible and its genetic components identified. In the planning stage of gene mapping the statistical methods and the type of family material to be collected are defined. Genetic and statistical analyses of the collected samples are performed to localize the disease-causing gene. Repeated analyses in different populations are usually performed to confirm the localization and to refine the genetic region. When the locus is refined to a reasonable size, genes within the region are identified by different means and tested for mutation in the affected individuals. When a gene is found to be mutated in the disease, additional cell or animal experiments should follow to confirm its biological functions.

Figure 1 is a simplified drawing of the various steps and their interaction within the gene mapping process. This summary will concentrate on the methods in the localization of disease gene loci. Examples of its application in diseases with focus on cleft lip and palate will be discussed.

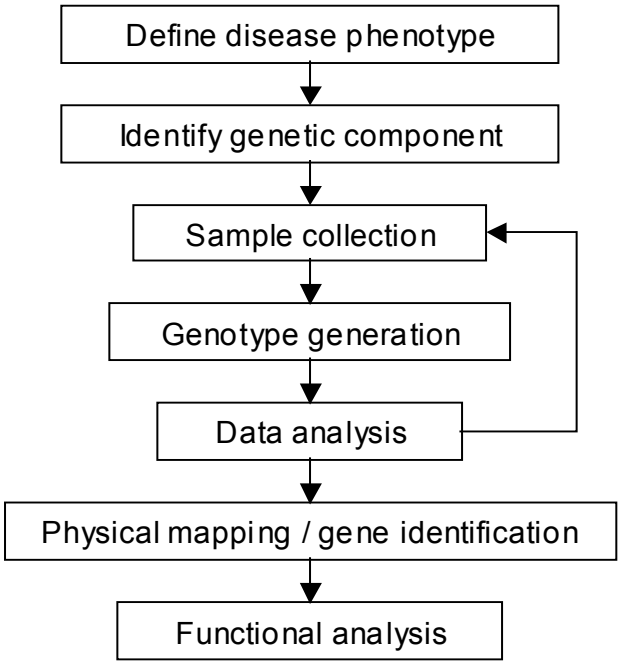

Figure 1. Positional cloning approach in the identification of human disease genes.

In the early 1930's, simple statistical methods for human genetic mapping became available (Haldane 1934, Fisher 1935). However, the application of these methods was limited to small nuclear pedigrees and their calculations were not as complex and comprehensive as today's. A breakthrough in the statistical methodology for genetic linkage was introduced by Morton (1955). He proposed the use of the maximum likelihood approach for linkage testing between two loci, and introduced the term LOD (logarithm of odds) score, which forms the basis of most of the linkage analysis being used today.

An obstacle in early human disease gene mapping was the lack of genetic markers, and blood groups and serum protein polymorphisms were the only available genetic markers. These markers were technically difficult to type and they only covered a small part of the human genome. This was overcome by the introduction of DNA restriction fragment length polymorphisms (RFLPs) in 1980 (Botstein et al 1980). RFLPs are based on single base pairs alterations that involve a cleavage site of a restriction endonuclease. In case of polymorphism the resulting restriction fragments on the maternally and paternally derived chromosomes will have different lengths/alleles, which can be identified by Southern blot analyses (Figure 2). As compared to protein polymorphisms, RFLPs are relatively abundant, evenly spread out in the human genome and can be readily typed by Southern blot analysis. An immediate success of this development was the mapping of the Huntington disease locus to the short arm of chromosome 4 (Gusella et al 1983). However, only two different alleles can be identified at each RFLP locus, giving a limited frequency of heterozygosity in the population.

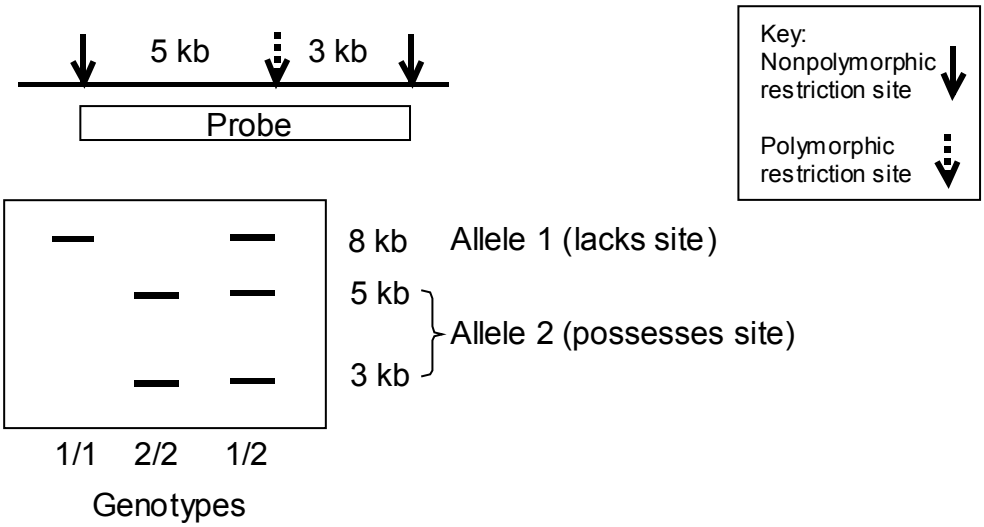

Figure 2. Assay of RFLP by Southern blot analysis. A RFLP locus is cleaved by a restriction endonuclease. The cleaved products are size-fractionated by gel electrophoresis and the fragments of interest are revealed by hybridization with a labeled probe.

The next generation of DNA markers constitute the variable number of tandem repeats (VNTRs) (Jeffreys et al 1985, Nakamura et al 1987). These highly informative markers are scattered throughout the genome and usually reflects variations in repeat motifs of 10-20 bases per unit. A drawback of the RFLP and VNTR markers is that their typing involves the relatively time consuming Southern blot analysis and there is a requirement of large amounts of DNA for the analyses. With the introduction of the Polymerase Chain Reaction (PCR) technology in the 1980's (Saiki et al 1985, Mullis et al 1986), genotyping of microsatellite markers quickly became the method of choice in genetic mapping. Short tandem repeat polymorphism (STRP) or microsatellites, were described by Weber and May, and Litt and Luty in 1989. They consist of interspersed DNA elements of the form (dC-dA)n/(dG-dT)n, and constitutes one of the most abundant human repetitive DNA families. These repeat units are polymorphic in length among individuals, which made them an enormous pool of potential genetic markers. The variations in their length were directly demonstrated by amplifying the DNA segment by PCR and then resolving the product on polyacrylamide DNA sequencing gels (Figure 3). PCR detection of DNA polymorphisms requires minute amounts of DNA and offers improved sensitivity and speed compared with traditional methods of Southern blotting and hybridization.

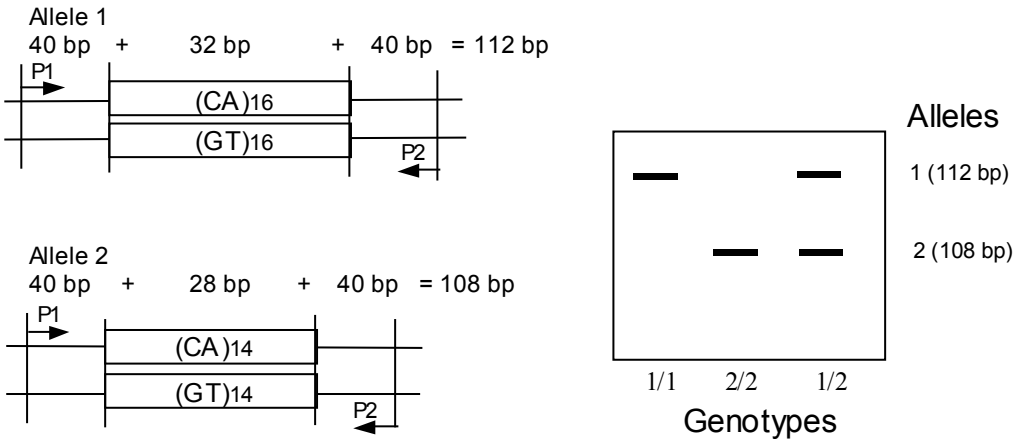

Figure 3. Genotyping of STRP by PCR. In this example, the PCR primers P1 and P2 are used to amplify STRPs with 16 or 14 copies of CA/TG dinucleotide repeats. The resulting PCR products of sizes 112 and 108 bps are revealed by polyacrylamide gel electrophoresis. For clarity reason, artifacts created by dinucleotide repeats (stutters) are omitted from the drawing.

The dinucleotide CA (GT) repeats however, are not without drawbacks. Many produce PCR artifacts called "stutter" that form a ladder of bands accompanying the main allele. This makes typing of suspected homozygotes particularly difficult. To resolve this problem, other repeats such as the trinucleotide and most importantly tetranucleotide repeats have been developed, which produce clear PCR products since the alleles are separated by three or four base pairs. These features make them indispensable in modern genotyping using computerized allele analysis.

The coordination of the development of the above-mentioned genetic markers for the human genome and the mapping of these polymorphisms along the chromosomes are enormous tasks. Fortunately, they have been taken up as main parts of the Human Genome Project and have been expedited by efforts from different genome centers worldwide.

The Human Genome Project and Genetic Maps

A coordinated effort by the U.S. Department of Energy and the National Institutes of Health started the U.S. Human Genome Project (HGP) in 1990. The goals of the project were to identify the estimated 80-100,000 (or even 140,000) human genes and the sequences of the 3 billion base pairs of human DNA, to develop related technologies and to resolve ethical, legal, and social issue that may arise from the project.

One of the specific goals in the first 5-year plan of the HGP was to develop a comprehensive human genetic map, which would be used in the identification of the genes associated with genetic diseases and other biological properties and as a backbone for building physical maps. For the purpose, a human genetic map consisting of 600 to 1500 informative genetic markers with a resolution of 2 to 5

centiMorgan (cM) would be needed. This goal was completed in 1994 as one of the first achievements of the HGP. A genetic map with an average density of 0.7 centiMorgan was compiled by Murray et al (1994), which consisted of 5,840 loci, out of which 3,617 were PCR-based STRPs.

Rapid development in molecular technologies partly led to a revision of the goals of the HGP in 1993 (Collins and Galas 1993). As the construction of the genetic map was to finish on schedule, focus was shifted to the usability and accessibility of the map data. In addition, emphasis were put on the development of technologies that would allow non-experts to perform genotyping for medical research purposes and increase the ability to analyze complex genetic diseases.

Online Resources for Gene Mapping

To ensure accessibility of various genetic mapping data, including efficient data submission and retrieval, the Internet based on the World Wide Web (WWW) protocol became the major vehicle for genetic information traffics. One of the most well known online databases among geneticists is the online version of the Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov/omim/) compiled by McKusick (1998). This database is a catalogue of human genes and genetic disorders with links to other databases such as literatures, mapping and sequence databases. The compilation was initially published as a printed version during the 1960s, describing approximately 1500 genetic disorders. At the end of 1999, the OMIM held more than 10,000 entries and the number is still increasing.

The prime sources of information necessary for human gene mapping are databases of genetic markers and genetic maps describing their locations. Many genome centers develop their own sets of databases using different types of markers and genetic maps. To standardize the genotyping work by these centers such as the sizing of marker alleles, most of the genetic maps have been generated with the CEPH (Centre D'Étude du Polymorphisme Humain) families. The Center is now referred to as Foundation Jean Dausset-CEPH and can be accessed at http://www.cephb.fr. The database provides genotype information based on 61 well characterized families (Dausset et al 1990) and presently contains genotypes for more than 12,000 genetic markers submitted to the CEPH by over 100 laboratories around the world. These data form an essential guide for standardization in genotyping for different experiments, projects and laboratories.

Whole genome maps using PCR based DNA markers can be found at the Généthon Database (http://www.genethon.fr). The Généthon map consists of 5,264 dinucleotide STRPs with a mean heterozygosity of 70%. The map comprises 2,335 positions of which 2,032 could be ordered with a high level of accuracy and with an average interval of 1.6 cM.

As discussed above, the tri- and tetra-nucleotide repeats have their advantage over the dinucleotide repeats. The Cooperative Human Linkage Center (CHLC) (http://www.chlc.org) develops and compiles genetic maps mainly based on tri- and tetranucleotide repeats. Different kinds of genetic maps and specific information on

primers such as sequences, allele sizes from selected CEPH pedigrees and heterozygosity can be found at the center.

Other databases worth mentioning include the Center for Medical Genetics at the Marshfield Medical Research Foundation (http://www.marshmed.org/genetics), which, apart from providing genetic mapping information, also creates sets of genetic markers for whole genome linkage analysis projects. The genetic location database (LDB), developed by the Wessex Human Genetics Institute, is intended to integrate various genetic, cytogenetic, radiation hybrid and physical maps. This site can be reached at http://cedar.genetics.soton.ac.uk/public_html/. The Genome Database, GDB (http://gdbwww.gdb.org) is another essential database for gene mapping by providing detailed marker and mapping information. A summary of these online resources is given in Table 1.

Table 1. Useful websites with databases for human gene mapping.

Database	Description of contents	World wide web address
OMIM	Inherited diseases and genes	http://www.ncbi.nlm.nih.gov/omim
CEPH	Genotypes of CEPH families	http://www.cephb.fr
Généthon	Genetic maps (dinucleotide repeats)	http://www.genethon.fr
CHLC	Genetic maps (tetranucleotide repeats)	http://www.chlc.org
Marshfield	Genetic maps, genome screening sets	http://www.marshmed.org/genetics
LDB	Integrated maps of markers and genes	http://cedar.genetics.soton.ac.uk/public_html
GDB	Genetic markers and maps	http://gdbwww.gdb.org

Linkage Analysis

In physical mapping, the distance between loci is measured by the actual number of base pairs. Genetic mapping, however, is based on genetic recombination between linked loci. During meiosis, homologous chromosomes pair up in close approximation and crossover occurs, in which the double helix structure of one maternal and one paternal chromatid physically break up to join the end of the other chromatid (Figure 4A). The result is the formation of recombinant gametes. Therefore, in a pedigree, the offspring can be either recombinant or non-recombinant for different loci from their parents (Figure 4B).

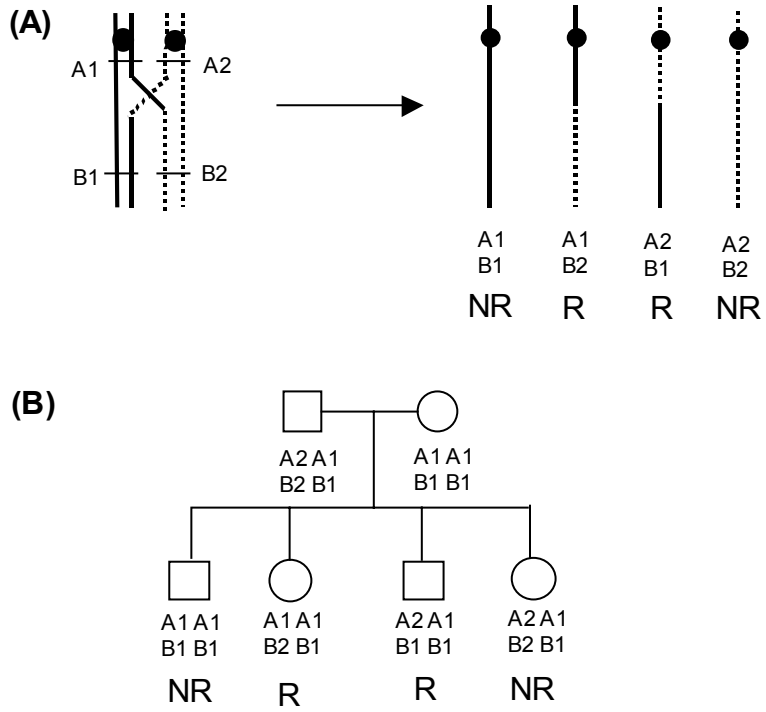

Figure 4. Genetic recombination. (**A**) During meiosis, crossover involving two of the four chromatids of two homologous chromosomes will lead to the formation of recombinant (R) and non-recombinant (NR) gametes, denoted by alleles 1 and 2 at loci A and B respectively. (**B**) An example of segregation of alleles at loci A and B in a pedigree. The offspring can be either recombinants (R) or non-recombinants (NR) from their father.

The recombination fraction (θ) in a pedigree is defined as the proportion of children who are recombinant between the loci in question, such as illustrated in Figure 4B where the children who inherited A1B2 or A2B1 are recombinant between loci A and B. If loci A and B are physically very close together on the same chromosome, the chance of forming a crossover between them is low. The further apart they are the more likely can recombination occur, and higher recombination fractions will be observed in the pedigree.

If the two loci are located on different chromosomes, they have a random chance of being segregated together; therefore, their recombination fraction is 50%. When the two loci are located on the same chromosome, assuming random crossover, the highest chance of recombination is also 50%, because there will also be a 50% chance for having non-recombinant offspring. Therefore, a recombination fraction of 0.5 indicates that the two loci are located far apart on the same chromosome or they are located on different chromosomes (unlinked).

Two-point Linkage Analysis

Linkage refers to the association in inheritance of two or more loci due to the close proximity of their locations in the same chromosome. In two-point linkage analysis,

the loci can be two genetic markers, whereas in human disease gene mapping one of the loci is the disease phenotype, as shown in the pedigree drawing in Figure 5. If the disease-causing gene is located close to the genetic marker, a low recombination fraction between the phenotype and genotype should be observed. If they are far apart or are on different chromosomes (unlinked), the recombination fraction between them would instead approach 0.5. The pedigree in Figure 5 represents a situation where the recombination in the offspring can be identified unambiguously. In most cases when it is not possible to determine and count recombinants and non-recombinants, statistical analysis of the overall likelihood for linkage in the pedigree is required.

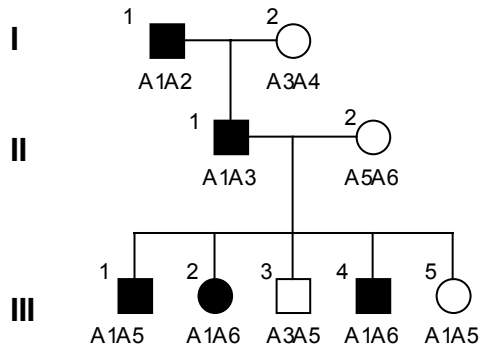

Figure 5. Two-point linkage analysis in disease gene mapping. The family having an autosomal dominant disease (filled symbols) is typed with marker A. It can be observed that allele A1 is likely to be associated with the disease phenotype, such that individuals III-1 to III-4 are non-recombinants and III-5 is recombinant.

In the statistical analysis of two-point linkage, two alternative hypotheses are tested i.e. that the two loci are linked (at recombination fraction = θ) and that they are unlinked (recombination fraction = 0.5). The ratio between the probabilities of these two hypotheses is the odds of linkage, which is expressed in logarithm with a base of 10, commonly known as the LOD score (Z).

Two-point LOD scores are generally calculated over a range of θ values from zero to 0.5 so that a maximized LOD over θ is obtained. The advantage of LOD score analysis is that the results from different pedigrees calculated with the same parameters can be added together to form a set of cumulative LOD scores for the total sample group.

For linkage analysis in autosomes (chromosomes 1 to 22), LOD scores of 3 (odds 1000:1) or above are accepted as significant linkage, a figure that apparently seems very stringent. However, one should consider that there is a prior probability for two loci to be linked in the human genome anyway, and for the 22 pairs of autosomes a value of 1/50 is generally accepted. Therefore, by applying the Bayesian approach, a LOD score of 3 is the joint probability between the prior and the conditional probabilities of linkage. The conditional probability of linkage therefore corresponds

to the conventional statistically significant p value of 0.05. To exclude linkage, on the other hand, would only require a LOD score of –2.

To perform linkage analysis by counting recombination is possible only in very simple and straightforward pedigrees. In most pedigrees and especially when there is missing genotype data, linkage calculation could only be performed by computer. One of the most well known computer programs for two-point linkage analysis is the MLINK program, which is part of the LINKAGE program package (Lathrop et al 1984). This program package, including accessory programs, is available for download at the ftp site of the Laboratory of Statistical Genetics at the Rockefeller University (ftp://linkage.rockefeller.edu/software/linkage). FASTLINK (Cottingham et al 1993) is an improved version of the LINKAGE program that offers several advantages including a much higher speed of operation. The program is available at the Computational Biology Branch of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/CBBresearch/Schaffer/fastlink.html).

Multi-point Linkage Analysis

If several genetic loci can be analyzed simultaneously (multi-point analysis) instead of analyzing one at a time (two-point analysis), the efficiency of linkage analysis would be greatly improved. Analyzing a group of linked markers can compensate for the lack of informativeness in certain markers; therefore, the total amount of information that can be extracted from a family is increased. In addition, multi-point analysis can be used to locate a disease gene within a framework of markers.

Early multi-point linkage analyses based on the Elston-Stewart algorithm (Elston and Stewart 1971) was limited to the analysis of a few markers. Lander and Green (1987) described the use of the Hidden Markov Models in calculating inheritance distribution, which allowed a more comprehensive multi-point linkage analysis in families of moderate sizes. The method was improved by Kruglyak et al (1995a) in the speed of computation and subsequently implemented in the GENEHUNTER computer program (Kruglyak et al 1996), which is available for download at the homepage of the Whitehead Institute for Biomedical Research (http://waldo.wi.mit.edu/ftp/distribution/software/genehunter/gh2/). In addition to rapid computation of multipoint LOD scores with dozens of polymorphic markers, the GENEHUNTER program allows calculation of the information-content for each region and the construction of multi-marker haplotypes in the pedigrees. The only limitation for the GENEHUNTER algorithm is the size of the pedigree, which is dictated by the power of the computer.

The methods for linkage analysis which generate LOD scores are classified as parametric or model-dependent. In these analyses, the parameters describing the genetic model of the disease including the mode of inheritance, the frequency and penetrance of the disease allele, the frequency of phenocopies and the mutation rate need to be stated correctly before analyzing the phenotype and genotype data. Otherwise, either the power of the analysis would be affected or distorted results would be produced. Therefore, when these parameters of a disease are unclear, other

kinds of linkage analysis that do not rely on the genetic models of the disease might be preferred.

Non-parametric Linkage Analysis

Correct parameters for linkage analysis are not always available. In addition, nowadays when human genetic linkage is shifting from simple Mendelian to complex diseases, other methods of genetic analysis are required. Non-parametric or model-free analyses examine the genetic factors on a phenotype without specifying a genetic model. In general, an increase in the sharing of marker alleles among persons having the same phenotype is searched against a background of random distribution in the population. Nonparametric linkage analysis in a pedigree studies the distribution of alleles among affected members that are inherited from the same ancestor, a status known as identical by descent (IBD) (Figure 6).

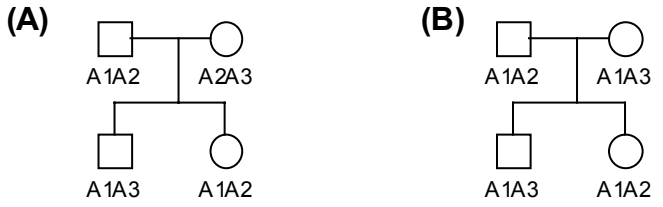

Figure 6. Identity by descent and identity by state. In (A), both sibs share the A1 allele inherited from the father (identical by descent, IBD); whereas in (B) the two A1 alleles originate from different parents; therefore the sibs are only identical by state (IBS) for A1.

Assuming random distribution, two siblings share 0, 1, or 2 of their parental alleles IBD in ¼, ½ and ¼ of the cases respectively. If a group of sib pairs affected with the same disease shares more parental alleles than would have been expected under random segregation, the marker may be associated with the disease. The significance of such an association could be tested by statistical means. One of these statistical algorithms is executed by the computer program MAPMAKER/SIBS (Kruglyak and Lander 1995b) which is intended for sib-pair analysis. The method was later modified for structured pedigree settings and combined into the GENEHUNTER program. The statistics is expressed as the non-parametric linkage (NPL) scores with its corresponding *p* values.

Cleft Lip and Palate

The term cleft lip and palate is commonly used to represent two types of malformation i.e. cleft lip with or without cleft palate and cleft palate. Cleft lip and palate are common congenital malformations. The reported incidences varying widely depending on the individual study designs and on the populations under studies. As reviewed by Tolarova and Cervenka (1998), figures between 1 and 2 per 1000 live

births have been reported. The incidence of cleft lip and palate in Scandinavia varied from 1.7 to 2 per 1000 live births (Henriksson 1971, Jensen et al 1988, Hagberg et al 1998). Among all the cleft cases, cleft lip and palate were not always the only malformations in an individual. Milerad et al (1997) showed that 21% of cases with cleft were associated with additional malformations that required follow-up or treatment. Tolarova and Cervenka (1998) revealed in a Californian population that only 62% of the cleft cases occurred as isolated abnormalities; the rest were associated with sequences, chromosomal abnormalities, syndromes or other malformations. When only isolated cleft cases were considered, the prevalence was 1.1 per 1000 births.

An understanding of the normal human maxillofacial development is necessary before the discussion of cleft lip and palate. During the fourth week of gestation, the maxillary processes emerge from the first branchial arch on each side and the nasal placodes form from the frontal prominence. By the fifth week, all the primordia for the lip and palate are present. The medial, lateral nasal and the frontonasal process are formed from the nasal placodes and the maxillary processes continue to enlarge. During the seventh week the medial nasal, frontonasal and maxillary processes fuse to form the primary palate, which becomes the medial portion of the upper lip, alveolus and the anterior part of hard palate up to the incisive foramen. When the primary palate is completely formed, the maxillary processes enlarge intraorally to form the palatine processes. During the eighth weeks of gestation, the palatal shelves fill up the space on both sides of the tongue. During the ninth and tenth weeks, the mandibular arch enlarges and the tongue drops. The palatal shelves transpose horizontally and fuse with each other and with the anterior part of the palate. Palatal fusion occurs anteroposteriorly and the process is completed by the 11^{th} to 12^{th} weeks (Shapiro 1976).

Failure in the fusion of the nasal and maxillary processes leads to cleft of the primary palate, which can be unilateral or bilateral. The degree of cleft can vary from a slight notch on the lip to complete cleft of the primary palate. Cleft of the secondary palate is medial. It varies from bifid uvula to complete cleft palate up to the incisive foramen. When it is associated with the primary palate, a complete uni- or bilateral cleft lip and palate is formed.

Syndromic Cleft Lip and Palate

A large number of syndromes are associated with cleft lip and palate. A search in the OMIM database using the keyword “cleft lip” yields 154 entries and with “cleft palate” 289 entries. This indicates that cleft lip and cleft palate can be induced by defects along many different developmental pathways. A few of the genes responsible for syndromic cleft lip and palate have been identified. The gene *p63* is mutated in the ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome type 3 (EEC3, OMIM 604292). SIX3 (homologue of drosophila sine oculis homeobox 3) is mutated in holoprosencephaly type 2 (HPE2, OMIM 157170); and SHH (homologue of drosophila sonic hedgehog) is mutated in HPE3 (OMIM 142945) respectively. On the other hand, many of the syndromic cleft genes are mapped but not identified and in some cases, only clinical descriptions are available.

Van der Woude Syndrome (VWS)

Lower lip pits have been reported since 1845 (Demarquay 1845) and a familial form was reported by Murray in 1860. Van der Woude (1954) described an autosomal dominant form of lip pits and oral clefts in five pedigrees, therefore the syndrome is now known as the van der Woude Syndrome (VWS; OMIM 119300). It is the most common form of syndromic orofacial clefts, accounting for 2 % of all cleft lip and palate cases (Rintala and Ranta 1981). This syndrome is characterized by cleft lip with or without cleft palate, cleft palate, pits on the lower lip and hypodontia. The expressivity of VWS is highly variable; for example, the lower lip pits may appear as mucous cysts or as conical elevations only (Ranta and Rintala 1983). The inheritance of VWS is autosomal dominant with an almost complete penetrance (Janku et al 1980, Shprintzen et al 1980).

An interesting feature in VWS is the segregation of both cleft lip with or without cleft palate and cleft palate in the same family (Test and Falls 1947). Since different embryonic processes are involved in the formation of primary and secondary palate, this association links the VWS gene to both processes. A possible explanation would be that the VWS gene is involved in the control of the fusion of both primary and secondary palate.

In 1987, a patient with multiple malformations, developmental delay, submucous cleft palate and lower lip pits was found to have a constitutional deletion of chromosomal region 1q32-q41 (Bocian and Walker 1987). Since lip pits are the main features of VWS, it was suggested that a submicroscopic deletion of chromosome 1q might be the cause of VWS. Localization of VWS to chromosome 1 was confirmed by Murray et al (1990) when six VWS families were found linked to chromosome 1q. Sander et al (1994) demonstrated linkage to 1q32-q41 in 15 European VWS families, in which a microdeletion of the marker D1S205 segregated in one of them. This finding refined the VWS region and suggested that VWS is caused by haploinsufficiency of a gene in 1q32-q41. To look for new patients with microdeletion of the VWS region, Schutte et al (1999) employed a novel polymorphic marker D1S3753 from the VWS region, and a new family with microdeletion was identified.

The VWS region has been refined to a 1.6-cM interval between D1S491 and D1S205 by linkage studies in affected families (Schutte et al 1996) (Figure 7). Based on genomic sequencing of BAC (bacterial artificial chromosome) and PAC (P1-derived artificial chromosome) clones, the physical distance of the VWS region was found to be 350 kb (Schutte et al 2000) (Figure 7). The VWS gene has not been identified yet. In addition, the 1q region is so far the only locus for VWS from the published linkage and deletion studies (Houdayer et al 1999).

Recently, a large Brazilian VWS family with multiple cases affected with cleft palate was studied by Sertié et al (1999). Linkage to 1q32-q41 in this family was confirmed. The authors suggested that the VWS gene at 1q32-q41 mainly caused lip pits in the family, while susceptibility to cleft palate in the family was affected by an additional gene acting as a modifier to the gene at 1q. A partial genome search was performed to

locate this modifying gene and suggestive linkage (Z=2.05) was found in chromosome 17p11.2-p11.1. The authors concluded that the 17p region contains a modifying gene for cleft palate in the Brazilian VWS family, but whether the gene is a major modifier in VWS or only one of several modifying genes needs to be verified in other VWS families.

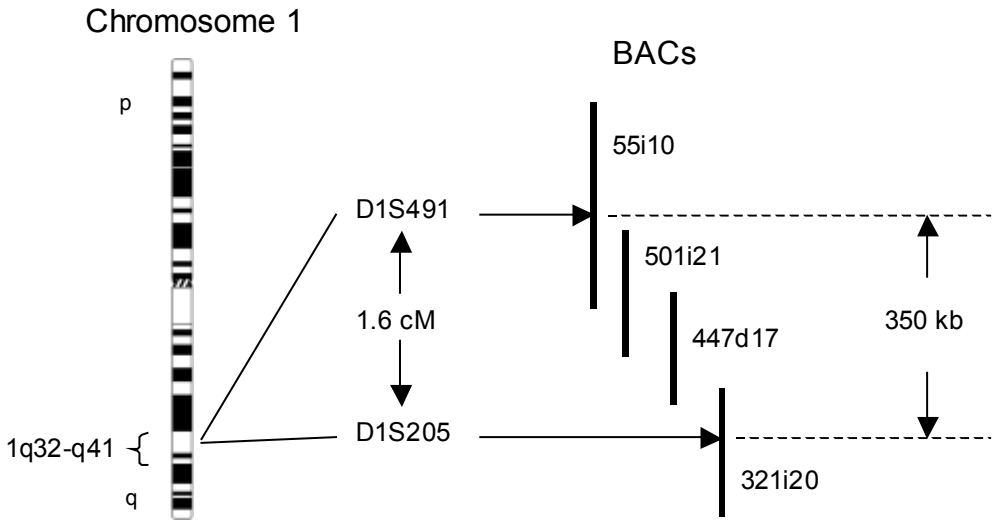

Figure 7. The VWS region at chromosome 1q32-q41.

Popliteal Pterygium Syndrome (PPS)

Popliteal pterygium syndrome (PPS, OMIM 119500) is a rare congenital malformation syndrome with an incidence of 1/300,000 (Froster-Iskenius 1990). The syndrome was first described by Trélat in 1869. The mode of inheritance is autosomal dominant with incomplete penetrance and variable expressivity. As reviewed by Froster-Iskenius (1990), cleft palate or cleft lip and palate are the main features of PPS, occurring in more than 90% of the cases. Paramedian sinus or pits on the lower lip, syngnathia (intraoral tissue bands), popliteal pterygium or syndactyly can be found in half of the cases and a distinct toenail abnormality in one-third. Due to the high variability of PPS, different diagnostic criteria have been suggested. Hunter (1990) suggested that syndactyly, especially that of the toes, was diagnostic for PPS. Gorlin et al (1990) suggested that a piece of triangular overgrowth of skin over the nail of the first toe, if accompanied with cleft palate with or without cleft lip, should be diagnosed as PPS even when popliteal pterygium is not present.

PPS and VWS share common features of cleft lip, cleft palate and lip pits, Bixler et al (1973) proposed that these two syndromes represent variants of the same condition. In a large family with PPS, affected members in the earlier generations resembled VWS (Soekarman et al 1995). The authors therefore suggested that PPS and VWS might be allelic variants of the same gene. So far, studies of PPS were mainly clinical and the gene for PPS has not been mapped. A recent study provided suggestive evidence of

linkage (Z = 2.7) in four PPS families to the VWS locus on 1q32-q41 (Lees et al 1999).

Non-syndromic Cleft Lip with or without Cleft Palate (CLP)

Although cleft lip and palate can be associated with syndromes, the majority of clefts especially cleft of the primary palate occurs as a non-syndromic form (CLP). CLP appears to have complex etiology and the susceptibility to CLP is apparently influenced by both genetic and environmental factors. How these factors interact and contribute to CLP is still under investigation. An extensive review by Wyszynski and Beaty (1996) pointed out the deficiencies in previous case-control studies in the search for environmental factors in human orofacial clefts, which might explain the lack of conclusions on this issue. Recent studies by Hwang et al (1995) and Shaw et al (1996) combined the studies of both genetic and environmental factors, demonstrating an interaction between maternal smoking and risk allele of the transforming growth factor alpha (TGFA) in the fetus in determining the risk for CLP.

The frequently observed clustering of CLP cases in families points to the importance of genetic predisposing factors. However, the exact mode of inheritance of CLP is still unclear. Studies based on different sampling and analytical methods have postulated several models.

Mode of Inheritance

By observing familial inheritance patterns of CLP, Fogh-Andersen (1942) proposed a monogenic model with reduced penetrance. Based on the non-Mendelian recurrences of CLP in families, Fraser (1970) suggested that the best fitting model should be the multifactorial threshold model (MF/T) (Figure 8). MF/T was developed by Falconer (1965) to describe diseases with complex etiologies involving both genetic and environmental components. According to this model, susceptibility to CLP is contributed by multiple factors (genetic and environmental), and when these contributing factors accumulate up to a certain threshold value in an individual, cleft occurs. The model also predicts that the more severely affected individuals will have a higher proportion of affected relatives because they accumulate more predisposing factors beyond the threshold compared to the mildly affected ones. The problem of gene mapping in such a model is the contribution of individual factors e.g. one of the genes, might not be significant enough to be detected.

Support for the MF/T model was found when the relative recurrent risk in a group of 424 three-generational CLP families was measured (Carter et al 1982). However, when the same group of families was analyzed with the complex segregation analysis (Marazita et al 1986), the MT/F model was clearly excluded, instead the best fitting model was an autosomal major gene inheritance plus multifactorial contributions.

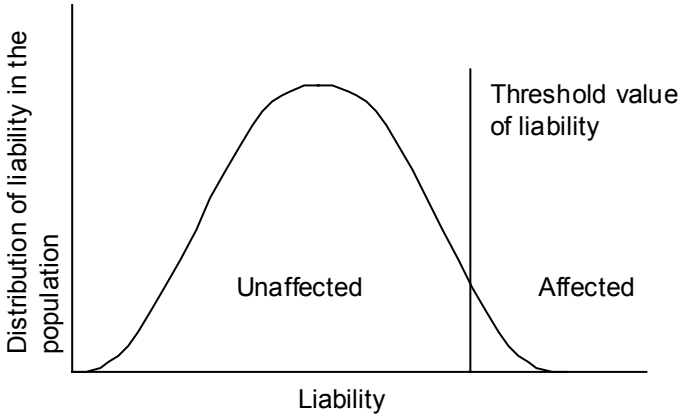

Figure 8. The multifactorial threshold model (MF/T). Multiple factors contributing to the liability of a certain disease are distributed among the general population. The individuals in whom the liabilities have cumulated above a certain threshold became affected.

Using complex segregation analysis, similar results supporting a major gene inheritance in CLP were obtained in groups of Danish (Marazita et al 1984) and Chinese families (Melnick et al 1986). Pietrzyk et al (1985a) also suggested that the best fitting inheritance model for CLP was autosomal dominant with reduced penetrance, although the importance of environmental factors became obvious when the relative recurrent risk of CLP was analyzed (Pietrzyk et al 1985b).

A complex segregation analysis of 79 CLP families by Hecht et al (1991a) showed that although the MF/T model was consistent with the data, the high heritability in the families could best be explained by a major locus with reduced penetrance. The study established a set of inheritance parameters for CLP, which have been widely adopted by different groups in the linkage studies of CLP families.

Mitchell and Risch (1992) re-analyzed the mode of inheritance in several sets of CLP families by considering the power from different kinds of statistics and the effect of multiple factors including gender and severity of cleft. Only by applying the relative risk analysis on monozygotic twins and distant relatives was the power high enough to differentiate between various modes of inheritance. The results showed that MF/T and a model of multiple interacting loci were more appropriate for CLP, and it was concluded that each locus contributed less than a six-fold increase of relative risk to first-degree relatives. Using a similar approach, Farrall and Holder (1992) also suggested that a major gene with an oligogenic background contributed to a relative risk of nine in CLP. In such a case, a sample of 50 affected sib pairs would have 83% chance to map that major gene. After analyzing a larger group of families, Mitchell and Christensen (1996) restated that a single locus could not contribute to more than three fold of relative risk in CLP. According to this model, using a non-parametric linkage approach, 300-500 affected sib pairs would be required to detect the CLP susceptibility gene.

Candidate Genes in CLP

Although a consensus for the mode of inheritance of CLP has not been reached, several studies employing different strategies including linkage and association analyses were able to identify a number of susceptibility loci in CLP (Table 2). The results from some of these studies contradict to each other and until now, the gene(s) responsible for CLP have not yet been identified.

Orofacial Cleft 1, OFC1 (OMIM 119530)

The first positive linkage result in CLP was reported by Eiberg et al (1987) who showed that the blood clotting factor F13A1 on chromosome 6p23 was linked to a group of mixed CLP and isolated cleft palate families. Although the result was not supported by another linkage analysis (Hecht et al 1993), further evidence of a gene for CLP on 6p23 was provided when constitutional balanced translocations and deletions involving 6p25-p23 were found in three patients with orofacial clefts respectively (Davies et al 1995). A recent linkage analysis in a group of Italian families also supported the involvement of the 6p23 region (Scapoli et al 1997).

Orofacial Cleft 2, OFC2 (OMIM 602966)

An association between CLP and specific allelic variants of the transforming growth factor alpha gene (TGFA) locus in chromosome 2p13 has been observed in several studies of different populations (Ardinger et al 1989, Holder et al 1992, Chenevix-Trench et al 1992, Jara et al 1995) and in a linkage disequilibrium study (Feng et al 1994). Although the association of TGFA alleles with CLP has been repeatedly demonstrated in population based studies, this region was excluded by several linkage studies in CLP families (Hecht et al 1991b, Vintiner et al 1992 and Wyszynski et al 1997a). However, when a group of Italian CLP families that has already been linked to 6p23 was analyzed, evidence of linkage to 2p13 was also found (Pezzetti et al 1998). The authors suggested that this locus (*OFC2*) interacts with *OFC1* in causing CLP in this group of families.

Orofacial Cleft 3, OFC3 (OMIM 600757)

Linkage of CLP to chromosome 19q13 was first reported by Stein et al (1995) and was substantiated by linkage disequilibrium findings (Wyszynski et al 1997b). A recent linkage analysis in a group of Italian CLP families also supported the region (Martinelli et al 1998).

Other Candidate Loci

Chromosome 4q is another candidate region for CLP since suggestive linkage to 4q25-q31 has been shown in a 5-generationed CLP family by Beiraghi et al (1994). Chromosomal abnormalities in patients with multiple malformations including cleft lip and palate have also been reported (Yu et al 1981, Najafzadeh et al 1983).

In addition to the candidate loci, a number of candidate genes are implicated in CLP, for example MSX1 (homologue of drosophila muscle segment homeobox 1) at

4p16.1, PAX9 (paired box gene 9) at 14q12-q13, TGFB3 (transforming growth factor beta 3) at 14q24 and RARA (retinoic acid receptor alpha) at 17q12. Until now pathogenic mutations in these candidate genes have not been demonstrated in CLP patients, therefore it is still unclear how these genes contribute to the susceptibility of CLP. The conflicting results obtained in different studies also indicate that CLP is unlikely to be a single gene disorder.

Table 2. Candidate loci in CLP. OFC1=orofacial cleft 1, OFC2=orofacial cleft2, OFC3=orofacial cleft 3, VWS=van der Woude syndrome, MSX1=homologue of drosophila muscle segment homeobox 1, PAX9=paired box gene 9, TGFB3=transforming growth factor beta 3, RARA=retinoic acid receptor alpha.

Symbol	Location	Evidence for involvement in CLP
OFC1	6p23	Chromosomal abnormalities, linkage
OFC2	2p13	Association, linkage
OFC3	19q13	Linkage, transmission disequilibrium
VWS	1q32	Syndromic cleft
	4q25-q31	Association, chromosomal abnormalities, linkage
MSX1	4p16	Animal models, association, linkage disequilibrium
TGFB3	14q24	Animal models
PAX9	14q12	Animal models, chromosomal abnormalities
RARA	17q12	Animal models, association

Familial Isolated Hyperparathyroidism (FIHP)

The calcium homeostasis in human is regulated by mainly parathyroid hormone (PTH), calcitonin and vitamin D involving mainly parathyroid glands, kidney, bone and gastrointestinal tract. The parathyroid hormone, which is secreted by the four parathyroid glands, increases the extracellular Ca++ level. When parathyroid glands become tumors, they secrete PTH excessively causing hypercalcaemia, a condition called hyperparathyroidism (HPT). The tumors maybe in the form of solitary adenoma, multiglandular hyperplasia and rarely carcinoma and all are treated by surgery. The majority (90%) of hyperparathyroidism are sporadic cases with unknown etiology and they are usually caused by solitary adenoma. The other 10% are hereditary which are associated with a number of syndromes such as multiple endocrine neoplasia type 1 (MEN 1) and hyperparathyroidism-jaw tumor syndrome (HPT-JT).

Familial HPT occurs commonly as part of the multiple endocrine neoplasia syndrome type 1 (MEN 1, OMIM 131100), which is an autosomal dominant endocrine tumor syndrome characterized by tumors of the parathyroids, the enteropancreatic endocrine tissues, and the anterior pituitary. MEN 1 was mapped to 11q13 and the gene has been identified (Chandrasekharappa et al 1997, ECM 1997). Primary HPT is the most frequent manifestation of MEN 1 and invariably involves multiple glands.

The other HPT-associated disease is the hyperparathyroidism-jaw tumor syndrome (HPT-JT) which is an autosomal dominant condition characterized by recurrent parathyroid adenoma/carcinoma, fibro-osseous tumors of the mandible and/or maxilla, Wilms tumor, renal hamartomas and cysts. HPT-JT was shown unlinked to MEN1 (Jackson et al 1990) and later mapped to 1q21-q32; and the gene was designated *HRPT2* (OMIM 145001) (Szabo et al 1995). HPT in HPT-JT is usually caused by a single adenoma and men are predominantly affected (sex dependent penetrance). Although HPT in HPT-JT can be treated by removal of the adenoma, some HPT-JT patients later develop a second adenoma; and parathyroid carcinoma has been reported (Dinnen et al 1977, Kakinuma et al 1994).

To date, over 100 families with isolated HPT without other endocrinopathies have been described (Huang et al 1997). Familial isolated hyperparathyroidism (FIHP) is characterized by hypercalcaemia, high PTH levels, and uni- or multi-glandular parathyroid tumors. It has been described as a distinct condition unrelated to other multiple endocrine neoplasia syndromes, and the putative gene designated as *HRPT1* (OMIM 145000) (Wassif et al 1993). However, supports for FIHP as a variant of the multiple endocrine tumor type 1 (MEN1) has been found by linkage and mutation analyses in two large families (Kassem et al 1994, Teh et al 1998, Kassem et al 2000). Notably, these two families present with multi-glandular disease. It is therefore unknown if FIHP with solitary adenoma share the same etiology.

AIMS OF THE STUDIES

The aims of the present studies were to dissect the genetic basis of syndromic and non-syndromic cleft lip and palate by using genetic linkage analysis that was first applied to the mapping of a disease locus in families with a predisposition to hyperparathyroidism. The specific aims of these studies are:

1. To assign the chromosomal localization for the disease locus in two families with isolated hyperparathyroidism (FIHP).

2. To characterize loss of heterozygosity in tumors from families with FIHP and the hyperparathyroidism-jaw tumor (HPT-JT) syndrome assigned to the *HRPT2* locus in 1q21-q32.

3. To study linkage and microdeletions in families with the van der Woude syndrome with regard to the VWS locus in 1q32-q41.

4. To determine the involvement of a modifying locus in 17p11.2-11.1 for the cleft palate phenotype in VWS families.

5. To clinically characterize a family with signs of the popliteal pterygium syndrome (PPS), and to study the possible involvement of the 1q VWS locus.

6. To study the importance of candidate loci on chromosomes 1, 2, 4, 6, and 19 which have been implicated in familial non-syndromic cleft lip with or without cleft palate (CLP).

MATERIALS AND METHODS

Families

I. Families with isolated hyperparathyroidism and hyperparathyroidism-jaw tumor syndrome

Three families were included in this study (Fig.1, Paper I). Families 1 and 2 were Caucasian residing in Sweden and Australia respectively. Six and four members from each family were diagnosed with primary hyperparathyroidism without any other associated endocrinopathies. They were all treated by parathyroidectomy. On further investigation, there were no signs of multiple endocrine neoplasia type 1 (MEN1), jaw or renal tumors. In Family 3, all four affected siblings had parathyroidectomy and one was diagnosed with parathyroid carcinoma. Multiple renal cysts were found in two of the affected individuals.

II. Swedish families with van der Woude syndrome

By comparing records from the cleft palate team in Stockholm and the national malformation registry, four Swedish patients fulfilling the criteria for VWS were identified. At joint-consultation sessions, the patients and their family members were interviewed and examined by members of the team. Two of the patients were found to be familial cases. In Family 1 (Figure 1, Paper II), five family members were affected with VWS, and in Family 2 (Figure 2, Paper II) four were affected. The other two VWS patients were confirmed to be isolated cases, as extensive family studies failed to identify other affected members. In total, 27 individuals were available for the genotype analysis.

III. Finnish families with van der Woude syndrome

All patients with orofacial clefts in Finland are referred to the National Cleft Center in Helsinki. The VWS patients in this study were ascertained by hospital records at the Center. The diagnosis of VWS in the families was based on clinical examination, medical records, telephone interviews and information from other family members. Five families were available for this study (Figures 1-3, Paper III). In total, 56 individuals (25 affected) were available for the genotyping.

IV. Swedish family with features of popliteal pterygium syndrome

A database search for cleft lip and palate patients with associated malformations was performed through the Swedish Medical Birth Registry and the National Malformation Registry. Five multi-generation families with cleft lip and palate with associated malformations were selected for interview and all available family members were clinically examined. During the examination of one family, a boy reported to have cleft lip and palate and syndactyly, was found to carry signs related to PPS. Similar features were also found on his mother, who was reported to have cleft lip and palate. Other available family members were clinically examined and found to be unaffected (Figure 1, Paper IV).

V. Swedish families with non-syndromic cleft lip with or without cleft palate

All newborns with cleft lip and palate in the city of Stockholm and the greater Stockholm area are referred to the cleft palate team of Stockholm. Cleft lip and palate patients in this study were identified by records of the team and were compared with hospital records and the Malformation Registry of the National Board of Health and Welfare in Sweden. All participating families were interviewed and examined by members of the team. Those families having two or more cases with cleft lip and palate (multiplex families) were selected. Cases belonging to known syndromes or having a history of teratogen exposure were excluded, as well as those associated with additional malformations. The affection status of cleft lip and palate in most individuals was confirmed by clinical examination except that of a few unaffected family members by telephone interview. Efforts have been made to ascertain the status of the unaffected individuals but the possibility of microform clefts or submucous cleft palate could not be eliminated.

To date, more than 50 families have been recruited, in which the majority were of Swedish origin while a few were immigrant families. Since this study focused on non-syndromic cleft lip and palate (CLP); therefore, those families having members with isolated cleft palate were excluded. Finally, 19 CLP families of Swedish origin were available for genetic analysis. In total, 109 individuals were included in the genotyping, among which 44 were affected with CLP (Figure 1, Paper V).

Methods

Polymerase chain reaction (PCR) and Electrophoresis

The studies performed in Paper I to Paper V were approved by the local ethical committees. After obtaining informed consent from the participating individuals, peripheral blood samples were collected. In Paper I, paraffin-embedded parathyroid tissue blocks from the parathyroidectomy were collected for histochemistry and loss

of heterozygosity (LOH) analyses. DNA purification was done by Proteinase K digestion with standard methods (Sambrook 1989). In general, the DNA samples were analyzed by PCR amplification of polymorphic microsatellite markers (Figure 3). At each genetic locus, polymorphism was revealed by size-fractionation of the PCR products in polyacrylamide gel electrophoresis. The genetic markers used in the individual studies are detailed in the corresponding papers.

In Paper I and II, PCR with radioisotope labeling and manually operated electrophoresis were used. After PCR amplification of the DNA samples, the radioactive PCR products were denatured and loaded onto a polyacrylamide gel. High voltage was then applied to the gel and the PCR products migrated along the gel. The speed of migration for each product depended on its molecular weights. After electrophoresis, the gel was treated with acetic acid, washed, dried and exposed to a radiographic film. The exposure time varied from a few hours up to several days depending on the strength of the radioactive signals. The allele scoring was performed manually by comparing to a standard size marker loaded adjacent to the samples.

From Papers III to Paper V, PCR using fluorescent labeled primers were employed instead of radioisotope. In each PCR, the forward primer was labeled at the 5' end with fluorescent dye, which can be of different chemistries e.g. 6-FAM, HEX or TET. A fourth dye e.g. TAMRA was incorporated in a separate DNA mixture as size standards. When excited by laser these dyes will emit spectrums of light with different wavelengths (Figure 9).

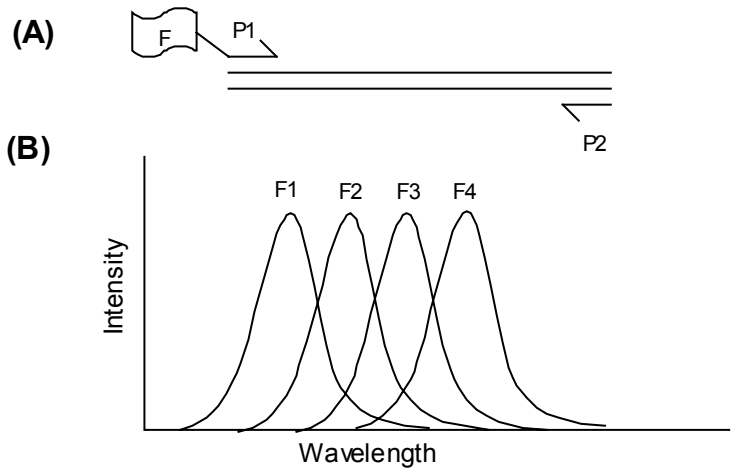

Figure 9. Fluorescent-labeling of PCR primer. (A) The forward primer P1 is labeled with a fluorescent dye F. (B) Fluorescent dyes with different chemistries (F1-F4) emit light with different wavelengths when excited by laser.

The electrophoresis was performed on an automated laser-fluorescent sequencing machine (ABI 377, PE Biosystems, CA, USA). The principle for the detection of the DNA fragments is by laser excitation of the fluorescent-labeled PCR products on the gel. The emission is picked up by a detector and the different colors are separated by a virtual filter. The digitalized signal is then collected by computer in real time (Figure 10). The collected data is analyzed by the GeneScan program (PE Biosystems) to

reveal the alleles from individual PCR products. The current system is designed to distinguish four different colors; therefore at least three different PCR products plus an internal size marker could be run on the same lane during electrophoresis (Table 1, Paper V). When PCR products of different size ranges are pooled, more samples can be mixed into one electrophoresis experiment.

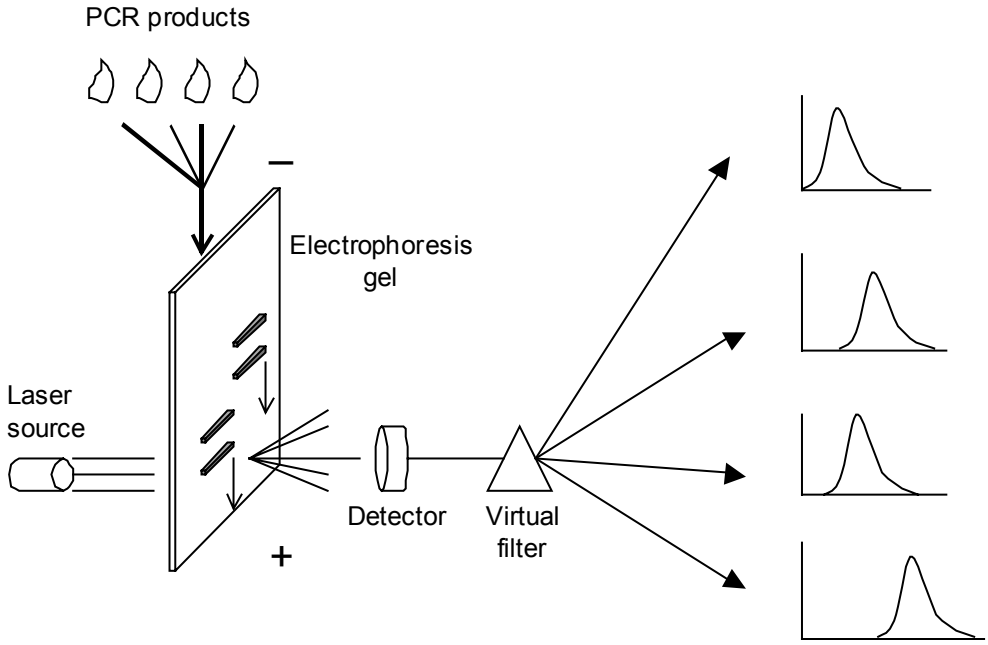

Figure 10. Laser-fluorescent electrophoresis. PCR products with different labeling are mixed and loaded onto the electrophoresis gel. When the fragments migrate along the gel under high voltage, they are excited by the laser and emit light of mixed wavelengths. The detector picks up the signal and feeds into a virtual filter, which electronically separates the different wavelengths. The digital signals are collected by computer for further analysis.

Statistical Analyses

Familial isolated hyperparathyroidism and hyperparathyroidism-jaw tumor syndrome (Paper I)

Families 1 and 2 were analyzed together as their clinical features were confined to FIHP. Markers from the 11q13 *MEN1* and the 1q21-q32 *HRPT2* regions were analyzed. Two-point linkage analysis was performed with the FASTLINK computer program (Cottingham et al 1993) and multi-point linkage analysis with the GENEHUNTER program (Kruglyak et al 1996). A conservative approach was used in the linkage analysis where all the unaffected individuals except two were labeled as having an unknown disease status. Those two individuals (II-3 and II-5, Family 1) labeled as unaffected were over 80 years old and repeated screenings for hyperparathyroidism were negative.

Loss of heterozygosity (LOH) analysis was performed by analyzing paired constitutional and tumor DNA samples using PCR amplification of markers. In this study, markers from 1q21-q32 and 11q13 were used. To test for LOH, the genotypes from polymorphic loci are compared between matched pairs of constitutional and tumor DNA. LOH is defined as at least 50% decrease in the intensity of one of the alleles in the tumor DNA when compared to the constitutional counterpart (Figure 11).

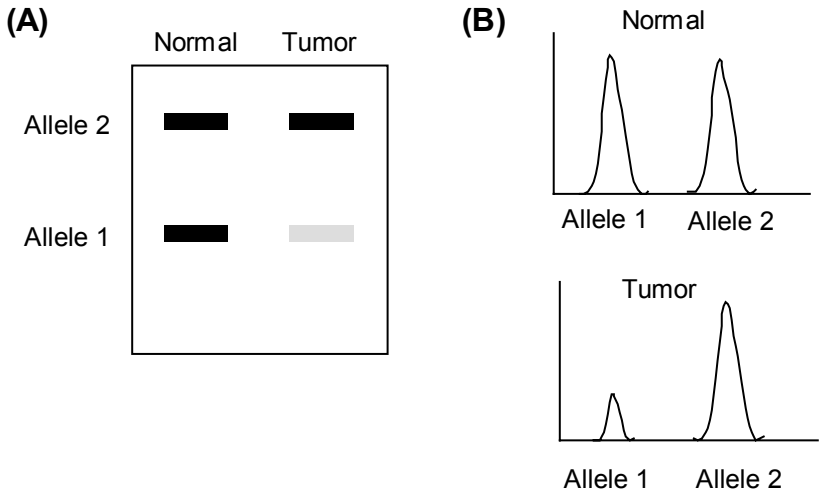

Figure 11. Loss of heterozygosity (LOH). (A) On an electrophoresis gel, a tumor with LOH will present a disappearance of one of the bands corresponding to the normal allele. (B) The same samples when analyzed with fluorescent PCR, a tumor with LOH will show a diminished peak corresponding to the normal allele.

van der Woude syndrome, VWS (Paper II and III)

All affected individuals were screened for the presence of a microdeletion in the 1q32-q41 VWS region. After genotyping by PCR using microsatellite markers from the region, those individuals showing homozygous genotype were compared to the genotypes of their parents. The disappearance of a parental allele would imply microdeletion involving that marker (Figure 12).

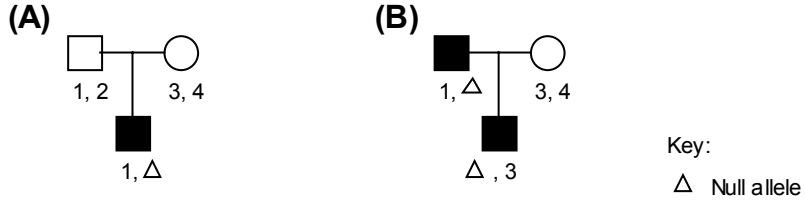

Figure 12. Microdeletion analysis. In both (A) and (B), the affected child is missing one allele from one of the parents, which indicates microdeletion involving that genetic marker. In (A) the null allele has occurred *de novo*, while in (B) it has been inherited from the father.

For the linkage analysis of VWS, the disease was modeled as autosomal dominant with 95% penetrance (Sander et al 1993). The disease gene frequency was taken as $1.5x10^{-5}$ (Rintala and Ranta 1981) and a mutation rate of $1.8x10^{-5}$ (Burdick et al 1985). Two-point linkage analysis was performed with the MLINK program of the LINKAGE 5.1 package (Lathrop et al 1984). Multi-point linkage analysis in Paper III was performed with the GENEHUNTER program (Kruglyak et al 1996).

For the study of a possible modifying locus in chromosome 17p11.2-p11.1, the cleft palate phenotype was modeled as an autosomal dominant trait with a penetrance of 70% and a disease gene frequency of 0.001 (Sertié et al 1999). In this analysis, only those individuals having cleft palate were considered affected, regardless of the presence of lip pits.

Popliteal pterygium syndrome, PPS (Paper IV)

Cytogenetic examination was performed for both the affected mother and son by karyotyping of cultured white blood cells. In each case, 30 cells in metaphase were examined at 400 to 500-band levels by G-banding. In addition, microdeletion analysis with markers from the 1q32-q41 VWS region was performed.

Non-syndromic cleft lip with or without cleft palate, CLP (Paper V)

Multi-point linkage analysis for the Swedish CLP families was performed with the GENEHUNTER computer program (Kruglyak et al 1996) at candidate regions on chromosome 1, 2, 4, 6 and 19 respectively. CLP was modeled as autosomal dominant with a penetrance of 0.3, a disease gene frequency of 0.001 and a phenocopy rate of 0.001 (Hecht et al 1991a). Since a higher penetrance was observed in some families, the linkage analysis was repeated with a penetrance of 0.7. Similarly, a recessive model was included to account for such possibilities in other families. The analyses were also performed with the assumption of genetic heterogeneity in the group of families. Finally, a non-parametric linkage analysis using the NPL (non-parametric linkage) was included. The allele frequencies of individual markers were estimated from 47 unrelated individuals from within the sample group.

RESULTS AND DISCUSSION

I. Familial isolated hyperparathyroidism and Hyperparathyroidism-jaw tumor syndrome

Linkage to the *MEN1* region in 11q13 was excluded in all three families by haplotype analysis and the negative LOD scores of less than -2 obtained from the linkage analysis. Two-point linkage analysis for Families 1 and 2 at 1q21-q32 produced positive LOD scores with most of the markers (Table 1, Paper I). Linkage of these two families to 1q21-q32 was confirmed by the maximum two-point LOD scores of 3.1 at D1S222, 3.4 at D1S249 and multipoint LOD scores of higher than 3 for the region D1S222 to D1S504. Recombination events in the families placed the disease locus at a 37-cM region between D1S215 and D1S249, overlapping with the *HRPT2* locus. Three parathyroid tumors from Family 1 showed LOH in 1q21-q32. In all cases, the wild-type alleles from the unaffected parent were lost.

In Family 3, all affected members shared the same haplotypes for 1q21-q32 from the father. The parathyroid carcinoma from individual II-5 showed LOH of the maternal alleles in the region, which together with the LOH results from Families 1, suggested that *HRPT2* is a tumor suppressor gene.

This study demonstrated the application of genetic analyses in determining the genetic location of a hereditary tumor syndrome and provided insight into its genetic etiology. Both linkage and LOH analyses of the FIHP families supported the hypothesis that a subset of FIHP is a variant of HPT-JT and the gene involved is a tumor suppressor gene. Recently, mutations of the *MEN1* gene in 11q13 were found in two FIHP families (Teh et al 1998, Kassem et al 2000), demonstrating that some FIHP families are MEN1 variants. These studies exemplified the genetic heterogeneity of FIHP and emphasized the importance of linkage analysis as a tool for genetic classification of human diseases. Since HPT-JT and MEN1 are associated with tumors in different organs, correct genetic classification of patients with hyperparathyroidism is essential for the proper screening of associated tumors.

II. Swedish van der Woude syndrome

Clinical examination of the two isolated patients revealed typical VWS features of lower lip pits and bilateral complete cleft lip and palate (Table 1, Paper II). However, the clinical features of the familial cases were more variable. All affected members in Family 1 have lip pits but only two have cleft lip and palate. Hypodontia was found in all the affected members in Family 2 but only two members have lip pits (Table 1, Paper II).

Microdeletion of the VWS region was not observed either in the isolated or in the familial VWS patients. Linkage analysis with microsatellite markers from within the VWS region at 1q32-q41 revealed positive two-point LOD scores in both families ranging from 2.56 to 2.88. A maximum cumulative LOD score of 3.75 at zero

recombination was obtained with the combined haplotype from D1S491 and D1S205, which confirmed linkage of the two Swedish VWS families to 1q32-q41. A recombination event between markers D1S471 and D1S491 was observed in individual III:2 of Family 1, which placed D1S491 telomeric to D1S471.

The inter- and intra-familial clinical variability in VWS illustrated the challenge in the diagnosis of this syndrome. A number of VWS cases might have been overlooked as microform lip pits accounted for 35% of all lip pits (Rintala and Ranta 1981). The importance of these VWS features on genetic counseling should also be stressed as the penetrance of VWS is close to 100%.

Negative results from the test of microdeletion at 1q32-q41 in the VWS patients implied that it is not a common mechanism for VWS. The two-point linkage analysis in this study utilized a combined haplotype from tightly linked markers to increase their informativeness and confirmed linkage of the Swedish VWS families to 1q32-q41. The observation of recombination between D1S471 and D1S491 clarified the genetic locations of these two closely linked markers, which was later confirmed by the genomic sequencing of the VWS region (Schutte et al 2000). Chromosome 1q32-q41 so far was the only VWS locus as shown by this and other linkage studies (Murray et al 1990, Sander et al 1993, 1995, Beiraghi et al 1999, Houdayer et al 1999, Sertié et al 1999).

III. Finnish van der Woude syndrome

Clinical data from the Finnish VWS patients revealed cleft palate or submucous cleft palate in 90% of the cases (Table 1, Paper III). Varied expressivity of VWS was found in the families and was greatly reduced in Family 60 (Figure 2, Paper III).

Linkage to 1q32-q41 was confirmed in three of the families (Table 2, Paper III). One large family (Family 57) was found unlinked to 1q32-q41, which provided the first evidence of genetic heterogeneity in VWS. Family 62 was uninformative for all nine markers from the 1q VWS region. Linkage to this region was excluded when additional markers around the region were typed. In two 1q-linked families (60 and 61), a common haplotype was observed and in an unaffected individual from Family 60, a recombination was observed. These evidences tentatively refined the VWS locus to less than 200 kb (Figure 4, Paper III).

During the genotype analysis, the inheritance of D1S3753 did not follow a Mendelian pattern, suggesting the presence of an undetected (null) allele or deletion. The null allele was found in both affected and unaffected individuals, meaning that it may not be related to VWS. Five control families with no history of orofacial clefts were therefore tested with this marker and the null allele was present as well, thereby excluding the deletion of this marker as a cause of orofacial clefts.

When the Finnish families were tested for the modifying locus at 17p11.2-p11.1, multi-point LOD scores of less than –2 were found in four families (Table 3, Paper III). When all five families were considered together, a 20-cM region at 17p11 could be excluded. The two Swedish families in Paper II were also tested for this region and negative LOD scores were obtained (data not shown).

The Finnish population is well known for its unique and homogenous genetic background (Peltonen et al 1999). More than 30 diseases were found to be enriched or having unique clinical features in the Finnish population. In many of these diseases, both distinct phenotypic and genetic profiles have been revealed. Therefore, families from Finland are valuable resources for genetic studies. The high prevalence of cleft palate in the Finnish VWS families also pointed to the renowned Finnish disease heritage.

One of the purposes of analyzing the Finnish VWS families was to refine the VWS locus at 1q32-q41. This was tentatively attained by observing the recombination event and the common haplotypes in two families. We remained cautious about the recombination due to the low expressivity of VWS in the family. The significance of the shared haplotypes also requires confirmation by genealogical studies.

The most important finding from the Finnish families was the evidence of genetic heterogeneity in VWS, which might have an impact on the genetic studies of this allegedly monogenic condition. Our results showed that VWS might be caused by more than one gene. In order to identify this second VWS locus in the Finnish families, a genome wide linkage analysis is underway using the high throughput fluorescent based PCR strategy.

The Brazilian VWS family studied by Sertié et al (1999) was characterized by a clustering of cleft palate. Similar clustering was observed in the Finnish VWS families, which made them suitable to test for the modifier locus for cleft palate at 17p11.2-p11.1. Our results demonstrated that the 17p region does not contain a major modifier gene for cleft palate in the Finnish or Swedish VWS families. Further studies in other families would clarify the role of this locus in the susceptibility of cleft palate in VWS.

IV. Popliteal pterygium syndrome

The proband (IV:2, Figure 1, Paper IV), reported to have cleft lip and palate and syndactyly, was previously affected with lower lip pits which were surgically repaired. Intra-orally, prominent buccal frenums were noticed, which were remnants of intermaxillary tissue bands after their surgical removal. Surgical scars were found on the second and third fingers showing previous correction of syndactyly. The great toes were short, broad and laterally deviated. A piece of skin overgrowth was found on the great toe nails (Figure 2, Paper IV). Varying degrees of soft-tissue syndactyly and hypoplasia were observed on the other toes. The mother (III:3, Figure 1, Paper IV) presented with similar orofacial and great toe malformations but without syndactyly of the fingers. Based on the cleft lip and palate, lip pits, syndactyly and the toe malformation, the diagnosis of PPS was made (Hunter 1990, Gorlin et al 1990).

Cytogenetic analysis of the mother and son did not reveal any constitutional chromosomal abnormalities. The proband was heterozygous for all five markers at the VWS region in 1q32-q41 (Figure 1, Paper IV), suggesting that microdeletion of the region was unlikely.

The well established systems of national registries for birth and malformation in Sweden allowed the ascertainment of patients for this study. On the other hand, our results also demonstrated the importance of careful clinical examination and integration of knowledge in clinical diagnosis. Our attempt to investigate the genetic basis of PPS in relation to VWS was based on their clinical similarities. Results from the cytogenetic and microdeletion analyses showed that PPS in this family was not caused by chromosomal abnormalities or microdeletion of the 1q VWS region. However, linkage to the VWS region could not be excluded, as the size of this family was too small for linkage analysis. It is possible that the affected mother (III:3, Paper IV) carries a *de novo* germline mutation which was then transmitted to her affected son (IV:2, Paper IV) and giving rise to the PPS phenotype in both individuals. A recent study showing suggestive linkage to the VWS locus in three PPS families (Lees et al 1999) supported our hypothesis. These findings not only strengthened the possible link between VWS and PPS, but they also hinted on a broader clinical spectrum of these two syndromes.

V. Non-syndromic cleft lip with or without cleft palate

Among the 19 Swedish CLP families, four were with isolated cleft lip, 10 were cleft lip with cleft palate and the rest with both kinds of cleft (Figure 1, Paper V). In addition, a mixture of unilateral and bilateral cleft was observed in seven families and no consistent pattern was noticed.

Multi-point LOD scores generated from the candidate regions on chromosomes 1, 2, 4, 6 and 19 under the autosomal dominant mode of inheritance with a disease penetrance of 0.3 were less than –2, except in 6p23 which were less than –1 (Figures 2-6, Paper V). LOD scores from these regions were more negative when a disease penetrance of 0.7 was assumed. In the recessive model, slightly positive LOD scores were obtained for chromosomes 1, 2 and 6 but statistical significance were not reached. Linkage analyses with the dominant and recessive models were re-calculated by assuming genetic heterogeneity but the LOD scores obtained did not reach statistical significance. The non-parametric analysis revealed only non-significant positive NPL scores in 6p23.

These results showed that when the Swedish families were considered as a homogeneous group and calculated with an autosomal dominant mode of inheritance, linkage to all the candidate regions was excluded. The results from this study were consistent with previous studies with similar settings where several candidate regions were excluded (Hecht et al 1991b, 1993, Vintiner et al 1992, Blanton et al 1996, Wyszynski et al 1997a). Taken together, these findings might imply the presence of genetic heterogeneity among the individual groups of CLP families in each study.

The CLP candidates on chromosome 2, 6 and 19 were all derived from linkage analysis of the respective sample groups under the assumption of genetic heterogeneity; therefore, the same assumption was applied to the analysis of the Swedish CLP families. The calculation was performed with the heterogeneity option in the GENEHUNTER program, in which the highest LOD scores under heterogeneity (HLOD) were computed. The HLOD is obtained by maximizing the LOD scores at each position with the probability of a certain proportion of families

(alpha) being linked to the locus in question. The method was derived from the HOMOG statistics (Ott 1986), which evaluates the significance of variations in LOD scores between families. If the differences in LOD between families are small, heterogeneity in the group of families may not be differentiated. The insignificant GENEHUNTER results in this study might be caused by such reason. Therefore, the Swedish CLP families were analyzed with the original HOMOG program (unpublished data). Our results showed small (insignificant) LOD differences between the families, which explained the insignificant GENEHUNTER results.

LOD scores obtained from the recessive mode of inheritance were inconclusive in all regions. However, the analysis with recessive inheritance was hypothetical since the model was not supported by population data. The higher LOD scores obtained with the recessive model compared to the dominant models might not imply it as a better fitting model for CLP. Instead, the inconclusive LOD scores obtained with the recessive model could be interpreted as the results of a lower analytical power when the mode of inheritance was not correctly specified. The insignificant NPL scores obtained also revealed its lower analytical power when compared to the parametric analysis. A recent study showed that even for diseases with complex inheritance and heterogeneity, parametric linkage analysis could provide higher power than non-parametric analysis (Abreu et al 1999). However, the deployment of several analytical approaches in this study was justified not only by the lack of consensus in the mode of inheritance for CLP, it was also in line with the discussion by Lander and Schork (1994) in the genetic dissection of complex traits.

Several conclusions could be drawn from this study. In order to distinguish genetic heterogeneity, families of larger size would be needed to produce more significant LOD scores, either it be positive or negative. To search for CLP susceptibility genes by a non-parametric approach, the total sample size needs to be much bigger (Mitchell and Christensen 1996). However, linkage results from individual families are equally important in terms of excluding linkage. The data obtained from this study could be utilized when candidate genes from these regions become available, so that certain families could be prioritized for mutation analysis.

Until now, the characterization of CLP families in genetic studies focuses on clinical classification of cleft lip, cleft lip and palate and isolated cleft palate. As heterogeneity in CLP is evidenced, a more detailed characterization of the conditions might identify a more homogenous group of CLP families. The importance of ascertainment in isolated CLP cases has been discussed by Tolarova and Cervenka (1998). Recent clinical studies provided new ideas on the clinical characterization of CLP. The study by Gosain et al (1999) revealed a high incidence (36%) of submucous cleft palate in a group of apparently isolated cleft lip cases, in which two-thirds of the submucous cleft could only be diagnosed by nasoendoscopy and digital palpation after general anesthesia. In another study by Martin et al (2000), dehiscence of the orbicularis oris muscle was discovered by ultrasonography in the first-degree relatives of isolated cleft lip patients, which partly explained the apparently low penetrance of CLP. Improvement in the clinical characterization of CLP will greatly enhance future genetic analysis of the condition.

CONCLUSIONS

These studies demonstrate the application of genetic analyses in human diseases:

1. In the two families with FIHP, the hyperparathyroidism trait was mapped to the *HRPT2* locus in chromosomal region 1q21-q32 indicating that FIHP in these families represent a variant of the HPT-JT syndrome.

2. Somatic loss of the wild type alleles for the 1q21-q32 region was demonstrated in tumors from FIHP and HPT-JT families suggesting that the *HRPT2* gene is a tumor suppressor.

3. The VWS locus in 1q32-q41 was confirmed in the Swedish and in three of the five Finnish families. In two of the Finnish families, linkage to the VWS region in 1q was excluded, demonstrating for the first time evidence of genetic heterogeneity in VWS. Microdeletions of the VWS 1q region is not a common cause of VWS in the Swedish and Finnish patients.

4. The suggested locus in the 17p11.2-p11.1 region is not a major modifying locus for the cleft palate phenotype in the Swedish and Finnish VWS families.

5. Microdeletion of the VWS region at 1q32-q41 is unlikely to be the cause of PPS in the Swedish family but it may still be linked to 1q32-q41.

6. The group of Swedish families with CLP is unlinked to the candidate regions at chromosome 1, 2, 4, 6 and 19 and there is no evidence of genetic heterogeneity. This may imply a new locus or loci in the families although linkage in some of the families might be hidden by the insignificant genetic heterogeneity.

FUTURE DIRECTIONS

Our understanding of the molecular basis of early embryogenesis is still limited and the number of genes involved in craniofacial development is yet unknown. The lack of comprehensive knowledge in this area of biology remains one of the obstacles in the identification of genes responsible for developmental defects. In VWS, although positional cloning approach might eventually identify the gene(s), the process could be greatly facilitated if some prior knowledge of the genes in the region were available. The strategy, termed positional candidate gene approach (Collins 1995), employs combined positional cloning and candidate gene analysis for disease gene identification. In the example of VWS, genes or gene fragments isolated from the VWS region could be used to compare sequence homologies and expression patterns in other organisms to select candidate genes for mutation analysis.

Sequencing of the human genome is due to complete in 2003 and a "working draft" covering the majority of the genome will be ready by the end of 2001 (Collins et al 1998). Complete sequencing of the 33.5 million base pairs in the human chromosome 22 (Dunham et al 1999) represents a milestone of the project. These data will become powerful tools for the studies of human diseases by providing the basis for disease gene identification.

Developments in the technologies of molecular genetics are constantly improving the efficiency of human disease gene identification. Robotic handling of reagents is now commonly used to implement the concept of high throughput genotyping. This series of studies illustrated the transition from isotopic to fluorescent labeled PCR, which allowed the rapid genotyping of a large number of samples as shown in Paper V. Optimization of multiplex fluorescent labeled PCR allows simultaneous amplification of several markers. The Center for Medical Genetics of the Marshfield Medical Research Foundation is constantly developing sets of markers for genome screening. Its goal is to develop a set of markers for multiplex PCR that could cover the whole genome in less than 100 PCR experiments.

A recent trend in human gene mapping is the rapid discovery and the development of technologies in typing single nucleotide polymorphisms (SNPs). SNPs are the most common polymorphisms in the human genome, which occurs on average every 1000 bases. Similar to RFLPs, SNPs are bi-allelic; but the difference is SNPs can be typed on solid-state high-density arrays (DNA chips) instead of gel electrophoresis (Wang et al 1998). This new strategy allows large-scale rapid genotyping of SNPs, which also compensates for the relatively low heterozygosity in this type of markers.

When a high-density (e.g. SNP) genetic map and efficient genotyping technology (e.g. DNA chip) are available, analysis of complex diseases such as CLP would be greatly facilitated. Powerful mapping statistics such as the transmission disequilibrium test (TDT) (Spielman et al 1993) could be used in either candidate gene analysis or in genome wide scan (Risch and Merikangas 1996). TDT is similar to a case-control association study, however in TDT the parental genotypes are used as internal controls, thus avoiding the problem of population stratification which occurs in case-control studies. The sample units in TDT are case-parents triplets, which are more straightforward to collect than multiplex families. One drawback of

this method is a sharp decrease in power when the distance between the marker and the disease gene increases. TDT has already been applied in CLP candidate gene studies e.g. Stein et al (1995), Maestri et al (1997), Wyszynski et al (1997b) and Martinelli et al (1998) with varying success. In the future, studies of this kind will benefit from the availability of high-density SNP maps.

The traditional concept of single gene mutations in monogenic diseases is constantly evolving when locus and phenotypic heterogeneities are increasingly observed. For example, locus heterogeneity of FIHP is evidenced when the condition is associated with at least two syndromes (Paper I, Kassem et al 2000) and similarly when a subset of VWS families was shown unlinked to 1q32 (Paper III). Phenotypic heterogeneity might be the explanation for the observed genetic relationship between VWS and PPS (Paper IV, Lees et all 1999). Complex diseases such as CLP are likely to be caused by a number of susceptibility genes, in which a one-to-one correlation between mutation and phenotype might not hold true. In a mouse model of CLP, the susceptibility to CLP was found to be caused by two major loci with epistatic interaction plus three other loci with smaller influence (Juriloff and Harris 1999). Logically the genetic background of human CLP would not be simpler than that. The combination of high throughput genotyping with DNA chips based on dense SNP maps and powerful statistical methods such as TDT might in the future reveal the susceptibility genes for CLP including those with minor effects.

In summary, this series of studies illustrates the application of a few methods of genetic analysis in mapping human disease genes, especially in conditions related to cleft lip and palate. From the results obtained by individual studies, differences in the applicability and limitation of each approach as well as the effects from the types of family material are observed. The identification of human disease genes in most cases is a tedious process, which involves the contribution from different specialties including the clinical, genetic, biological, statistical and computational fields. The goals of genetic analysis in human diseases, however, do not stop at gene identification and functional analysis. An understanding of the molecular basis of human diseases is essential for the effective diagnosis, prevention and treatment of these conditions.

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GLOSSARY

Allele: one of several alternative forms of a gene or DNA sequence at a specific chromosomal location.

Ascertainment: the scheme by which individuals are selected, identified, and recruited for participation in a research study.

CentiMorgan (cM): a unit of genetic distance which is usually equivalent to a 1% probability of recombination during meiosis.

Chromosome: a linear structure found in all nucleated cells consisting of both DNA and proteins. Genes are organized on the strands of DNA. The normal human complement of chromosomes is 46 per cell.

Complex trait: a trait having a genetic component that is not strictly Mendelian (dominant, recessive or sex-linked) and characterized by an increased risk to the relatives of an affected individual as compared with the population.

Crossover: the physical process that results in the exchange of genetic material between two paired chromosomes during a recombination event.

Epistasis: two or more genes interacting with one another in a multiplicative fashion.

DNA chip: a microarray of oligonucleotides or cDNA clones fixed on a glass surface, commonly used in hybridization assay for sequence variation or in profiling gene expression.

Dominant: (in human genetics) describe any trait that is expressed in a heterozygote.

Exclusion mapping: genetic mapping with negative results, showing that the locus in question does not map to a particular location.

Gene: an individual unit of hereditary. It is a specific instruction that encodes a protein or RNA product. Each gene is located on a specific place (locus) on a specific chromosome.

Genetic model: the overall specification of how genes act to influence a given trait.

Genetic marker: polymorphic DNA or protein sequence derived from a single chromosomal location, used in genetic mapping.

Genome: the total genetic complement of an organism.

Genotype: the observed alleles at a genetic locus for an individual.

Haplotype: the linear, ordered arrangement of alleles on a chromosome.

Heterozygous: an individual is heterozygous at a locus if (s)he has two different alleles at that locus.

Homozygous: an individual is homozygous at a locus if (s)he has two identical alleles at that locus.

Identity by descent: two alleles are identical by descent (IBD) when it can be determined with certainty that they have been inherited from a common ancestor.

Identity by state: two alleles are identical by state (IBS) when they share the same state. Alleles that are IBS are not always identical by descent.

Informative meiosis: in linkage analysis, a meiosis is informative if the genotypes in the pedigree allow us to decide whether it is recombinant or not.

Karyotype: a summary of the chromosomal constitution of a cell or person.

Linkage: the tendency of genes or other DNA sequences at specific loci to be inherited together because of their physical proximity on a single chromosome.

Locus: a unique chromosomal location defining the position of an individual gene or DNA sequence.

Locus heterogeneity: determination of the same disease or phenotype by mutation at different loci.

LOD score (z): a measure of the likelihood of genetic linkage between loci. The log (base 10) of the odds ratio for linkage.

Microsatellite: small run (usually less than 0.1 kb) of tandem repeats of a very simple DNA sequence, usually 1-4 bp.

Modifier gene: a gene whose expression can influence a phenotype resulting from a mutation at another locus.

Mutation: a change in the DNA. A mutation occurring in a germ cell is a heritable change in that it can be transmitted from generation to generation.

Penetrance: the probability of expressing a phenotype given a genotype.

Phenocopy: a trait that appears to be identical to a genetic trait but which is caused by non-genetic factors.

Phenotype: the observable characteristics of a cell or organism.

Physical distance: distance between genes or sequences measured in kilobases, megabases or centiRays.

Physical mapping: a method of mapping genes or DNA sequences on a chromosome that does not rely on meiotic segregation (genetic mapping).

Polygenic character: a character determined by the combination of a number of genetic loci.

Polymorphism: the existence of two or more variants at significant frequencies (> 1%) in the population.

Primer: a short oligonucleotide, often 15-25 bases long, which base-pairs specifically to a target sequence to allow a polymerase to initiate synthesis of a complementary strand.

Recessive: a character is recessive if it is manifest only in the homozygote.

Recombinant: in linkage analysis, a person who inherits from a parent a recombination of alleles that is the result of a crossover during meiosis.

Recombination fraction (θ): the frequency of crossing over between two loci.

Restriction fragment length polymorphism (RFLP): a polymorphic difference in the size of allelic restriction fragments because of the presence or absence of a particular restriction site.

Restriction site: a short DNA sequence which is recognized by a restriction endonuclease.

Sib-pair analysis: a form of linkage analysis in which markers are tested for linkage to a disease or phenotypic trait by measuring the extent to which affected sib pairs share marker haplotypes.

SNP (single nucleotide polymorphism): any polymorphic variation at a single nucleotide.

Sporadic case: an individual who is the only member within a pedigree possessing the trait of interest.

Transmission disequilibrium test (TDT): a statistical test of allelic association with controls from within the same family.

Variable number of tandem repeat polymorphism (VNTR): array of tandemly repeated sequences that often vary between people in the number of repeat units.

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