GENE MAPPING OF COMPLEX DISORDERS

Gilles de la Tourette syndrome and Hereditary Paragangliomas.

Peter Heutink

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GEN LOCALISATIE VAN COMPLEXE AANDOENINGEN
Gilles de la Tourette syndroom en erfelijke paragangliomen

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PREFACE

During recent years enormous progress has been made in human genetics. Large numbers of disease genes have been identified and this has opened new perspectives for diagnosis and treatment. For an increasing number of disorders the defective gene product is identified by 'positional cloning'**(Chapter 1)**. This strategy has proven to be very powerful for single gene disorders and the successes have tempted investigators to apply this methodology to more complex disorders. The problems that are encountered for complex disorders are discussed in **Chapter 2.**

The purpose of the research presented in this thesis was to gain insight into the genetics of two complex disorders: Gilles de la Tourette syndrome (GTS) and hereditary paragangliomas. Although both clinically and genetically GTS and hereditary paragangliomas are two completely unrelated disorders, very similar approaches were used to unravel the genetic causes of these disorders **(Chapter 3)**.

Gilles de la Tourette syndrome (GTS) is a neuropsychiatric disorder with unknown etiology. The syndrome is characterized by chronic, multiple, intermittent motor and vocal tics. Affected individuals frequently display associated behavioral problems like obsessive compulsive behavior, coprolalia and echolalia. Sex and age affect the risk of expressing GTS. Analysis of family data is consistent with a single autosomal mode of transmission with incomplete penetrance. The clinical characteristics of GTS are discussed in **Chapter 4.** A simulation study on a large set of families with GTS is described. The outcome of the simulation studies were used to test the power of these families to detect a linkage under the assumption of genetic homogeneity and under the assumption of genetic heterogeneity. The results of the ongoing genome search for the GTS gene are presented here **(Chapter 5)**.

Paragangliomas of the head and neck are mostly benign and slow growing. Familial occurrence of paragangliomas is consistent with an autosomal dominant mode of inheritance with incomplete penetrance. Penetrance is age-dependent and strongly related to the sex of the transmitting parent, since clinical manifestations are expressed exclusively through the paternal line. In **Chapter 6** the clinical characteristics of hereditary paragangliomas are described. For hereditary paragangliomas an autosomal dominant mode of inheritance was assumed. Expression of the phenotype is influenced by genomic imprinting. Strong evidence for the proposed mode of inheritance was found and the responsible gene was localized to chromosome 11q23-qter. Additional fine mapping of the responsible gene is presented **(Chapter 7)**.

In **Chapter 8** the results obtained in the mapping studies on both disorders are discussed.

CHAPTER 1

POSITIONAL CLONING OF DISEASE GENES

1. POSITIONAL CLONING

During the last decade enormous progress in human gene mapping has been made. For more then 700 human disorders a chromosomal location has been determined (1) and for an increasing number of these disorders the encoding genes and gene mutations are being identified, opening new perspectives in early diagnosis, carrier detection, genetic counseling and prenatal diagnosis. Also the characterization of these genes enables a better understanding of the pathogenetic mechanisms of many genetic disorders (2,3). The development of recombinant DNA techniques has been a major driving force in this progress. Until a few years ago the molecular analysis of candidate genes in inherited disorders of man proceeded mainly on the basis of identification and characterization of the gene product. This requires knowledge of the responsible protein defect and the purification of the normal encoded protein. Antibodies raised against the purified proteins can be used to isolate complementary DNA (cDNA) clones from an expression library. Alternatively, on the basis of the amino acid sequence of the (normal) protein, oligonucleotides can be synthesized to screen cDNA libraries for the corresponding gene. The corresponding messenger RNA (mRNA) sequence can be used to localize and isolate the complete gene from genomic DNA. Detailed descriptions of the underlying biochemical defect and recognition of the (mutant) proteins contributed to the success of this approach, often referred to as functional cloning (4-7) (Fig 1).

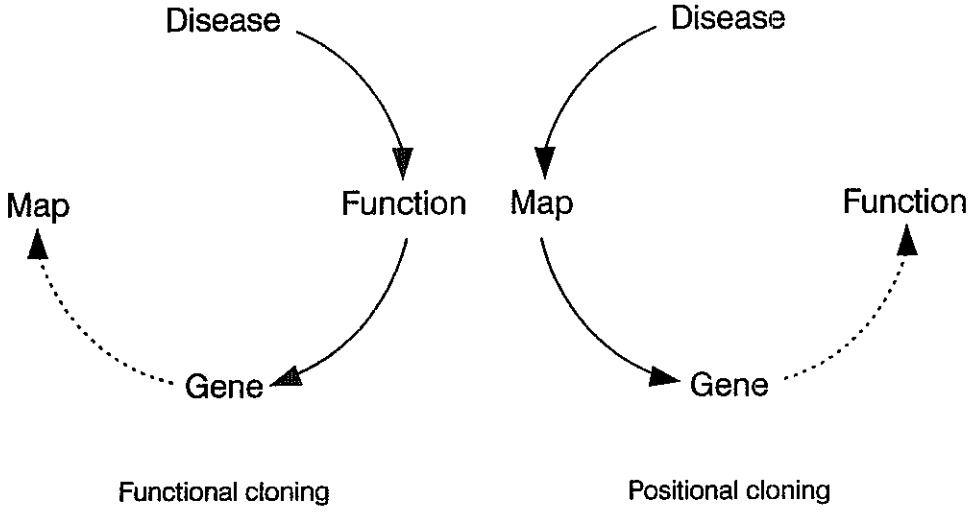

Fig 1. Contrasting methods of gene identification (Adapted from Nature Genet 1;3-6 1992).

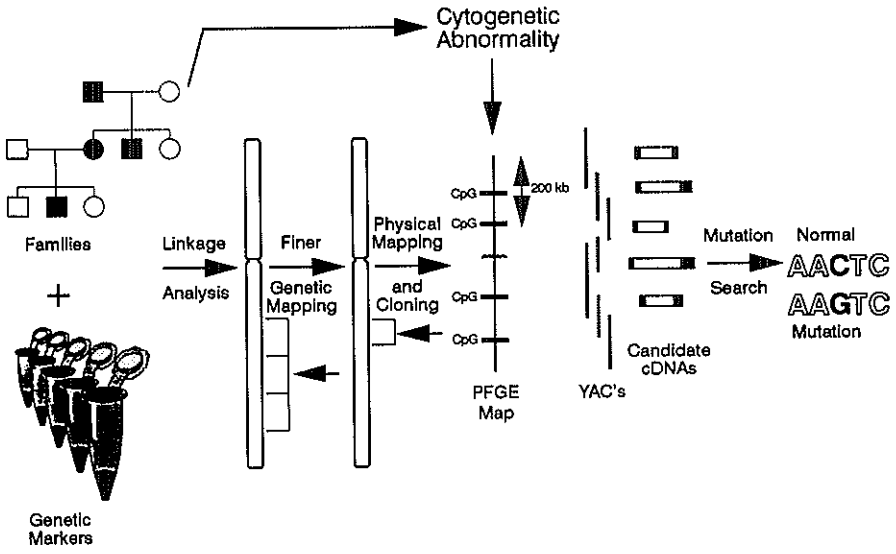

Fig 2. Scheme of the approach generally utilized for positional cloning. adapted from Nature Genet 1:3-6 (1992).

However, for almost 90% of the inherited disorders in man the responsible protein defect is not known. A mapping strategy has been developed which is referred to as 'positional cloning' (5) and which enables the identification of a gene without prior knowledge about its function (Fig 1 & 2). The first step in this strategy is to establish the chromosomal localization of the gene responsible for the disorder (4,5,8). The study of chromosomal aberrations in patients can be of great help in the search for a chromosomal localization. When no chromosomal aberrations are associated with the disease the gene must be mapped by linkage analysis. For this approach families have to be collected in which the disease gene is segregating. After the ascertainment of family material the linkage analysis is dependent on the availability and informativety of polymorphic DNA markers.

1.1 DNA POLYMORPHISMS

Botstein *et al.* (9) were first to suggest that polymorphisms in DNA sequences visualized as Restriction Fragment Length Polymorphisms (RFLPs) could be used as informative genetic markers to map human diseases and since then there has been an exponential growth in polymorphic markers (10). During the last few years RFLPs were supplemented with new classes of polymorphic markers like Variable Number of Tandem repeats (VNTRs) and markers that can be detected by the polymerase chain reaction (PCR). VNTRs are characterized by restriction fragments

that contain short tandemly repeated DNA segments. The repeat unit length varies between 11 and 60 base pairs (bp) (11). VNTRs are usualy more informative than RFLPs but their use in linkage studies is limited because they show a strong clustering toward the telomeres (12).

Several other classes of markers have been developed that can be detected with the use of PCR. PCR can be combined with denaturing gradient gel electrophoresis (DGGE) which allows detection of differences of DNA sequence anywhere along a migrating fragment as opposed to RFLPs which only detect sequence differences at restriction endonuclease recognition sites (13). DGGE markers are highly abundant but their informativity is restricted and the testing is labor intensive. Alumorphs are markers that reveal length polymorphisms in Alu repeat elements by the use of various combinations of PCR primers and enzymatic restriction digests (14). Random Amplified Polymorphic DNA markers (RAPDs) make use of primers with arbitrary nucleotide sequences. These primers detect polymorphisms by the presence or absence of specific nucleotide sequences (15). A large number of fragments are amplified with a single primer pair. A major advantage of Alumorphs and RAPDs is that they can detect a large number of polymorphisms in a single experiment. However, the informativity of these markers is not very high therefore their use is limited to an initial genome screening using large family samples.

Currently the most widely used markers for linkage studies are Simple Tandems Repeat sequences (STRs) or microsatellite markers which are often highly polymorphic in humans (16). STRs are based on polymorphisms in di-,tri, and tetranucleotide repeats in genomic DNA. STRs do not show the extensive clustering of VNTRs and are very abundant. The estimated number of repeat sequences in humans is between 35.000 and 130.000. The mutation rates in these repeat sequences is not more then 10^{-4} mutations per chromosome per generation. This value is sufficiently low to establish a reliable Mendelian segregation pattern, even in large families (17). Polymorphic dinucleotide repeats are now being identified at a high rate (17,18) and placed into high density linkage maps of the human genome where on average a highly polymorphic marker is available every 2 - 6 cM (18,19). Up to date information on these markers is available to the scientific community through the Human Genome Database (GDB) (10).

Informativity of a marker is of great importance in linkage analysis. For a mating to be informative in linkage analysis at least one of the parents must be heterozygous for both genetic loci. Therefore, the usefulness of a marker for linkage analysis depends on the number of alleles and the frequency of the different alleles in the population (degree of polymorphism). A high degree of polymorphism is

associated with a high probability of heterozygosity. A measure for the degree of polymorphism is the Polymorphism Information Content (PIC). This PIC value reflects the probability of a parental mating to be fully informative for the marker (20,21).

1.2 LINKAGE ANALYSIS

In families segregation of genetic markers can be studied through linkage analysis, a likelihood method which correlates the segregation of a disease with that of a well localized polymorphic marker (8,20,21). For each autosomal locus an individual carries two alleles. Since two randomly chosen loci are most likely localized on separate chromosomes or localized far apart on the same chromosome, the alleles of two loci are generally transmitted independently. However, if the alleles from two loci received from an individual tend to be transmitted together to offspring they are considered to be linked and located close to each other on the same chromosome. The genetic distance between two loci can be expressed as the recombination rate (Θ). The closer two loci are, the smaller the chances for a recombination event to occur between them. Two loci at a genetic distance of less than 50% recombination ($\Theta < 0.5$) are genetically linked. The basics of linkage analysis for human diseases is to test the hypothesis (H1) that a polymorphic locus is linked to a disease locus at a recombination fraction smaller than 50% against the hypothesis (H0) that these loci are unlinked, i.e the recombination fraction is 50% ($\Theta=0.5$). The most widely used method for linkage analysis is the maximum likelihood method. This method estimates the most probable recombination fraction between two loci and calculates the likelihood that an observed recombination fraction is significantly smaller than 50%, i.e it compares the likelihood (P1) that the data have arisen if H1 is true, versus the likelihood (P2) that the data have arisen if H0 is true. The ratio of P1:P2 is a measure for the likelihood that the data have arisen under linkage. This ratio is usually converted into the lod score (^{10}log of the odds). For Mendelian disorders a lod score of 3.0 (corresponding to an likelihood ratio of 1000:1) is the accepted threshold for the existance of linkage and a lod score of -2.0 as a threshold for non-linkage. The likelihood of a disease gene and a marker locus being linked can be calculated by the use of computer programs such as the LINKAGE program package (22). Linkage analysis of a disease locus and a genetic marker is usually performed over a number of recombination fractions and from the results a likelihood curve can be constructed. Based on this curve the intervals with evidence for linkage or evidence for non-linkage can be determined. Lod scores from different families can be

summed (21).
Even if a marker has a high degree of polymorphism not every meiosis will be informative. In multi-point linkage analysis information from several genetically linked marker loci can be combined in a single analysis (23). Meioses that are not informative for one locus might be informative for other loci in the same region. In this way more information can be extracted from the same family material. Multi-point linkage analysis can be useful to construct genetic maps and to determine the position of a genetic locus into such a map. The probability for a particular order of markers is compared with the probabilities for an alternative order. By repeated comparisons for all possible orders the order with the largest likelihood can be determined. For larger genetic distances multiple crossover events could occur. An even number of crossovers would go undetected and therefore mapping functions have been developed to convert genetic distances to recombination frequencies. The frequency of double crossovers is usually lower than would be expected on the number of single crossovers. This phenomenan of interference is incorporated in several mapping functions (21).
Once a disease gene has been localized on a certain chromosome the use of more polymorphic markers in the candidate region can narrow the region in which the defective gene must be localized. Recombination events between disease and polymorphic markers determine the borders of the candidate region in which the gene must reside. The use of multi-point linkage analysis and haplotype analysis can determine between which markers the gene must be localized.
The resolution of fine mapping is determined by the number of informative meioses that can be detected. Usually this resolution is limited to 1-3 centiMorgan (cM). One cM corresponds to a crossing over frequency of 1 in 100 meioses. There is no constant relation between genetic distances and physical distances on the human genome but 1cM corresponds to an average physical distance of approximately 10^6 base pairs. The total human genome is estimated to be approximately 3300 cM (24). The maximum resolution that can be obtained by linkage mapping varies between disorders because the number of families that can be ascertained for frequent disorders is usually higher then for rare disorders.
Evidence for non-linkage can be used to determine those genomic regions from which the gene can be excluded. With the results of two-point and multi-point analysis exclusion maps can be constructed that represent the progress in the systematic screening of the genome for linkage. An excellent and detailed overview of linkage analysis has been given by J. Ott (21).

1.3 GENE ISOLATION

When the maximum resolution using the available family material has narrowed down the candidate region to 1-3 cM, the candidate region can be cloned into partly overlapping clones from genomic DNA libraries. These libraries can be constructed in cloning vehicles such as cosmid vectors, Yeast Artificial Chromosomes (YACs) or bacteriophage P1 vectors (25-27). The genomic clones can be used to screen for expressed sequences in the candidate region. A number of methods are available to identify expressed sequences within a large genomic region. A few successful strategies are the screening of cDNA libraries, exon trapping and the identification of CpG islands, which are often localized in the regulatory regions of genes (ref. 4 for overview). None of these methods will detect all the genes that are expressed in the region and several methods must then be used simultaneously. Finally, the responsible gene has to be identified from all the expressed sequences. By comparing the sequence of the candidate gene between patients and control individuals the mutation(s) that lead(s) to the disease phenotype can be determined. Positional cloning of a disease gene can be time consuming and labor intensive. This is illustrated by the search for the gene responsible for Huntington disease. In 1983 this was the first Mendelian disorder with an unknown genetic defect to be localized with the new DNA markers. Recombination events pointed to two different locations on chromosome 4p but despite the efforts of various research groups during a period of ten years the gene has only very recently been cloned (28,29). In many instances 'positional cloning' has been succesful and a number of examples are listed in Table 1.

Important factors that contributed to the success of the positional cloning approach are gross rearrangements of DNA, cytogenetic rearrangements or instable repeat sequences. These have been found in almost all disorders for which the disease gene has successfully been cloned. The cloning of the cystic fibrosis gene was the first example of positional cloning without the help of gross DNA rearrangements or instable repeat sequences. Other disorders for which the responsible genes have been identified like retinitis pigmentosa, Marfan syndrome, Alzheimer disease and a hereditary form of hypertension have characteristic biochemical features that made it possible to propose candidate genes that indeed proved to be the responsible genes (65-71).

Table 1. Examples of genes cloned by positional cloning

Disease locus	Localization	Reference
Adrenoleukodystrophy	Xq28	30
Aniridia	11p13	31
Charcot Marie Tooth type 1A	17p11.2	32
Choroideremia	X21.2	33
Chronic granulomatous disease	Xp21.1	34
Cystic fibrosis	7q31.2	35
Duchenne muscular dystrophy	Xp21.2	36
Epidermolytic hyperkeratosis	12q11-q13	37
Familial polyposis coli	5q	38,39
Fragile X syndrome	Xq27.3	40
Glycerol kinase deficiency	Xp21	41,42
Greig craniopolysyndactyly syndrome	7p13	43
Huntington's disease	4p16.3	29
Kallman syndrome	Xp22.3	44
Kennedy's disease	X	45
Menkes syndrome	Xq12-q13	46,47
Myotonic dystrophy	19q13.2-q13.3	48-53
Neurofibromatosis type 1	17q11.2	54,55
Norrie's disease	Xp11.4	56,57
Nefrogenic diabetes insipidus	Xq28	58
Retinoblastoma	13q14.1-q14.2	59
Testis determining factor	Y	60
Thomson's disease	7q	61
Waardenburg syndrome type 1	2q35	62,63
Wilms tumor	11p13	64

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CHAPTER 2

COMPLICATING FACTORS IN LINKAGE ANALYSIS

Linkage analysis was initially developed for human disorders with recessive or dominant transmission of a single disease gene. A number of factors may complicate these Mendelian inheritance patterns and the linkage analysis for these disorders.

2.1 INCOMPLETE PENETRANCE

For a large number of inherited disorders only a proportion of the individuals who carry the disease genotype actually express it. This incomplete penetrance can be the result of factors which are not directly related to the mutation. These can include the genetic background of the individual, environmental factors or mere chance. Examples are cancer syndromes like retinoblastoma in which a recessive mutation is inherited and a second somatic mutation in the normal allele is a prerequisite for a tumor to develop (1). Another example is Alzheimer's disease that has a late onset. In family studies individuals carrying the disease genotype often do not show expression of the phenotype at the time of ascertainment (2). For most disorders the underlying cause for the incomplete penetrance is not known.
Incomplete penetrance has an important effect on the linkage analysis. In diseases with simple Mendelian transmission, unaffected children of an affected parent are as informative in the analysis as affected children. When penetrance is incomplete, unaffected children will not be fully informative due to the uncertainty about their genotype for the disease gene. In the linkage analysis these unaffected individuals will appear as recombinants if incomplete penetrance is not taken into consideration.

2.2 UNCERTAINTY OF DIAGNOSIS

Another factor that complicates the linkage analysis is clinical misdiagnosis. This is especially a serious problem in psychiatric disorders (3-7) where a wide range of behavioral characteristics are included among the diagnostic criteria. Families often contain individuals who express only some of the characteristics in which instance the diagnosis may be doubtful. To avoid incorrect linkage information these individuals are usually regarded in the analysis as having an unknown phenotype. Alternatively the linkage analysis can be performed with several alternatives for the affection status of critical individuals.
The appearance of phenocopies i.e. the situation in which individuals of normal genotype display the mutant phenotype, has the same effect on the linkage analysis as misdiagnosis.

2.3 GENETIC HETEROGENEITY

Genetic heterogeneity is another major problem that can be encountered in a linkage study. Locus heterogeneity is the situation in which the same phenotype is caused by single mutations at independent loci. Examples of disorders showing locus heterogeneity are retinitis pigmentosa, Charcot-Marie-Tooth disease, tuberous sclerosis and Alzheimer disease (8-13). In linkage studies often a number of relatively small families are used and the data from these families are pooled in order to aquire sufficient statistical power to detect linkage. In case of locus heterogeneity the linkage of a chromosomal locus in one family might remain undetected due to evidence for non-linkage in another family. In the search for the Alzheimer disease gene the subdivision of the clinical phenotype into a late and early onset form led to the isolation of one gene responsible for early onset *alz*heimer and more recently for the localization of a second gene (12-13). Moreover, clinically very heterogeneous disorders can be genetically homogeneous. Different mutations at a single locus may disrupt the same biochemical function in varying ways. An example for this allelic heterogeneity is the DMD gene where different mutations cause either the severe and progressive Duchenne Muscular Dystrophy or the much milder Becker Muscular Dystrophy (14,15).

A way to circumvent the described problems of heterogeneity is to use a single large pedigree for which it can be assumed that a single mutation is segregating. The ascertainment of such large pedigrees, however, has also several disadvantages. Ascertainment of very large families might be difficult for disorders with a high mortality rate and for extremely rare diseases. Furthermore, attempting to expand a family as much as possible carries the risk of introducing phenocopies or bilineality. Moreover, one should also keep in mind that the locus causing the disease in a single pedigree might not be representative for the more common form of the disorder in the general population. At the other end, studying families in a single geographic region or from a genetically isolated population might reduce the chances of genetic heterogeneity.

The recent history of linkage studies has shown the strength of the methodology for single gene disorders. For complex disorders all above mentioned problems have given either analytical problems or problems in reproducing the results in various populations.

2.4 POLYGENIC INHERITANCE

This is the situation in which a number of different loci have to interact to cause a

disease phenotype. Such interaction can be additive, in which situation none of the alleles are sufficient to cause the complete phenotype but each allele is responsible for part of the phenotypic spectrum of the disease. Another possibility is a synthetic trait where a number of mutant alleles are essential to cause the disease phenotype. If one of the alleles is normal a normal phenotype is the result. Although polygenic inherited disorders have long been regarded as too complicated for successful mapping using the available methodology, recent advances in the elucidation of the genetic background of diabetes mellitus and hypertension susceptibility genes has shown that the combination of different approaches and the use of strictly defined phenotypic criteria do allow gene mapping of polygenic disorders (16-19).

The mapping of polygenic disorders and multifactorial disorders subject to environmental influences could benefit from the use of animal models which mimic the human disease (17,18). Controlled experiments with inbred strains under controlled environmental conditions can be designed. Large numbers of animals can be used and crosses can be arranged at will, so that the complexity of the segregation pattern of the genetic trait under study is reduced.

2.5 GENOMIC IMPRINTING

Genomic imprinting is a phenomenon which confers functional differences on the maternal and paternal alleles. The term was first introduced by Crouse in 1960 (20) who described a process in the fungus fly *Scaria coprophila* that leads to a series of selective eliminations of the paternal X chromosome in somatic cells and in the germline. This process is the basis of sex determination in this species. Probably the best known examples of genomic imprinting are the mule and the hinny. If a horse and a donkey are crossed there is a characteristic difference in the phenotype of the offspring (mule or hinny) depending on which of the parents is a horse or a donkey. In both situations the offspring receives a complete haploid set of chromosomes from each of the parents, implying that epigenetic differences between the parental genomes cause the phenotypic differences of the offspring.

The phenomenon of genomic imprinting is now widely recognized in mammals where it appears to play an important role in development. In pronuclear transplantation and parthenogenetic activation experiments in mice, zygotes were constructed with the nuclear genes derived entirely from either the father or the mother. Zygotes with paternally derived chromosomes showed a relatively normal development of membranes and placentas but a very poor development of embryonic structures (21-23). The zygotes with maternally derived chromosomes

showed a relatively good development of the embryos but had poor development of membranes and placentas. Both situations are lethal and the developmental failure is caused by differences in nuclear DNA of maternal and paternal genetic material (24). In humans a similar situation exists in the form of hydatiform moles (25-27). Moles are regarded as placental tumors found in pregnancies without embryonic tissue. They are usually diploid with their chromosomes derived from two haploid sets of paternal chromosomes. Human fetal triploids show two distinctive phenotypes depending from which parent the two sets of chromosomes are inherited. If two sets of chromosomes are derived from the father, large cystic placentas are observed and usually there is no fetal growth (27). Two sets of maternal chromosomes cause small underdeveloped placentas and fetal development is severely retarded. These observations show that the combined presence of maternal and paternal genomes is vital for normal development and that both genomes should be present in balanced amounts.

Uniparental disomy, i.e. the situation in which offspring receives a diploid set of chromosomes or a chromosomal region from a single parent, has been extensively studied in mice using genetic complementation studies (28). A genome map has been constructed containing all currently known imprinted regions. The mouse chromosome 7 has been studied in detail for a number of imprinting phenomena. Maternal duplication or paternal deficiency of the T9H breakpoint region results in a late onset fetal lethality due to retarded placental and fetal growth. Maternal duplication or paternal deficiency for a region distal to the T50H breakpoint is also lethal with death occurring early in embryogenesis (28).

Imprinting of mouse chromosome 7 was studied by DeChiara *et al.* (29) through a targeted disruption in the insulin-like growth factor II (Igf2) in mice. Transmission of that mutation through the male germ line resulted in heterozygous progeny with growth deficiency, having only 60% of the normal size. Transmission of the disrupted gene through the maternal line resulted in a phenotypically normal progeny. Expression studies on Igf2 showed that only the paternal allele is expressed in embryos while the maternal allele is silent, indicating that the maternal allele is marked by an imprinting process.

Recently maternal imprinting was described for the mouse Snrpn gene on chromosome 7, encoding a small nuclear ribonucleoprotein (snRNP)-associated polypeptide that is maternally imprinted (30). The human homologue of this gene is located at chromosome 15q12 (31). In that region the gene responsible for Prader-Willi syndrome (PWS) is localized. This syndrome is characterized by infantile hypotonia, childhood hyperphagia, mental retardation and hypogenitalism (32). PWS is a deletion syndrome associated with paternally derived deletions of

chromosome 15q12 (33). Angelman syndrome (AS) is also a deletion syndrome in which chromosome 15q12 is involved but these deletions are typically maternally derived. The phenotype of AS patients is different from PWS patients. AS patients display severe mental retardation, microcephaly, excessive laughter and seizures, but other features may also be present (34).

In PWS patients without a cytogenetically detectable deletion maternal disomy for the critical region on chromosome 15 has been observed. These observations strongly suggests that lack of a paternal contribution of this critical region leads to PWS. Conversely, absence of a maternal contribution can lead to AS (35,36). PWS and AS were described to occur in a single family and the origin of the parental allele was consistent with imprinting (37). The critical regions for PWS and AS are close to each other but distinct (38). The human Snrpn gene is located in the critical region for PWS but not in the critical region for AS. The gene is not expressed from the paternal allele (30,31), this indicates that, like in the mouse the gene is maternally imprinted and that it is a candidate gene for PWS.

Genomic imprinting appears to be responsible for irregular patterns of inheritance and variable expression of a number of other human disorders (39). These disorders show differences in phenotype, age of onset and severity that seem to be related to the sex of the parent transmitting the gene. In Huntington'sisease (HD) in 5-10% of the families a severe rigid juvenile form of the disorder is transmitted through the father. In 10-20% of families with myotonic dystrophy (MD) a severe congenital form of the disease is transmitted through the mother. In the fragile-X syndrome the expansion of a CGG repeat from a premutation to a full mutation in the FMR-1 gene can only occur after passage of the premutation through a female meiosis. It never happens after passage through a male meiosis. Genomic imprinting has been put forward as a possible explanation for these findings (40-42).

In a number of cancer syndromes genomic imprinting may play a role in the onset of the disease. In sporadic Wilms tumor, rhabdomyosarcoma and osteosarcoma preferential maternal loss of paternal alleles has been observed (43-45). Uniparental disomy is reported in Beckwith-Wiedermann syndrome (46,47). Another example is the t(9;22) translocation associated Philadelphia chromosome positive leukemia (48). The translocated chromosome 9 is preferentially of paternal origin and the translocated chromosome 22 preferentially of maternal origin. These translocations occur somatically and genomic imprinting must be involved either in formation of the translocation or in the specific selection of the parental origin of the translocation.

In retinoblastoma and hereditary paragangliomas 'skipped' generations are

observed in families. Usually the 'skipped' individuals are children of affected females (49,50).

2.5.1 The imprinting mechanism

A genomically imprintable gene is transmitted in a Mendelian manner, but expression of the phenotype will be determined by the sex of the transmitting parent. The maternal and paternal chromosomes appear to epigenetically modified in a different way during parental gametogenesis and are therefore differentially 'imprinted' with the consequence that they are not functionally equivalent in the embryo. This effect of the imprint persists during embryogenesis and sometimes apparently throughout the lifetime of an individual (48). During gametogenesis of the progeny the original imprint must be erased and a new imprint gained depending upon the sex of the transmitting parent (39).

The mechanism involved in genomic imprinting is unknown but must lead to modifications of the nuclear DNA of cells in order to produce these phenotypic differences. Hypermethylated genes or genes residing in heterochromatin regions are usually repressed (51). Allele specific differences in methylation patterns have been detected in a number of tissues and site specific changes in DNA methylation pattern are known to influence gene expression (52,53).

In the mouse methylation of a number of genes occurs around the time of X chromosome inactivation but it is not clear whether methylation precedes or follows this inactivation (54). Methylation does not seem to play a role in the silencing of the maternal allele of Igf2 (55). In this case the chromatin of the repressed allele is potentially active for transcription rather then being heterochromatic and inactive. One of the two promoters is unmethylated and DNAse I sensitive sites are present on both chromosomes indicating that there are no gross differences in the chromatin state of both alleles. Low levels of primary transcripts from the imprinted allele have been detected. An explanation for these observations is that epigenetic modifications outside the promotor region or other unidentified modifications at the promotor might be responsible for the imprinting. Several kilobases upstream from the first exon methylation differences were found. Possibly there are transcriptional elements for pairs of genes on homologous chromosomes which have a role in determining the specific allelic expression. These elements could interact with an imprintor gene. Recent evidence for an imprintor gene in mice has been described (56). This gene could directly imprint a set of genes or alternatively modify the imprinting mechanism.

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CHAPTER 3

INTRODUCTION TO THE EXPERIMENTAL WORK

The primary goal of the experimental work in this thesis was to localize the responsible genes for GTS and hereditary paragangliomas. Since the biochemical mechanisms of both disorders are not yet known the functional cloning strategy could not be used. Positional cloning is an alternative but is not straightforward because the segregation patterns of both disorders do not follow simple Mendelian principles.
The inheritance pattern of GTS is complicated by incomplete penetrance, associated behavioral problems such as chronic multiple tic syndrome (CMT), obsessive compulsive symptoms (OCS) and a number of associated psychiatric symptoms. Clinical expression of the phenotype follows a waxing and waning course over time (Chapter 4).
The incomplete penetrance observed in hereditary paragangliomas is dependent on the sex of the transmitting parent and can be explained in terms of genomic imprinting (Chapter 6).

3.1 DETERMINING THE MODE OF INHERITANCE

Prior to performing a linkage study, segregation analysis can be used to determine the mode of inheritance of a genetic trait. Segregation analysis applies complex models of inheritance to clinical diagnostic data in families. The observed proportions of affected relatives of patients among each type of relative is matched to different modes of inheritance (1). The analysis uses maximum likelihood procedures to test for single locus and polygenic components of inheritance and takes into account factors as incomplete penetrance, age of onset variation and phenocopies. In Chapter 4 the results of segregation studies on GTS are reviewed. The segregation pattern of GTS is consistent with an autosomal dominant trait with incomplete penetrance. There is evidence that both CMT and OCS are milder expressions of the phenotype. The outcome of segregation studies allows the definition of genetic models that can be used in linkage analysis. If the mode of inheritance or the definition of the phenotype of a genetic trait is uncertain, different models can be defined with different diagnostic schemes or genetic parameters. Linkage analysis is then performed for all models. In effect the lod score will be maximized over diagnostic schemes and/or penetrance values. Weeks (2) noted that 'maximizing the lod score over models' will tend to inflate the lod score and this inflation should be taken into account. Before actually starting a systematic genome search it is essential that the models being used are spelled out clearly. They should not be allowed to change.
For GTS three diagnostic models were defined based on the results of segregation

Table 1. Models used in linkage analysis for Gilles de la Tourette syndrome[1]

	Diagnostic models	penetrance ♂	penetrance ♀	phenocopies ♂	phenocopies ♀
1.	GTS only.	0.81	0.31	0.005	0.003
2.	GTS including CMT.	0.90	0.56	0.01	0.006
3.	GTS including CMT and OCS.	0.90	0.71	0.01	0.006

[1]Mode of inheritance: Autosomal dominant with incomplete penetrance and variable expression. Gene frequency: 0.003

studies of Pauls & Leckman (3) reflecting the uncertainties of the possible phenotypes (Table 1).

For hereditary paragangliomas an alternative approach was used. There is general agreement on the clinical diagnosis of hereditary paragangliomas. Penetrance is influenced by age of onset and by the sex of the transmitting parent. For linkage analysis an autosomal gene with incomplete penetrance was assumed. A second model implicated genomic imprinting by assuming complete absence of the disease phenotype when the gene was inherited from the mother (Chapter 7.1).

3.2 SIMULATION STUDIES

After defining the genetic model(s) to be tested in the linkage analysis, simulation studies can be performed to establish the statistical power of the available family material to detect linkage. For simple Mendelian disorders the statistical power of a family to detect a linkage can easily be calculated by counting the number of meioses. For complex disorders computer programs are available that can generate genotypes for a hypothetical marker (4). Simulation studies with these hypothetical marker data can be used to determine the probabilities to detect linkage with the ascertained family material.
Simulation studies are a way to predict the results that can be expected from a linkage study. In the case of genetic heterogeneity simulation studies can be used to determine the power of the family material to detect a linkage under different proportions of genetic heterogeneity. Other applications of simulation studies are the investigation of the effect of changing diagnosis, and risk calculations, and the determination of the contribution of each family member to the power of the

family. If the informativity of the available family material is sufficient to detect linkage the actual mapping of the disorder under investigation can be undertaken.
In Chapter 5.1 an extensive simulation study for GTS is described. The statistical power to detect linkage of these families was tested under the assumption of genetic homogeneity as well as under the assumption of genetic heterogeneity. A sensitivity analysis to determine the impact of changing diagnosis was performed and the statistical power to detect linkage under an incorrect diagnostic model was tested.
For hereditary paragangliomas a simulation study was carried out on a single large five generation pedigree to determine its power to detect linkage. By 'peeling' off individuals from this pedigree in subsequent rounds of simulations, the key individuals who contribute most to its statistical power were determined. (Chapter 7.1).

3.3 LINKAGE STUDIES

In linkage studies several strategies can be used in an attempt to identify the disease gene.
Chromosomal abnormalities such as translocations or deletions in patients can give an indication of the localization of the disease gene. By testing polymorphic markers in the region of the abnormality in families with the disease of interest the involvement of the region can be tested (Chapter 5.4).
For complex disorders the linkage analysis could be simplified if the disorder would co-segregate with a disorder that has already been localized or inherits in an Mendelian way. The candidate-gene approach appears to be an attractive way for linkage analysis. The hypothesis of the involvement of a certain gene in the etiology of the disorder of interest can be directly tested. Those candidate genes that have been cloned, and localized and are polymorphic, can easily be included in a systematic screening of the human genome. Linkage studies are carried out to test the hypothesis that the disease gene and the candidate gene are the same by demonstrating that an allele from the candidate gene is co-segregating with the disease. If this segregation is not strictly correlated the candidate gene cannot be the disease gene.
The linkage studies on GTS presented in this thesis focused on the systematic screening for polymorphic markers linked to the disease locus. The linkage results of the actual mapping studies are described in Chapter 5.2-5.4. The linkage results from the ongoing collaborative genome search for GTS are incorporated in exclusion maps (Chapter 5.3-5.4).

For hereditary paragangliomas highly significant evidence for linkage and for genomic imprinting was obtained and additional fine mapping is described including the construction of a high resolution linkage map (Chapter 7.1-7.3).

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CHAPTER 4

CLINICAL STUDIES ON GILLES DE LA TOURETTE SYNDROME*

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4.1 INTRODUCTION

In 1885 Gilles de la Tourette described a neurological condition with uncoordinated movements associated with uncontrolled speech, echolalia and coprolalia (1). The condition received his name upon the suggestion of his teacher Charcot. Georges Gilles de la Tourette suggested a genetic etiology in his first observations of 9 patients with motor and vocal tics. He found a positive family history in five families and a family history for tics in two families. However, in the next 65 years psychologic theories predominated the clinical view on the Gilles de la Tourette syndrome (GTS) (2-8). Familial occurrence was mainly of interest from a family dynamic point of view (9). Arthur and Elaine Shapiro and their collaborators contributed to over 50 papers and two books to renew interest in the neuro-biological mechanisms in GTS (10-13). An excellent comprehensive review on the latest clinical and neuro-biological insights is given by Robertson (14).
Most GTS cases are idiopathic, i.e. without a known cause. However, the discovery by Seignot of the therapeutic effects of the dopamine receptor blocking agent haloperidol suggested a disturbance of the central dopaminergic system, especially the basal ganglia and the frontal cortex, in the pathogenesis of GTS (15). Now noradrenergic, serotonergic, GABA-ergic and endorphin systems in the brain are also suggested to be involved in the pathogenesis of GTS (13).
The diagnostic criteria for GTS in the revised third edition of the Diagnostical and Statistical Manual of mental Disorders (DSM-IIIR) are listed in Table 1.

Table 1. Diagnostic criteria for the Gilles de la Tourette syndrome according to DSM-IIIR

A.	Both multiple motor and one or more vocal tics have been present it some time during the illness, allthough not necessarily concurrently.
B.	The tics occur many times a day (usually) in bouts, nearly every day or intermittently throughout a period of more than one year.
C.	The anatomic location, number, frequency, complexity, and severity of the tics change over time.
D.	Onset before age 21.
E.	Occurrence not exclusively during Psychoactive Substance Intoxication or known central nervous system disease such as Huntington's chorea and postviral encephalitis.

These criteria exclude tics occurring during psychoactive substance intoxication or known central nervous system diseases like Huntington's chorea and postviral encephalitis (16). Conditions mimicking GTS have been reported secondary to external or environmental events like neuroleptic treatment (17-19), carbon monoxide poisoning (20), gasoline inhalation (21), AIDS (13), post-encephalitic syndromes especially when treated with L-DOPA (22,23,24), head trauma (25), and angiographic complications (26). Although diffuse CNS damage was documented in these cases, involvement of the basal ganglia appeared to be the greatest common denominator. The initially alarming reports that GTS, tics and also obsessive-compulsive behaviors could be caused by neurostimulant drugs per se, seem to be unwarranted (13,27-30). However, it is still unresolved, whether biologic relatives of GTS patients have an increased risk to develop GTS after neurostimulant treatment (13,31-33).

4.2 EPIDEMIOLOGY

Patients with a positive family history for GTS and or tics were initially described in Jewish, Italian and Northern-European families (11,34,35). However, subsequently similar observations were made in nearly every population in the world, including Western Europe, Russia, Korea (36), China (37,38), Japan (36,39), India and the Middle East (40,41).

The incidence of tics in relatives of GTS patients must be compared with the tic frequency in the normal population. For the population three percent seems a minimal estimate (42). However, only limited population studies on tics are available. Simple, transient tics occur in 12 to 16% of American and European children at school age (43,44). The difference in the reported incidence can be explained by the observation that the frequency of tics decreases with age.

The first estimates on the prevalence of Gilles de la Tourette syndrome were based on heterogeneous clinical samples. Koester reported 2 GTS cases in 2500 outpatients and Ascher reported 4 GTS cases in 59000 in- and outpatients (45,46). Lucas *et al.* calculated an incidence rate of 4.6/1,000,000 based on population data in an urban area in the USA (47). Burd *et al.* reported prevalence rates of 1 per 10,000 girls and 9.3 per 10,000 boys at school age and much lower prevalence in adults: 0.22 per 10,000 for women and 0.77 per 10,000 for men, based on questionnaires send to health professionals in North Dakota (48,49). The sex ratio of almost 10:1 in favor of boys in childhood is much lower in adults (about 3.5:1). In a single school district in Los Angeles, Comings *et al.* found prevalence rates of 1 in 95 for boys and 1 in 759 for girls (50).

These epidemiologic data have been difficult to interpret and to compare for several reasons. GTS is considered to be a relatively rare condition and unfamiliarity with the disorder may lead to underdiagnosis. Furthermore, large population samples need to be studied using proper diagnostic criteria to obtain reliable data. Moreover, the extent to which more or less severe cases are included will dramatically influence the prevalence rates.
Caine *et al.* identified 41 GTS cases from ± 140.000 children at school age in Monroe county (Rochester, NY, USA) using DSM-III criteria (51). Of these cases, 24 had already been diagnosed as GTS before and 17 new cases were diagnosed during the study. Of the 24 earlier diagnosed cases 19 (79%) had associated behavioral problems versus 1 (6%) of the new cases. Obviously more severe or more complicated cases will become diagnosed (and come to medical attention) earlier as compared to mild cases. Furthermore, in cases where associated behavioral problems are more striking than the tics, the medical sophistication of the patient's family and teachers may be the key in determining whether an affected child is correctly diagnosed or merely considered unmanageable. These considerations underscore the need to use proper diagnostic criteria, including attention for classification of mildly affected patients and correction for ascertainment bias, before the "natural incidence" of tics and of GTS in population samples can be reliably established.

4.3 EARLY OBSERVATIONS ON FAMILIAL INCIDENCE

It took almost 80 years before Gilles de la Tourette's notion about genetic influences on the etiology of GTS was supported by other clinicians. Since the onset of GTS genetic research in the late sixties and early seventies positive family histories have been found in about 10% of GTS cases (11,52,53). However, these studies were not systematic and relied on incidental clinical samples of GTS and family histories obtained during clinical work-up (31,53). Additional cases were not systematically evaluated or seen by the investigator, neither were systematic studies performed of all first and second degree relatives. The small recurrence risks in families suggested that "a family history of tics does not characterize patients with Tourette Syndrome" (11). Defense mechanisms consisting of partial introjection of an aggressive parent with tics and the identification with these behaviors have been suggested to explain familial occurrence rather than inheritance (54).
The first report of multiple diagnosed GTS cases in a nuclear family with 6 children concerned two sisters with childhood onset GTS and a formal diagnosis at

the age of 28 and 34, respectively (55). These sisters had six children; four had definite and obvious multiple motor tics, one also had vocal tics.
Frost *et al.* carried out the first extended pedigree study in the family of a GTS patient with tics since age 6 (56). Fourteen of 17 maternal relatives were available and diagnosed according Feighner's Research Diagnostic Criteria using an extensive, structured psychiatric interview (56). None of the interviewed relatives had GTS, but 6 had tics (1 first degree, 2 second degree and 3 third degree relative(s)). One paternal cousin had a temporary tic syndrome for 2 years.
The systematic evaluation of symptoms in relatives was a major methodological improvement and increased the percentage of positive family histories in selected GTS samples to 60-80% (34,57,58). Tics were then found in about 10% of the relatives. In some families both parental lines included affected individuals (34). Several cautious hypotheses about the heredity of GTS emerged at this time, suggesting that GTS was very likely heterogeneous, some forms probably non-genetic, others either caused by autosomal recessive (57) or autosomal dominant inheritance with phenotypic variation and subclinical expression in some of the relatives (35,59).
While it was still impossible to utilize these data for quantitative analyses on patterns of heredity they initiated the next phase in the search of the genetics of GTS. Improved methodology now prescribed studies of randomly selected families in which all relatives and spouses could be systematically interviewed using standardized methods and reliable diagnostic schedules. Other evidence described for the involvement of genetic mechanisms in the etiology of GTS came from adoption and twin studies.

4.4 ADOPTION STUDIES

Adoption studies have been relevant to studies on the interaction between genetics and environment since they provide a way to analyze the expression of genetic factors in different surroundings. There is only one study on the occurrence of tics among families of 22 adopted GTS patients, compared with the family histories of the biological families of 641 patients using the family history method (13). There was no history of tics in the adoptive families of GTS patients. From the 641 GTS patients of the biological families 227 had a positive family history in at least one of their first degree relatives. By including second and third degree relatives this number was raised to 302 patients.
A limitation of this method was that precise data on the biological parents and other relatives of the adopted-away patients were generally not available.

However, the proportion of the differences observed is a strong argument for a genetic mechanism for GTS.

4.5 TWIN STUDIES

Monozygotic (MZ) twins are genetically identical, dizygotic (DZ) twins are genetically as different as normal sibs. If a disorder is inherited in a Mendelian way MZ twins are expected to be 100% concordant.
There are observations on DZ and MZ twins with variations in age of onset, type and severity of symptoms between twins of the same sibship (34,56,57,60-68). These studies showed a pairwise concordance in monozygotic twins of ± 50-70% for GTS and ± 75-90% for GTS and tics together (13,56). In the dizygotic twins the concordance rates for both conditions were respectively ± 10% and ± 20% (13,56). Twin studies showed no 100% concordance rate for GTS and/or tics in MZ twins suggesting that nongenetic circumstances like perinatal and postnatal factors may influence the expression of the GTS in individuals at risk (69,70). All the affected discordant MZ twins in one of these series had a lower birth weight as compared to the unaffected twins (69). Moreover, in a series of MZ twins, concordant for GTS and tics, the twins with the lowest birth weight consistently had the most severe symptoms (71).
Twin studies are relevant to determine the phenotypic relationships between GTS, tics and associated behavioral problems. Concordance in MZ GTS twins has been reported for obsessional traits (50,52,62,65,66,68) and for attention deficit disorder (32). The delineation of the phenotype, that may be more complex than was supposed initially, now became the most important issue in the diagnostic assessment of GTS families.

4.6 SYSTEMATIC FAMILY STUDIES

The aim of the systematic family studies, started around 1980, was to obtain insight into the mode of inheritance by means of segregation analysis and to collect family material for DNA linkage studies (72). Well documented extended families are the cornerstone of this approach. Direct interviews of all available family members proved to be a more sensitive method to identify affected relatives and establish more reliably the momentary unaffected status of others (73). Supported by the American Tourette Syndrome Association groups in the USA and Europe have collected extended families in a collaborative effort to map the GTS gene.

4.6.1 Family studies in the Netherlands

Ten extended families, comprising 286 subjects, participated in the study. The size of the available families varied from 6 to 60 subjects. All families were Dutch caucasian, except for one Norwegian family of 32 subjects. Diagnoses of all subjects were based on a semi structured interview. (D.L. Pauls, Yale University. New Haven). This schedule includes an extensive questionnaire about tics in addition to a standardized psychiatric interview. Furthermore, a Dutch version of the Leyton Obsessional Inventory was filled in by all Dutch subjects over the age of 15 years. GTS and other Tic syndromes were diagnosed using the information from observation and the structured interview. Obsessive compulsive symptoms (OCS) was assessed in two ways: 1. the method of structured interview and 2. Dutch version of the Leyton Obsessional Inventory.

There were four probands (all male, mean age 11 ± 2.6 years) without any OCS and six probands (three males, three females, mean age 21 ± 10.8 years) with OCS. Of 233 biological relatives 29 suffered from GTS. The number of biological relatives (n=40) with Chronic-multiple tics (CMT) was scarcely higher. Just above 60% of these subjects had not any of the characteristics of the Tic syndromes. Fifty-one biological relatives (22%) were affected by one or more obsessive-compulsive symptoms. Of 53 non-biologic relatives none had GTS and only one showed chronic tics. A detailed clinical description and information about the pedigree structures is provided by Dr. B.J.M. van de Wetering ().

4.6.2 Definition of the GTS phenotype

Within the consortium consensus was reached that chronic and transient tics had to be considered as milder expressions of the gene (35,74-76). Clinical observations and the twin studies indicated that GTS patients have obsessive-compulsive symptoms much more frequently than expected according to the population prevalence for obsessive-compulsive disorder (OCD) (14). On the other hand children with OCD show much more tics than their matched controls (77). This increased risk for OCD in GTS and for tics in OCD may very well reflect a variable expression of a common genetic cause for both conditions. Therefore it was decided to document systematically OCD and OC traits in the families that were part of the study. Other associated behavioral symptoms like attention deficit disorder with hyperkinesia (ADDH) and a variety of psychiatric conditions like anxiety disorders and drug abuse have also been suggested as part of the phenotype and are therefore also systematically recorded (78-83).

Statistical analysis is consistent with a single major gene responsible for the susceptibility for both GTS and chronic tics in families with a GTS proband (84-88).

The mode of inheritance observed in most of these studies is consistent with an autosomal dominant gene with incomplete penetrance and variable expression. The penetrance for GTS and tics in males is estimated about 0.99 and in females about 0.6 i.e. 99% of the males and 60% with the gene will have at least chronic tics or full-blown GTS (89,90). When OCD is included as part of the phenotype, the penetrance is raised to 0.70 in females suggesting that OCD is the more frequent expression of the gene in females (89-72). Though ADDH was found to be increased in relatives of GTS probands, an etiological relationship between ADDH and GTS could not be confirmed by segregation analysis (93,94).

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CHAPTER 5.1

LINKAGE STUDIES ON GILLES DE LA TOURETTE SYNDROME: WHICH FAMILIES AND PHENOTYPES SHOULD BE ANALYZED FURTHER?

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Submitted

ABSTRACT

For a linkage study it is important to ascertain family material sufficiently informative to detect linkage. The statistical power of a linkage sample can be determined via computer simulation. For complex traits uncertain genetic parameters such as incomplete penetrance, frequency of phenocopies, and variable expression of the phenotype have to be taken into account. One can either include only the most severe phenotype in the analysis or apply multiple linkage tests for a gradually broadened disease phenotype.
Gilles de la Tourette syndrome (GTS) is a chronical neurological disorder characterized by multiple, intermittent motor and vocal tics. Evidence suggests that GTS and milder phenotypes are caused by a single dominant gene.
During recent years six research groups have started genetic studies on GTS. A large set of families is available within this consortium. Until now the power of these families to detect linkage had not been systematically investigated. We report here the results of an extensive simulation study on this large set of families. We conclude that the available family material is informative enough to detect linkage even in the case of extensive genetic heterogeneity. Repeated interviews in the most informative family revealed the large impact of instability in clinical diagnoses. We compared the effectiveness of linkage tests with only the GTS phenotype versus multiple tests that included various milder phenotypes.
The scenario of multiple tests yielded superior power. Our results show that computer simulation can indicate the strategy of choice in linkage studies of multiple, complex phenotypes.

INTRODUCTION

Gilles de la Tourette syndrome (GTS) is a chronic neuropsychiatric disorder with unknown etiology. The syndrome is characterized by multiple, intermittent motor and vocal tics. Affected individuals frequently display associated behaviors like obsessive compulsive symptoms, attention deficit and hyperactivity disorder, coprolalia and echolalia (1). Expression of the phenotype follows a waxing and waning course and is influenced by sex and age. Patients are often capable of suppressing tics for limited periods of time.
Analysis of family data is consistent with an autosomal dominant mode of inheritance with incomplete penetrance (2-4). It has been suggested that a number of milder behavioral problems should be considered as variant expressions of the presumed genetic defect responsible for GTS. The chronic multiple-tic syndrome

(CMT) is generally agreed to be a variant phenotype of GTS. There is also evidence for a genetic relationship between GTS and obsessive compulsive symptoms (OCS). In recent years six research groups have initiated a collaboration, under the auspices of the American Tourette Syndrome Association, in order to localize the gene(s) responsible for GTS. Among the human genes that have recently been characterized no obvious candidate genes for GTS have been identified. Chromosomal regions 18q22.1 and 9p23-pter, implicated by structural rearrangements in GTS patients have failed to generate positive evidence for linkage (5,6). In a systematic global genome search the collaborating research groups have tested more then 600 genetic markers (5-12). No strong and definite evidence for linkage was obtained. Assuming locus homogeneity, and considering CMT as a variant phenotype of GTS, an exclusion map based on a well localized subset of markers showes exclusion of at least 80% of the human autosomes.

In linkage studies for bipolar disorder and schizophrenia promising findings could not be confirmed or supported (13-15). The failure to localize genes for psychiatric disorders via linkage analysis has generated a broad discussion in the literature, not only about the appropriateness of single-gene assumptions for complex disorders (16-24), but also on the question how genetic disease entities should be delineated in the intricate diagnostic classification schemes of today's psychiatry. Some authors propose to include only the most extreme phenotypes in the linkage analysis, assuming that these phenotypes are most likely based on genetic factors (25,26). Others have carried out multiple analyses, gradually broadening the phenotype definition to include milder or less specific diagnoses (27). The former suggestion might lead to severe loss of information, while the practice of multiple testing will undoubtedly give rise to an increased frequency of false positive linkage findings if no statistical corrections for multiple testing are made. Decisions on the strategy of choice have been made arbitrarily.

Linkage studies on GTS are subject to the same complexities as those for other psychiatric disorders. The question arises whether GTS should be considered as yet another example of a complex disease where linkage analysis will fail to produce dependable conclusions. Alternatively, the accumulated family material might simply be not large enough to produce convincing evidence for linkage.

In an attempt to characterize the available family material more accurately with respect to size, diagnostic uncertainties and sensitivity to clinical and genetic assumptions, we have carried out an extensive simulation study. The use of computer simulations to evaluate the adequacy in size of a linkage sample is becoming more common (28); its use has also been suggested for the assessment of the expected frequency of false positive findings in a multiple test situation (29).

Terwilliger and Ott proposed efficient procedures to reduce the time needed for this analysis (30).

In this simulation study we are addressing three major questions concerning the collaborative data set of GTS families. Firstly, what is the probability of generating conclusive evidence for linkage in these combined families assuming locus homogeneity? Secondly, which of these families contains sufficient linkage information for mapping under extensive locus heterogeneity? Finally, what approach should be taken in the analysis with respect to the spectrum disorders, CMT and OCS? We systematically investigated whether a narrowly defined phenotype would give better probabilities to detect linkage compared to a strategy where three diagnostic categories with a broadening in the spectrum of included clinical characteristics was used.

Our findings are of relevance to researchers involved in the mapping of psychiatric disorders, but the approaches presented here are also applicable in mapping projects of other complex disorders.

MATERIAL AND METHODS

Family material

In the study reported here we used pedigree information and diagnostic data on thirty-two GTS families ascertained by six different research groups: Erasmus University Rotterdam, The Netherlands (12 families), Yale University School of Medicine, New Haven, CT, USA (2 families), Marshfield Medical Research Foundation, Marshfield, WI, USA (1 family), Hospital for Sick Children, Toronto, Canada (1 family), University of Iowa, Iowa City, Iowa, USA (16 families).

All families have been previously included in linkage studies, as described elsewhere (4,10-18,31) except for the family that was contributed by the research group from Toronto. This set of families was also used in a segregation analysis which will be reported separately. A detailed description of the pedigrees is available upon request.

All diagnosed subjects were personally interviewed by investigators from the contributing centers. For diagnostic assessment a structured questionnaire was used with a section on GTS and CMT (32). A separate questionnaire was used for OCS. Diagnoses were confirmed by independent clinical investigators who had no prior knowledge of family history.

Statistical analyses

The assumptions concerning the mode of inheritance of GTS made in the analyses

presented here, were identical to those adopted in our previous linkage studies (9,11-18). GTS was taken to be caused by an autosomal dominant mutation, incompletely penetrant, with a population frequency of 0.003. Probabilities to express the complete GTS phenotype were 0.81 and 0.31 for male and female gene carriers respectively, and 0.005 and 0.003 for males and females without this genetic defect (phenocopy frequency). Persons with CMT or OCS were treated in various ways, depending on the diagnostic model chosen. Three different diagnostic models were applied in these analyses: a broad, intermediate, and narrow model. In the broad model, it was assumed that the GTS gene also predisposes to development of both the CMT and the OCS phenotypes. Probabilities to manifest these phenotypes were 0.9 and 0.6 for CMT (males and females respectively), and 0.9 and 0.71 for OCS. In the broad model, phenocopy rates for both phenotypes were fixed at 0.01 for males and 0.006 for females. In the intermediate model, subjects with OCS were treated as 'phenotype unknown', which implies that they did not contribute directly to the linkage analysis, although their marker genotypes may have aided in the reconstruction of marker genotypes for unavailable persons. In the narrow diagnostic model, subjects with CMT were also treated as 'phenotype unknown'.
Penetrances for persons homozygous for the abnormal GTS allele were kept identical to those for heterozygous individuals.
Male and female recombination fractions were assumed to be equal.

The computer simulations that we carried out will be described in three separate steps: i) the construction of marker data for a hypothetical marker either closely linked or unlinked to the GTS gene, ii) the linkage analysis of those marker results as if it concerned real marker data, and iii) the evaluation of the resulting lod scores.

Marker data were generated for all family members for whom DNA was available in reality, using the computer program SLINK (33). Markers of various informativeness (2, 4, and 8 alleles), with a polymorphism information content (PIC value), of 0.375, 0.70 and 0.86 respectively, were simulated to be either linked to the GTS gene with 5 % recombination, or unlinked. For each family 100 or 400 distinct replicates were prepared depending on the size of the family. Simulations were carried out separately for each of the three diagnostic models (for the linked marker only; in the absence of linkage, the choice of the diagnostic model will not influence the construction of the hypothetical marker data).
Analysis of the resulting data was carried out for each replicate of each family

separately, with a slightly modified version of the MLINK option of the LINKAGE package, version 5.03 (33). All simulated data were analyzed under the diagnostic scheme used for simulation, but also under the other diagnostic models. Lod scores for each replicate were calculated for recombination fractions ranging from 0.0 to 0.5 in steps of 0.01. The resulting lod score lists were manipulated via the computer programs SIMSUM and SIMCOMP (unpublished programs by L.A. Sandkuijl) to yield expected lod scores for individual families and for sets of families. Expected lod scores for individual families were calculated for each marker and each model. For each replicate of a given family, the maximum lod score was identified. The mean of those maxima (over 100 or 400 replicates) was taken to represent the expected lod score in that family.

The expected maximum lod score in a set of families was obtained via a bootstrap procedure, as proposed by Terwilliger and Ott (34). For each family of a given set, a replicate was selected at random. Lod scores for the selected replicates were summed for each value of the recombination fraction, and the resulting lod score curve was taken to represent one simulated replicate of that set of families. Unless otherwise specified, analyses of sets of families were based on 10,000 bootstrapped replicates.

Corrections for multiple testing were as proposed by Risch (35): lod score threshold = 3 + LOG(t) where t represents the number of tests carried out.

RESULTS

Individual families.

Expected lod scores varied widely between the thirty-two families, and, for a given family, between different diagnostic models. With a four-allele marker thirteen of the families yielded mean lod scores of 0.5 or more under at least one of the diagnostic models (figure 1). For these families, the broadest diagnostic model yielded the highest lod score, but differences between the broad model and the narrower ones showed marked variation between families. In family S18 all linkage information appeared to be based upon persons with tics, while in family S14 information depended almost exclusively on GTS patients. Family S14 was the most informative family under all diagnostic models. Under the narrow model, and with only a two-allele marker, this family already generated an average lod score of 2.4. With the more informative markers, this average lod score increased to 4.8 for a four-allele marker and to 6.0 for an eight-allele marker. Kindreds S8, S10, and S13 also yielded mean lod scores close to (S8), or over 3.0 with a four-allele marker, but only for the broader diagnostic models. For the thirteen most informative

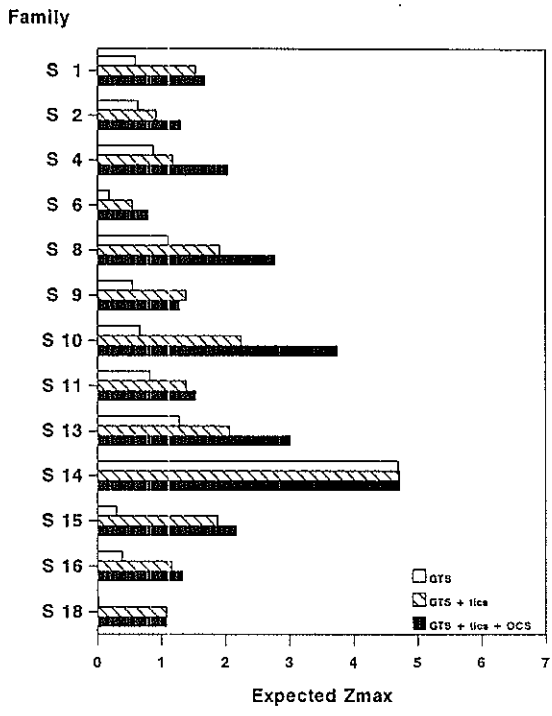

Fig 1. Mean lod scores per family under three diagnostic models for a four-allele marker, PIC=0.7

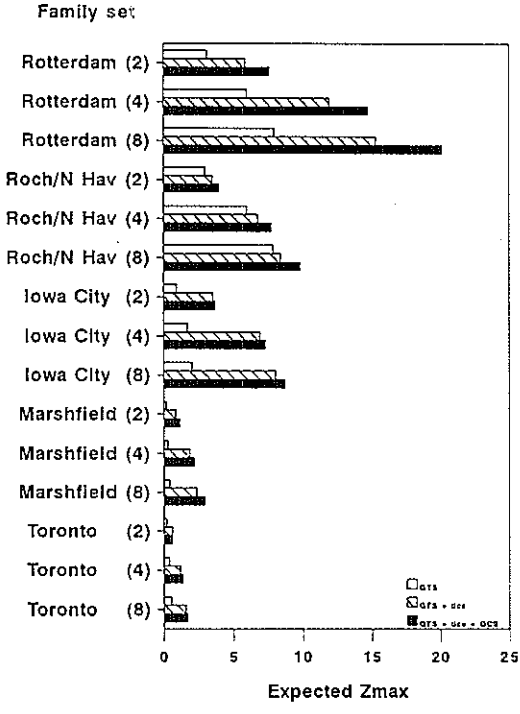

Fig 2. Mean lod scores under genetic homogeneity for three diagnostic models. results are presented per family set ascertained at the six collaborative research centers. Values between brackets indicate the informativety of the simulated marker. (2) Two-allele marker with PIC=0.375. (4) Four-allele marker PIC=0.7. (8) Eight-allele marker PIC=0.86.

families, use of a two-allele marker led to an average loss in lod score of 50.6 % when compared to results for a four-allele system, while lod scores increased by an average 30.9 % when the most informative marker was used.

Combinations of families.

In order to assess the probability to map a GTS gene for each of the research groups contributing to the collaborative GTS mapping effort, the results of the simulations were grouped by contributing center. In each of these sets of families, the average expected lod score was calculated (figure 2). The family sets from Rotterdam and Rochester/New Haven could already generate significant lod scores with a two-allele marker under the most restrictive diagnostic model. When more informative markers and the broadest diagnostic scheme were applied, the expected maximum lod score rose to well above the generally accepted threshold for significance; the mean Z_{max} amounted to 20.1 and 9.8 for data sets from Rotterdam and Rochester/New Haven, respectively.

Use of incorrect model for analysis.

The results presented so far were obtained by generating marker data for a given diagnostic model, and analyzing the data under the identical model. We have also analyzed the data for the Dutch families under different, 'incorrect' models, thereby obtaining an indication for the loss of statistical power when incorrect diagnostic models are used.

Ten thousand different combinations of the simulated data for the Dutch families were analyzed with the broad, intermediate and narrow diagnostic models seperately. For each model, the frequency of a lod score above three was scored. In addition, we applied the three analysis models simultaneously on 10,000 simulated family sets, and scored how frequently a lod score above 3.477 was obtained under at least one of the diagnostic models. In this latter analysis, a higher lod score threshold was chosen, in order to compensate for the increased probability of false positive linkage findings due to multiple tests (35).

Results are presented in Table 1. For marker data generated under the broadest model, the analyses yielded almost always significant results with the broader models, but with the narrow model only in approximately 50 % of cases. Using the broad diagnostic model in the analysis when the simulated, 'true', model was narrow resulted in a dramatic loss of power: significant results were obtained in only 3.2 % of all replicates.

Similar analyses were carried out on data simulated under the absence of linkage. Here, as many as 500,000 replicates were evaluated, in order to count the rare

occurrences of false positive linkage findings. The frequency of incorrect linkage findings was remarkably low: between 19 and 25 per 500,000 (Table 1).

Impact of diagnostic instability.

For one of the families, family S14, the diagnoses have been re-evaluated via repeated interviews. Diagnoses have changed for several family members, and additional diagnoses have been obtained for subjects who had not been interviewed previously. For ten subjects, hitherto regarded as unaffected, the diagnosis of GTS was established upon their second interview. For three subjects, previously regarded as unaffected, the diagnosis of CMT was established in the second interview. Diagnosis for one subject changed from GTS to unaffected and diagnosis of one subject changed from CMT to unaffected.

In order to evaluate the possible impact of diagnostic instability, we carried out simulations in these families under the assumption that the recently updated diagnoses are correct. Subsequent lod score calculations were carried out once with the updated diagnoses, and once with the older diagnostic information. The intermediate diagnostic model was used in both the simulations and the analyses. In these analyses not a single lod score exceeded the threshold of three in the analyses with the older diagnoses (figure 3). The average loss in peak lod score due to use of the older diagnoses was 84.5 %. In an attempt to reduce this damaging effect of diagnostic instability, we carried out additional analysis under the narrow diagnostic model. An approximately equal reduction in lod score of 86.3 %. was obtained (data not shown).

Table 1. Expected frequency of lod scores over 3.0 in the Dutch data set for various models of simulation and analysis.

Model of simulation	Model of analysis			
	Broad	Interm.	Narrow	Combined*
Broad	99.9 %	98.2 %	48.0 %	99.9 %
Intermediate	84.4 %	99.7 %	73.2 %	99.5 %
Narrow	3.2 %	13.0 %	78.4 %	70.5 %
Unlinked	$19/0.5x10^6$	$22/0.5x10^6$	$25/0.5x10^6$	$20/0.5x10^6$

* For the combined tests, a lod score threshold of 3.477 was applied.

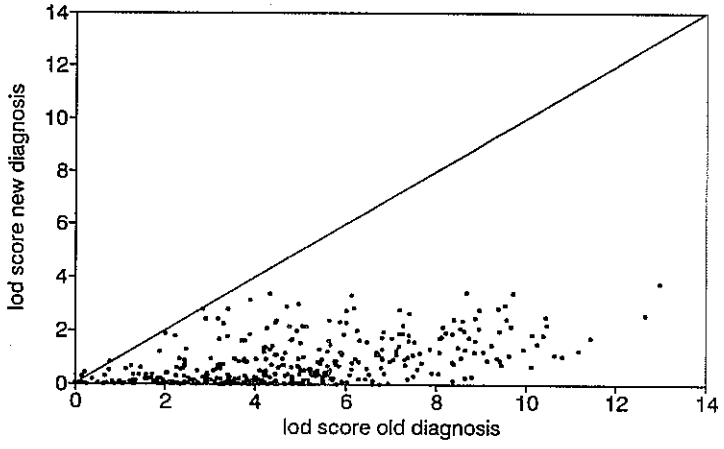

Fig 3. The effect of changed diagnosis in family S14. Simulation of a four-allele marker (PIC=0.7) under the intermediate diagnostic model with new diagnoses. Analysis under the intermediate model with the old diagnoses (X axis) and the new diagnoses (Y axis).

DISCUSSION

In planning a linkage study, the use of computer simulations for evaluation of the adequacy in size of a linkage sample is becoming more common. In our ongoing collaborative effort to localize the gene(s) responsible for GTS simulation studies had not been applied. Now that more than 600 markers have been tested without evidence for linkage, two questions arise: Is the set of available families adequate to detect linkage and, what is the effect of diagnostic uncertainties on the power to detect linkage in this family set?

Our current results show that when locus homogeneity is assumed the power of the family set to detect linkage is very high with all three diagnostic models. Even when a two-allele marker is used, significant results can be obtained (figure 2). From the increase in the expected lod scores it can be concluded that an important part of the available information was derived from patients with the associated spectrum disorders, CMT and OCS.

In order to have a family set informative enough to detect linkage in each collaborating center, family material was shared between different groups so that all centers have access to adequate family material for their linkage studies.

By pooling all the available linkage data on more than 600 markers from the six

research centers more than 80% of the genome can be excluded as a site for the GTS gene, assuming that CMT is variant expression of the GTS gene defect. Most markers, however, have been tested in a subset of the families. Therefore we have to consider the possibility of locus heterogeneity. In our simulations we studied the power of each separate family. One family was informative enough to detect linkage under all diagnostic models, while three other families yielded evidence for linkage under the two broader diagnostic models. We conclude that even in the case of the most extensive genetic heterogeneity the family set is informative enough to detect linkage.

Other explanations for the failure to detect linkage are the diagnostic uncertainties for GTS. Associated behavioral problems as CMT and OCS are likely to be variant expressions of the GTS gene defect but are more difficult to diagnose. Furthermore, GTS shows a waxing and waning course of expression and patients are often able to suppress symptoms for limited time. This may lead to misdiagnosis or inclusion of phenocopies. Both the role of spectrum disorders and of uncertainty of diagnosis were studied in our simulation studies by the testing of three diagnostic models and the testing of the impact of diagnostic instability on the statistical power to detect linkage. As a remedy against false positive linkage findings it has been proposed to include only the most narrow phenotypes in a linkage analysis. This will result in a loss of information but it seems more likeley that the phenotype is the result of a genetic factor (Table 1). This method does not protect us however, against the consequences of diagnostic instabilities. An alternative solution that has been proposed is to define different models with a broadening in diagnostic criteria. This approach has been used by the GTS consortium research centers in their linkage studies. There is an increased risk on false positive results which can be circumvented by a correction for multiple testing. Risch (35) proposed to increase the lod score threshold of significance of 3.0 with a factor equal to the logarithm of the number of tested models. In this case three genetic models were tested implying an increase in lod score to 3.477. In the simulated data of the Dutch families we investigated whether the use of three diagnostic models would protect us better against false negative findings than the use of a single model that carries the risk of mis-specification.

If CMT and OCS are variant expressions of the GTS gene defect, analysis of the two broadest models was very powerful. In more than 98% of the replicates linkage was detected (Table 1). Analysis with the narrow model showed a considerable loss in information, the power to detect linkage was lowered to 48% as linkage information from individuals expressing CMT or OCS was lost. If OCS is not part of the GTS spectrum (intermediate model) the probabilities to detect

linkage using three analysis models are high, between 73% and 99.7%, but a dramatically reduction is observed when the most narrow model is correct. If analyzed correctly the probabilities of reaching a threshold of three are 78.4% but they drop to 3.2% if analyzed under the broadest model.

These results can be explained by the fact that individuals with CMT and OCS will be classified inappropriately as gene carrier, and frequently will be scored as recombinants in the analysis. The results indicate that choosing a single genetic model will lead to a dramatic loss of information except if by chance the correct model is chosen. As a remedy the application of three diagnostic models is very effective (Table 1). Whatever diagnostic model is correct, the probability of detecting linkage always exceeded 70%.

This situation does not necessarily apply to other disorders. In this specific case a large amount of the information is obtained from associated behaviors of GTS. For other disorders the gain in information using broader models might not be enough to compensate for the raise in the lod score threshold.

When multiple models are tested, an increased lod score threshold should be adhered to due to of an increased probability of false positives. We evaluated the appropriateness of the correction proposed by Risch. When only a single model was tested, the frequency of false positive findings was extremely low (less then 1 in 20,000, see Table 1). When all three tests were applied simultaneously with a threshold of 3.0 each, an approximate threefold increase in false positive findings was observed (1 in 7812, data not shown). The proposed correction dealt appropriately with this (only 1 in 25,000 cases the lod score threshold of 3.477 was exceeded).

Testing three diagnostic models is not a remedy against diagnostic instability. The waxing and waning course of phenotype expression and the possibility that patients suppress tics lead to diagnostic instability. This can result in a dramatic loss in lod score as we observed due to the updated diagnoses in one of the families (figure 3). Diagnostic instability could very well vary between different families, but could also be the result of the long time span between subsequent interviews. The time between the two series of diagnoses in this family was approximately five years. Recently, a second family was completely re-diagnosed after a period of only eighteen months without any changes in diagnosis. In order to account for the diagnostic uncertainties we propose to establish a lifetime diagnosis via repeated interviews. Individuals that have been diagnosed as affected will remain affected in the analyses even if in subsequent series of diagnosis no disease phenotype is observed. Linkage information from unaffected individuals could be omitted in the analysis by applying alternative analytic methods as

affected only analysis and sib-pair analysis.
In conclusion, the available family set is informative enough to detect linkage even in the case of extensive heterogeneity. In order to increase the chances of detecting linkage the four most informative families will be shared between all the members within the consortium. The consequences of diagnostic instability can be dramatic. As a remedy the members of the consortium agreed to re-investigate the available families periodically and to perform sib-pair analysis on a large set of sib-pairs.
By using simulation techniques the strategy of choice for a linkage study can be determined systematically instead of being made arbitrarily. In the case of GTS the simultaneous testing of three diagnostic models was shown to give the best probabilities on detecting linkage.

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CHAPTER 5.2

NO EVIDENCE FOR GENETIC LINKAGE OF GILLES DE LA TOURETTE SYNDROME ON CHROMOSOME 7 AND 18.

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ABSTRACT

Gilles de la Tourette Syndrome is an heritable neuropsychiatric disorder. In order to determine the chromosomal localisation of the locus involved, genetic linkage studies were initiated in six extended families. The Gilles de la Tourette gene has been tentatively assinged to chromosome 18q22.1. In our present study no evidence for genetic linkage on chromosome 18 and chromosome 7 was obtained. Data from the markers tested made it possible to exclude the whole of chromosome 18 and the chromosome 7q21.3-qter region as a site for the Gilles de la Tourette gene.

INTRODUCTION

Gilles de la Tourette Syndrome (GTS) is a neuropsychiatric disorder with an unknown etiology. The syndrome is characterised by recurrent, involuntary, repetitive multiple motor and vocal tics. In many patients associated behavioural problems like obsessive compulsive behaviour and copro- and echophenomena are observed (1).

There is strong evidence that GTS is genetically determined. The exact mode of inheritance, however, is still a matter of discussion. Both single major locus and multifactorial models have been proposed (1-6). The most widely held hypothesis states that a single autosomal dominant gene with reduced penetrance is involved (6). However, it has been discussedthat associated behavioural symptoms should be considered as variant expressions of the presumed genetic defect responsible for GTS. There is general agreement that the chronic tic syndromes, according to DSMIII-R criteria (7), in families afflicted with GTS are variant phenotypes of the GTS gene defect (2).

Comings *et al.* (8) presented evidence for the localisation of the GTS gene. They reported a 46 t(7;18)(q22;q22.1) reciprocal translocation in six relatives suffering from GTS. No support for linkage was found on the breakpoint on chromosome 7q22 with the COLA1 locus at 7q21.3-q22.1, suggesting a localisation of the GTS gene near the 18q22.1 breakpoint. Donnai (9) reported a GTS patient with a deletion of the long arm of chromosome 18 at 18q22.2. These findings led to the tentative assignment of the GTS gene to chromosome 18q22.1. At 18q21 a candidate gene, the Gastrin Releasing Peptide (GRP), has been localised coding for a neuropeptide like protein (10,11).

In order to determine the chromosomal location of the GTS gene, we started genetic linkage studies in five families of Dutch origin and in one family of Norwegian origin. In our present study we found no support for linkage, on either

chromosome 18 or chromosome 7, including the COLA1 locus (7q21.3-q22.1) near the translocation breakpoint.

MATERIAL AND METHODS

Family Material

Clinical and genetic studies were performed in five Dutch families and one Norwegian family. Complete pedigree data and methods of ascertainment on these data will be published elsewhere (van de Wetering *et al.* in preparation), and are briefly summarised here.

All subjects have been investigated using a standardized psychiatric interview with an added section on GTS and Tics (Yale Scale, Dr. D.L. Pauls, New Haven). Only individuals with GTS or Tic Syndrome, according to DSMIIIR criteria (7) were regarded as affected. The interviews were reviewed by an independent psychiatrist and a neurologist without prior knowledge of the family history. A total of 236 individuals have been investigated, of whom 48 were considered as affected.

Pedigrees of the families used in this study are not shown to protect the privacy of the inividuals that collaborated in this study. Pedigrees can be send to investigaters only, on request.

DNA Analysis

DNA was isolated from peripheral blood lymphocytes of family members as described by Miller *et al.*(12). Chromosomal DNA was digested with various restriction enzymes (Boehringer, Pharmarcia, BRL) according to the manufacturers instructions. Gelelectrophoresis of 8µg DNA samples on 0.7% agarose gels, and DNA immobilisation by alkaline blotting onto nylon membranes (Gene Screen plus) were done according to standard procedures (13). Hybridisation conditions were as described by Maniatis (13), washing was performed at 65°C at 0.1 x SSC final stringency. DNA was labelled by random hexamer priming according to Feinberg & Vogelstein (14).

Markers B74 (D18S3), OLVIIA8 (D18S7), OS-4 (D18S5), pHF12-62 (D18S1) and pERT25 (D18S11) were used as reference points for chromosome 18 as they had been mapped previously by physical methods and were used for the construction of a continuous linkage map for chromosome 18 (15). They were kindly provided by Drs. J.L. Mandel, H. Olek, H. Tateishi, R. White and U. Müller, respectively. Markers OLVIIE10 and GRP have previously been described (16,17) and shown to map to chromosome 18.

Markers pJ2(TCRB), Cgamma (TCRG), pMetH (MET), NJ3 (COLA1), pTHH28

(D7S371), pRMU7.4 (D7S370), pYNB3.1R (D7S372) C33 (D7S126), TM102L (D7S135) and pB79A (D7S13) were previously mapped on chromosome 7 and used in a linkage map (18). In our present study they were used as reference points for chromosome 7. These markers were kindly provided by Drs. T.W. Mak, R. White, P. Tsipouras, Y. Nakamura, L. Tsui and J. Schmidtke, respectively. Markers pXV-2C (D7S23) and TN127 (D7S144) have been described previously and shown to map to chromosome 7 (19,20).

Linkage analysis

Linkage analysis has been performed using the LINKAGE programs package version 5.03 (21,22). The GTS gene frequency was estimated to be 0.003, with a male penetrance of 0.999 and a female penetrance of 0.56. The correction for possible phenocopies was 0.0002 (6). Two point linkage analysis was performed with the MLINK program, multipoint analysis with the LINKMAP program using Haldane's mapping function for interference. No allowance was made for spontaneous mutations. In the multipoint analysis we assumed a constant sexratio for crossing over of 2.1 (Female/Male) for chromosome 18 (15). For chromosome 7 we assumed a constant sexratio for crossing over of 2.0 (18). A lod score of at least 3.0 was considered as evidence for genetic linkage, a lod score of -2.0 was considered as evidence for exclusion of linkage, for the assumed model of a single dominant gene with reduced penetrance (23).

RESULTS AND DISCUSSION.

Chromosome 18 markers were tested in six extended families and a linkage map was constructed using the continuous linkage map of O'Connell *et al.* as a basis (15). Mapping the genetic distances of corresponding markers B74, pHF12-62, OLVIIA8, OS-4 and pERT25 with our own material differed only marginally from the O'Connell mapping distances. Additional markers OLVIIE10 and GRP were then mapped, with our family material, into the fixed O'Connell linkage map.
Comings *et al.* and Donnai (8,9) postulated that the gene responsible for GTS resides on chromosome 18q22.1. Markers OLVIIE10 at 18q21.3, GRP at 18q21, OS-4 at 18q21-qter and pERT25 at 18q23 map into the region surrounding the t(7:18)(q22:q22.1) translocation breakpoint (24). Given the linkage map and the cytogenetic maps (15,24, fig 1.) the most likely localisation for the translocation breakpoint is between markers OLVIIE10 and OS-4.
Lod scores for the two point analysis of chromosome 18 between GTS and marker loci are shown in table 1. Only individuals presenting GTS or Tic Syndromes were

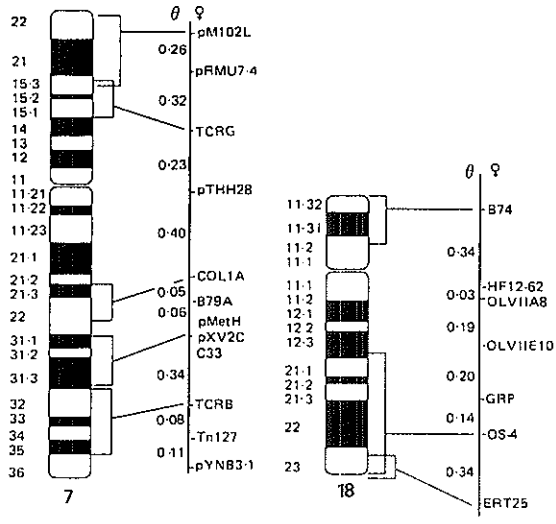

Figure 1. Female specific genetic maps of chromosomes 7 and 18. Physical locations are indicated where known. Marker order and map distances are based on the linkage maps of O'Connell et al[15] [18] and mapping studies with our family material. Map distances are presented as Θ, assuming a constant female/male crossing over ratio of 2.0 for chromosome 7 and 2.1 for chromosome 18.

included as affected in our present study. None of the markers tested showed evidence for linkage and they excluded linkage for the genetic distance mentioned in table 1. Using multipoint analysis (fig 2), we obtained lod scores of at least -5 for the translocation breakpoint region between markers OLVIIE10 and OS-4. A lodscore of at least -2 was obtained for the complete linkage map of chromosome 18. This value is generally accepted as evidence for the exclusion of linkage (23). We therefore conclude that chromosome 18 can be excluded as a site for the GTS gene. These results are not in agreement with the findings of Comings *et al.* and Donnai (8,9).

Another possible site for the GTS gene would be the breakpoint of the translocation on chromosome 7q22 reported by Comings *et al* (8). We have tested several RFLP markers on chromosome 7 with our family material, and a linkage map was constructed. Comparison of our linkage map with the primary linkage map of O'Connell *et al.*(18) gave marginal differences only except for marker C33, which we mapped at a theta of 0.01 telomeric of pMetH instead of theta 0.11 in the O'Connell map. Our results are consistent with the findings of Rommens *et al.* (19). To avoid possible mapping errors we did not include marker C33 in the multipoint analysis. Marker TN127, not on the primary linkage map of O'Connell *et al.*(18), was mapped using our family material and added to the combined linkage map

Table 1. Two point linkage data for chromosome 18.

	Recombination fraction (Θ.)							
Locus	.0	.05	.1	.15	.2	.3	.4	Exclusion*
B74	-23.46	-6.68	-4.24	-2.86	-1.96	-0.85	-0.26	25
pHF12-62	-15.89	-3.92	-2.18	-1.26	-0.69	-0.11	0.07	11
OLVIIA8	-14.33	-3.92	-2.22	-1.32	-0.77	-0.21	-0.02	11
OLVII10	-13.88	-5.39	-3.34	-2.20	-1.46	-0.62	-0.24	18
GRP	-10.26	-2.76	-1.84	-1.30	-0.91	-0.40	-0.11	5
OS4	-12.77	-4.32	-2.83	-1.93	-1.31	-0.51	-0.11	17
pERT25	-25.07	-8.04	-4.47	-2.61	-1.50	-0.44	-0.11	18

*: Centimorgans definitely excluded on either site of the tested marker, using Haldane's mapping function (23). A lodscore of -2 or less was assumed as definite proof for exclusion.

Table 2. Two point linkage data for chromosome 7.

	Recombination fraction (Θ)							
Locus	.0	.05	.1	.15	.2	.3	.4	Exclusion*
Tm102L	-22.62	-5.09	-2.48	-1.17	-0.43	0.19	0.22	11
TCRG	-12.68	-4.90	-3.29	-2.29	-1.60	-0.73	-0.26	18
pRMU7.4	-21.01	-5.06	-2.92	-1.76	-1.04	-0.58	-0.30	11
pTHH28	-11.04	-2.78	-1.43	-0.75	-0.36	-0.01	0.07	5
NJ3	-13.62	-2.73	-1.26	-0.54	-0.13	0.23	0.23	5
pB79A	-12.28	-3.28	-1.75	-0.93	-0.44	0.02	0.10	5
pMetH	-17.36	-3.91	-1.95	-1.00	-0.47	-0.01	0.06	11
pXV2C	-25.28	-6.47	-3.46	-1.93	-1.02	-0.13	0.11	17
C33	-1.54	0.04	0.20	0.23	0.21	0.12	0.03	0
TCRB	-21.18	-6.15	-3.63	-2.20	-1.28	-0.30	0.04	18
Tn127	-15.73	-4.98	-3.05	-1.99	-1.31	-0.52	-0.14	18
pYNB3.1	-7.28	-2.63	-1.45	-0.82	-0.43	-0.05	0.05	5

*: Centimorgans definitely excluded on either site of the tested marker, using Haldane's mapping function (23). A lodscore of -2 or less was assumed as definite proof for exclusion.

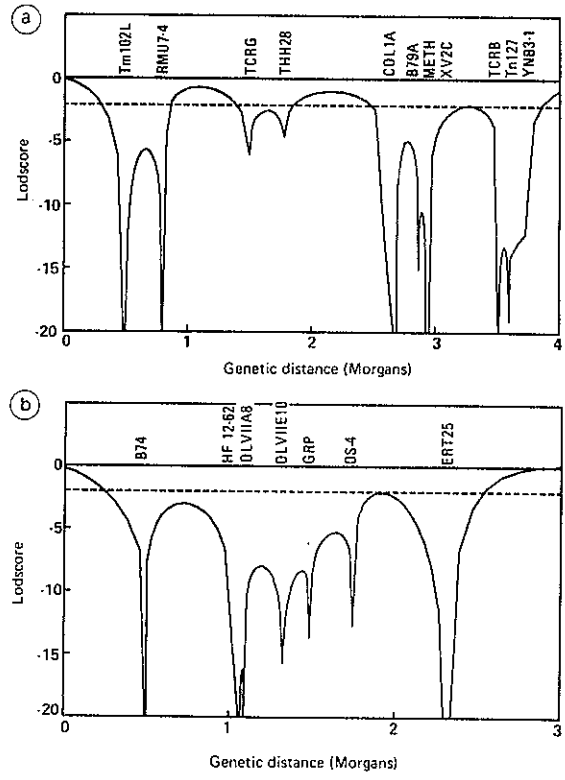

Figure 2. Multipoint linkage analysis showing the exclusion of the GTS locus from chromosome 7q21.3-qter (a) and chromosome 18 (b). Dotted line represents a value of -2.

(fig 1).

For none of the markers tested on chromosome 7 did we find evidence for linkage, including the COLA1 locus which is located proximal of the EPO locus at 7q21 and thus must be located proximal of the presumed breakpoint of the translocation (24). With multipoint analysis we have been able to exclude part of chromosome 7p and the 7q21.3-qter region including the translocation breakpoint region. The region around marker C33 is excluded by flanking markers pMetH, pXV2C and TCRB in the two point analysis as well as in the multipoint analysis, even if we assume that marker C33 is localised at a theta of 0.11 of pMetH.

With the exclusion of chromosome 18 as a possible site for the GTS gene and the exclusion of the 7q21.3-qter region of chromosome 7, we conclude that the 46 t(7;18)(q22;q22.1) reciprocal translocation is not linked to the gene responsible for the Gilles de la Tourette Syndrome. However, genetic heterogeneity could mask a

positive result. In this study, all families contributed to the negative lod scores on both chromosomes.

Currently, we are performing collaborative genetic linkage studies on other parts of the genome in order to find the location of the GTS gene (25). Chromosomal rearrangements in families suffering from GTS could facilitate the localisation of the GTS gene but should be followed by extensive linkage studies, in order to obtain definite proof for genetic linkage.

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CHAPTER 5.3

PROGRESS IN THE SEARCH FOR GENETIC LINKAGE WITH TOURETTE SYNDROME: AN EXCLUSION MAP COVERING MORE THAN 50% OF THE AUTOSOMAL GENOME

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SUMMARY

Gilles de la Tourette Syndrome is a neuropsychiatric disorder with an autosomal dominant mode of inheritance and reduced penetrance at a single genetic locus. Several research groups have genetic linkage studies underway to detect the chromosomal location of the gene that predisposes for this disorder. Strong and clear evidence of linkage has not yet been produced for Tourette Syndrome. This paper presents an overview of the methods and progress of the groups centered at Yale University and Erasmus University in excluding linkage from a large portion of the genome. Our labs have screened 228 genetic marker loci for linkage with a gene for this disorder in a series of affected families in the United States, Canada, the Netherlands, and Norway. More then 50% (and perhaps as much as 66%) of the autosomal genome has now been excluded on the assumtion that genetic heterogeneity is not an important factor in the Tourette Syndrome pedigrees pooled for this summary.

INTRODUCTION

Gilles de la Tourette Syndrome (GTS) is a familial, neuropsychiatric disorder with onset in childhood characterized by chronic, intermittent motor and vocal tics (Kidd *et al* 1980, Pauls *et al.* 1981, 1984). In addition to tics, affected individuals frequently display symptoms such as attention deficit hyperactivity disorder (ADHD) and/or obsessive compulsive disorder (OCD). Coprolalia and echolalia, which are complex vocal tics, are often also associated with the syndrome. Sex and age affect the risk of expressing GTS. Genetic analyses of family data have been consistent with the hypothesis that susceptibility to the disorder is most likely due to a single genetic locus with a dominant mode of transmission and reduced penetrance. Recent reviews of the disorder and the genetic models that have been considered can be found in the work of Price *et al.* (1987) and Pauls *et al.* (1990). In families affected by GTS, the chronic multiple tic syndrome (CMT) consisting of motor or vocal tics (but not both) is generally agreed to be a variant, milder phenotype of the GTS gene (Kurlan *et al* 1987, 1989). Research that has examined the relationship between GTS and obsessive compulsive disorder (OCD) suggests that OCD is etiologically related to GTS, and represents a variant phenotype of the disorder that may be more commonly expressed in females (Pauls and Leckman, 1986). However, there is no general agreement on whether individuals suffering from OCD alone should be included as affected with GTS. The nature of the relationship between ADHD and GTS is less clear. In the present report, we shall

report the results of testing for genetic linkage with GTS using a conservative diagnostic scheme in which affected status includes either the core GTS syndrome or chronic multiple tics.

Establishing strong evidence for genetic linkage between GTS and one or more marker loci would clearly demonstrate the existence of a major locus and help to obtain a better understanding of the pattern of inheritance of the syndrome, since it would then be possible to identify carriers of the disease-related gene independently of the observed clinical phenotype. Linkage studies can be a first step toward characterizing the products and function of the locus responsible for GTS during development and thus can lead to useful therapeutic remedies. Several research groups have started genetic linkage studies on GTS. Interim results of their work were presented at the Tourette Syndrome Association meeting in Cambridge, England in August of 1989. In the present collaborative paper we present an overview of the genetic markers typed and analyzed so far by the research groups led by Dr. K. K. K. at Yale University in New Haven,CT and by Dr. B.A.O. at Erasmus University at Rotterdam.

SUBJECTS AND METHODS

Families

In Rotterdam, clinical and genetic studies have been carried out in five families of Dutch origin and one family of Norwegian origin. The complete pedigree data and methods of ascertainment will be published elsewere (B.J.M. van de Wetering unpublished data). In brief, 236 individuals have been investigated by using a structured questionnaire (Pauls *et al.* 1980) extended to include a section on GTS and CMT. Only individuals with GTS or CMT, according to DSMIII(R) criteria, have been regarded as being affected. The interviews were reviewed by an independent psychiatrist and by a neurologist, neither of whom had prior knowledge of the family history. Also, genetic studies were performed on a North American family which was investigated using the same structured questionnaire (Pauls *et al.* 1980).

In New Haven, four large pedigrees have been collected with the help of R.Kurlan and D.L.Pauls. The three principal pedigrees which have contributed to the linkage results pooled together here include the Canadian (184 individuals in the pedigree structure analyzed) (Kurlan *et al.* 1986), Oregon (54 individuals), and the Michigan (43 individuals) families. Pauls *et al.* (1990) can be consulted for descriptions of the methods of ascertainment and diagnosis.

DNA Analysis

At New Haven, lymphoblastoid cell lines have been established on some 230 individuals out of the 281 who are are included in the Canadian, Oregon, and Michigan pedigree structures analyzed for linkage. The standard methods followed in establishing cell lines, extracting DNA, and phenotyping the RFLP systems used have been described elsewhere (Kidd *et al.* 1986). The 176 RFLP systems and classical blood groups and serum proteins tested for linkage in New Haven can be found in Table 1. The laboratory of R.S. Sparkes typed the classical genetic markers employing standard methods (Spence *et al.* 1977).

RFLP analyses at Rotterdam followed standard procedures (Maniatis *et al.* 1982) on genomic DNA isolated from peripheral blood lymphocytes of family members (Miller *et al.* 1988). DNA was labeled by primed synthesis according to the protocol of Feinberg and Vogelstein (1983). Those markers whose names begin with "mfd" were typed by the group of J.Weber according to procedures described by Weber and May (1989). Markers were typed on five Dutch families and on the Norwegian family, except for the mfd markers which were typed on the North American family and at least one of the larger Dutch families. The 67 genetic markers tested in Rotterdam, their cytogenetic map localization, and source are listed in table 1.

Our two labs overlap only in the typing of 15 markers. As can be seen from the distribution of typings by lab across chromosomes (table 1) our labs have generally concentrated on rather different chromosomes. The Rotterdam group has been primarily active up until now on chromosomes 7, 12, 18, and 19 while the New Haven group has focused so far on chromosomes 1, 2, 5, 10, and 17.

Linkage Analyses

Pairwise linkage analyses (at New Haven) were carried out using the LIPED program of Ott (1974) with test points generated on a 64 point test grid allowing male and female recombination frequencies to vary with the same 8 recombination fractions (0.0, .001, .05, .1, .2, .3, .4 and .5) on each axis at. Pairwise and multipoint linkage analyses have been performed using the Linkage analysis package (version 5.03) of Lathrop and Lalouel (Lathrop *et al.* 1984). Two point linkage analyses have been performed with the MLINK program (at Rotterdam), and multipoint analyses with the LINKMAP program using Haldane's mapping function. The genetic models used in the linkage analyses are described in table 2. Liability calculations, which are a function of sex and age, are carried out to provide appropriate weightings for individuals who are at risk of developing the disorder but who are not expressing symptoms that would result in a diagnosis of GTS.

Both of our research groups basically used the same genetic model for the inheritance of GTS. However, there are some parameter differences. The New Haven group used an age dependent penetrance for children up to 21 years of age, while the Rotterdam group did not use an age dependent penetrance but instead did not include children under the age of 15 in the linkage analyses unless these children were diagnosed as affected. The second difference is that the number of possible phenocopies allowed is a somewhat higher value in the New Haven analyses. While the models employed by our two labs are not identical, the differences in the parameters used should not result in any significant differences in the linkage results obtained.

Exclusion Map Figures

For each human autosome, a stick map (fig. 1) was constructed in which the symbols of the genetic loci tested for linkage with GTS are ordered along the map. Marker symbols lacking a precise localization are placed in boxes next to the chromosome map and arrows emerging from the boxes indicate the approximate placement of these loci along the genetic map. The scale is about 5 cM/line, the height of a locus symbol. Exclusion zones (where lod scores are $\leq$-2.0) are displayed to the left of each stick map by darkly shaded square blocks and half-sized blocks. A square exclusion block delimits about a 5 cM region giving a clear visual indication of eliminated areas. Within the boxes the numbers next to the marker symbols within the boxes show the size of the exclusion zone, if any, around that locus.

Only genetic loci that have been tested for linkage with GTS appear on the maps. Official human gene symbols (McAlpine *et al.* 1989) and D-numbers assigned by the DNA committee (Kidd *et al.* 1989) for anonymous loci have been employed. In some instances, typically for loci unassigned to a chromosome, official symbols are not yet available. All the locus labels on the maps (except for cen, pter, and qter) which have lower case letters are probe names rather than official locus symbols, which are always capitalized. In the space between each stick map and the exclusion zone block can be found the estimated map distances (in centimorgans) for the adjacent loci. Haldane's mapping function was used in converting recombination fractions to map distances. The exclusion zones reflect the pooling of results from pairwise and multipoint linkage analyses. A lod score of less than -2 was always used in determining whether exclusionary evidence was obtained. When map distances between tested loci are very short, the locus symbols are placed together on the same line next to the stick map even when their order may be known.

We employed a wide variety of sources in constructing the genetic maps. In addition to the work done by our own labs in building up the general linkage map, we consulted summaries provided by the individual chromosome committee reports published in Human Gene Mapping 10 (1989) to help scale the overall genetic length of each autosome and to position various isolated loci utilizing physical mapping data. We also utilized other published sources including O'Connell *et al.* (1987, 1989), Nakamura *et al.* (1988a,b), Lathrop *et al.* (1988, 1989). Although a considerable amount of progress is being made in mapping the human genome, many uncertainties persist, including the exact genetic map length of most chromosomes but especially those of chromosomes 3, 6, 14-16 and 19-22. Please note that when the map distance in a region is uncertain the continuity of the map line shown is interrupted by four dots arranged in a square (::) and that any estimated distances displayed nearby may be especially susceptible to revision. In general where sizeable differences occur because of uneven male and female recombination, the largest map distance estimates have been chosen.

RESULTS AND DISCUSSION

Table 1 reports the one-sided exclusion interval for each of the 228 marker loci tested on the basis of pairwise genetic linkage analyses and also shows which of our labs has tested each. Haldane's mapping function was used to convert recombination fractions to map distances. No strong positive evidence for linkage with GTS (i.e., lod scores > 3.0) has been obtained as yet, with the exception of one false positive finding which is discussed below. Table 1 also identifies the probes for the RFLP systems used, as well as the names of the researchers who have shared their probes with our labs. The chromosomal banding interval for each locus screened can be found in this table as well. Table 3 is a summary, by chromosome, of the computation of the total, nonoverlapping, exclusion zone. At least 59% and as much as 65% of the autosomal genetic map has been excluded by the pooled results from our two labs. Note that a simple summation of exclusion zones in table 1 will not always result in the totals for exclusion zones computed in table 3 or presented visually in the figure because many of the markers are closely linked. When the exclusion zones overlap, we have taken into account the linkage information and have eliminated the redundant exclusionary information.

An exact calculation of the proportion of the autosomal genetic map excluded from linkage with GTS cannot be carried out, for two reasons. The first difficulty is that the total length of the autosomal map is not known very precisely. Given our

Table 1. The 228 Autosomal Loci Tested for Linkage with Tourette Syndrome and the Exclusion Zones Around Each Locus

Symbol	Excluded cM[a]	Probe	Chromosomal band	Lab[b]	Source
ABO	1	serological	9q34.1-q34.2	Y	R.S.Sparkes
ACADM	25	MCAD	1p31	Y	K.Tanaka
ACP1	0.1	electrophoretic	2p25	Y	R.S.Sparkes
ADA	5	pADA211	20q13.11-qter	Y	D.Wiginton
ADH2	15	pADHbeta4	4q21-q23	Y	M.Smith
AK1	0	electrophoretic	9q34.1-q34.2	Y	R.S.Sparkes
ALB	5	B44	4q11-q13	Y	R.M.Lawn
ALPL	25	pS3-1	1p36.1-p34	Y	H.Harris
AMY@	0	pEB-8	1p21	Y	M.Meisler
APOA2	15	Mfd3	1q21-q23	E	J.Weber
APOB	25	AB1	2p24-p23	E,Y	J.Scott
APOC2	15	Mfd5	19q13.2	E	J.Weber
APRT	5	M13-APRT	16q24	Y	P.J.Stambrook
ARG1	0	Arg	6q23	Y	S.Cedarbaum
AT3	10	ATIII	1q23-q25.1	Y	S.H.Orkin
BF	10	electrophoretic	6p21.3	Y	R.S.Sparkes
C3	5	electrophoretic	19p13.3-p13.2	Y	R.S.Sparkes
CA2	11	H25-3.8	8q22	E,Y	R.E.Tashian
CALCA	5	pTT42	11p15.4	Y	B.D.Nelkin
CAT	1	ScaI-SnaICAT	11p13	Y	R.A.Gravel
CD8A	35	CD8	2p12	Y	A.Bowcock/P.Kavathas
CDC2	15	pOB231	10q21.1	Y	M.Lee
CHE2	0	electrophoretic	2q33-q35	Y	R.S.Sparkes
COL1A1	5	FG2	17q21.3-q22	Y	B.Sykes
COL1A2	5	NJ3 3.2	7q21.3-q22.1	E	P.Tsipouras
CRYB1	0	pSM 8A5	17q11.2-q12	Y	L-C.Tsui
CRYG@	10	p5G1	2q33-q35	Y	L-C.Tsui
CYP2E	0	mu101	10	Y	F.J.Gonzalez
DNTT	5	TdT	10q23-q24	Y	D.Baltimore
DRD2	10	hD2G1	11q22-q23	Y	O.Civelli
EGF	0	EGF 121	4q25	Y	J.C.Murray
EGR2	25	Zap 32,#367	10q21.1	Y	V.Sukhatme
ESD	0	pBM20-EL22	13q14.1-q14.2	Y	R.Bookstein
FGB	0	FbgB	4q28	Y	D.Chung

Table 1. (Continued)

Symbol	Excluded cM	Probe	Chromosomal band	Lab	Source
FNRB	10	pGem1-p32	10p11.2	Y	N.Simpson
FNRBL	5	pGEM1-32	19p	Y	N.Simpson
FY	10	serological	1q21-q25	Y	R.S.Sparkes
G10P1	0	p561	10q25-q26	Y	P.Szabo
GALT	15	electrophoretic	9p13	Y	R.S.Sparkes
GC	1	electrophoretic	4q12-q13	Y	R.S.Sparkes
GH1	10	pchGH-800	17q22-q24	Y	H.Goodman
GLO1	10	electrophoretic	6p21.3-p21.1	Y	R.S.Sparkes
GPT	0.1	electrophoretic	8q24.2-qter	Y	R.S.Sparkes
GRL	15	OB7	5q31-q32	Y	C.Weinberger
GRP	5	pB12	18q21	E	E.R.Spindel
GYPC	25	GPC	2q14-q21	Y	J.P.Cartron
HEXA	0	paHEX49	15q23-q24	Y	R.A.Gravel
HEXB	10	pHexX	5q13	Y	R.A.Gravel
HOX2	10	plasmid 3(BS3)	17q21-q22	Y	F.Ruddle/T.Miki
HP	10	Hp150 a,b	16q22.1	Y	B.Bowman
HRAS	5	J841Ha6.6	11p15.5	Y	E.H.Chang
IGF1	5	pIGF1	12q23	E	M.Jansen
IGF2	0	pHINS-311,phigf	11p15.5	Y	G.I.Bell
IL6	0	B2INF	7p21-p14	Y	P.B.Sehgal/F.Ruddle
INS	10	pHINS-310	11p15.5	Y	G.I.Bell
INT2	10	SS6	11q13	Y	G.Casey,G.Peters,Duke
JK	10	serological	18q11-q12	Y	R.S.Sparkes
KEL	1	serological	unassigned	Y	R.S.Sparkes
KRAS2	10	p640	12p12.1	E	R.A.Weinberg
LDLR	0	pLDLR-2HHI	19p13.2-p13.1	E	D.W.Russel
LPL	5	LPL35	8p22	E	M.C.Scholz
MBP	1	pP535	18q22-qter	Y	C.W.Campagnoni
MET	10	pmetH	7q31	E	R.White
MNS	10	serological	4q28-q31	Y	R.S.Sparkes
MPO	15	MPO-10A	17q21.3-q23	Y	S.C.Weil
MT2P1	10	pHM6	4p11-q21	Y	L-C.Tsui
MYC	0	p380-8ASaSs1.8	8q24	Y	C.Croce
NGFB	1	NGF	1p13	Y	J.Darby
NGFR	15	PE51	17q21-q22	Y	M.V.Chao

Table 1. (Continued)

Symbol	Excluded cM	Probe	Chromosomal band	Lab	Source
OAT	10	Hinc-Bam,SP35105	10q26	Y	J.Gusella/V.Ramesh/White
P1	0	serological	22q11.2-qter	Y	R.S.Sparkes
PAH	10	pPAH247	12q22-q24.2	E	S.L.Woo
PBGD	18	pUSE109,PBGD0.9	11q23.2-qter	E,Y	M.Goossens
PDYN	10	LHDG-1	20pter-p12	Y	M.Litt
PENK	25	Lam.ENK1;Mfd31	8q23-q24	E,Y	J.Weber;M.Litt
PGD	5	electrophoretic	1p36.3-p36.13	Y	R.S.Sparkes
PGM1	15	electrophoretic	1p22.1	Y	R.S.Sparkes
PGP	10	electrophoretic	16p13	Y	R.S.Sparkes
POMC	0	pLambda26/pLp3	2p23	Y	S.N.Cohen;L.Cavalli-Sforza
PPY	5	PPY	17p11.1-qter	Y	T.Takeuchi
PRIP	0	pEA974	20pter-p12	Y	N.K.Robakis
RAF1	5	p627	3p25	E	B.Seizinger
RARA	0	hKIR	17q21.1	Y	C.Weinberger
RARB	0	pCOD20	3p24	E	A.Dejean
RBP3	25	cTB-IRBP-9,H4	10q11.2	Y	Y.Nakamura,C.Bridges/Liou
REN	10	HRen	1q32 or 1q42	Y	J.M.Chirgwin
RH	15	serological	1p36.2-p34	Y	R.S.Sparkes
SFTP1	1	pPSP-35k-1A-27	10q21-q24	Y	J.Floros
SPTA1	5	3021	1q21	Y	B.Forget
SST	3	pgHS7-217	3q28	Y	G.I.Bell
TCRB	15	pJ2	7q35	E	T.W.Mak
TCRG	15	Cgamma	7p15	E	J.G.Seidman
TF	5	electrophoretic	3q21	Y	R.S.Sparkes
TH	10	TH7, J4.7-BamHI	11p15.5	Y	J.Mallet
THRB	5	pBH302, pHE-A2-S	3p24.1-p22	E,Y	W.E.C.Bradley,B.Vennstrom
TK1	1	TkHC9	17q23.2-q25.3	Y	P.Lin
D1F10S1	0	DR10	1q23-qter	Y	A.J.Driesel
D1S4	0	DR78	1p21-qter	Y	A.J.Driesel
D1S16	0	p2-32	1pter-p22	Y	N.Dracopoli
D1S17	15	p3-18	1pter-p22	Y	N.Dracopoli
D1S18	10	p3-39	1pter-p22	Y	N.Dracopoli
D1S19	15	p4-03	1pter-p22	Y	N.Dracopoli
D1S57	15	pYNZ2	1	Y	R.White
D1S75	15	OS-6	1q22-q23	Y	T.Miki/S.Takai

Table 1. (Continued)

Symbol	Excluded cM	Probe	Chromosomal band	Lab	Source
D1Z1	0	p308	1q12	Y	E.Jabs/B.Migeon
D1Z3	0	p308	1cen	Y	E.Jabs/B.Migeon
D2S1	25	L 2.30	2p25	Y	E.Bakker/P.Pearson
D2S3	10	C1-5,p1-30	2q35-q37	Y	M.Litt
D2S5	10	IMR32-6	2p16-p15	Y	S.Latt
D2S6	25	pXG-18	2p23-2p15	Y	P.Szabo
D2S9	0.1	Latt-3	2p25-p24	Y	S.Latt
D2S10	5	Latt-1 =IMR-1	2p25-p24	Y	S.Latt
D2S12	10	pHM20	2pter-p23	Y	R.Williamson/G.P.Bates
D2S45	10	pHHH133	2p	Y	Y.Nakamura
D2S47	15	TBA-B5-7	2p	Y	Y.Nakamura
D2S48	10	pEFD122	2pter-q32	Y	R.White
D2S49	10	pYNA15.1	2pter-p23	Y	R.White
D3S5	0	DR2	3q21-qter	Y	A.J.Driesel
D4S1	10	4c3.6/1.2	4q11-q21	Y	T.C.Gilliam/R.Williamson
D4S10	15	pKO82	4p16.3	Y	J.F.Gusella
D4S12	15	A1	4pter-q26	Y	R.Williamson
D4S35	0	pG9-20	4p11-q11	Y	T.C.Gilliam/J.Gusella
D4S112	0	E9P1	4q26-qter	Y	P.J.Scambler/G.I.Bell
D4S123	25	pIBS17	4pter-q21	Y	J.C.Murray
D4S171	10	Mfd22	4	E	J.Weber
D5S1	0	L1.7	5	Y	E.Bakker/P.Pearson
D5S4	10	L1.4	5pter-p15	Y	E.Bakker/P.Pearson
D5S6	1	M4	5q11.2-q13.3	Y	A.E.Retief/E.Dietzsch
D5S10	10	pD274EC	5pter-p15.3	Y	J.Wasmuth
D5S11	10	pN35E-A	5pter-p15.3	Y	J.Wasmuth
D5S12	10	pJ0209E-B	5p15.2-p15.1	Y	J.Wasmuth
D5S13	0	pJ0214H-B	5p15.3-p15.2	Y	J.Wasmuth
D5S18	1	pJ0120H-B	5p15.2	Y	J.Wasmuth
D5S19	5	pJ044E-B	5p14	Y	J.Wasmuth
D5S20	1	pJ071H-A	5p13	Y	J.Wasmuth
D5S21	10	pJ0110H-C	5p13-p11	Y	J.Wasmuth
D5S22	10	pJ0205E-D	5q34-qter	Y	J.Wasmuth
D5S36	5	pJO157E-A	5q32-qter	Y	J.Wasmuth/ATCC
D5S39	10	p105-153Ra	5q12-q14	Y	J.Wasmuth/ATCC

Table 1. (Continued)

Symbol	Excluded cM	Probe	Chromosomal band	Lab	Source
D5S76	10	p105-599Ha	5cen-q11.2	Y	J.Wasmuth/ATCC
D5S78	15	p105-798Rb	5q11.2-q13.3	Y	J.Wasmuth/ATCC
D5S88	35	CARLP II 6.3	5pter-p15	Y	P.Raeymaekers
D5S106	10	pLambda3.1	5	Y	L.Cavalli-Sforza
D6S2	5	pLambda2-2	6p21-qter	Y	L.Cavalli-Sforza
D6Z1	0	p308	6cen	Y	E.Jabs/Migeon
D7S8	1	3.11	7q31	Y	R.Williamson
D7S13	5	pB79a	7q22.3-q31.2	E	J.Schmidtke
D7S23	15	pXV2C	7q31-q32	E	X.Estivill
D7S126	0	C33	7q31-q32	E	L-C.Tsui
D7S135	10	pTM102L	7pter-p14	E	L-C.Tsui
D7S144	15	TN127	7q32-q34	E	L-C.Tsui
D7S370	10	pRMU7.4	7p	E	R.White/Y.Nakamura
D7S371	5	pTHH28	7p	E	R.White/Y.Nakamura
D7S372	5	pYNB3.1	7p	E	R.White/Y.Nakamura
D8S3	1	181.6b	8	Y	P.J.Scambler
D8S84	5	Mfd8	8	E	J.Weber
D8S85	5	Mfd18	8	E	J.Weber
D9S3	0	Dr6	9	E	A.J.Driesel
D9S10	3	pMCT136	9q	E	R.White/Y.Nakamura
D9S16	0	pMCOA12	9q	E	R.White/Y.Nakamura
D10S1	15	Dry5-1	10q22-q23	Y	T.Dryja/J.Gusella
D10S3	10	phage 10	10q22-q23	Y	A.Bowcock
D10S4	25	p1-101	10q22-q23	Y	M.Litt
D10S5	10	p9-12a/2dIII2.5	10q21.1	Y	H.McDermid/N.Simpson
D10S6	0	pBM1.1	10q26	Y	T.G.Krontiris/U.Francke
D10S15	5	pMCK2	10q11.2	Y	Y.Nakamura/R.White
D10S19	35	pTB10.171	10q21.1-q22	E,Y	Y.Nakamura/R.White
D10S20	15	OS3, OS2	10q21-q26	Y	T.Miki/S.Takai
D10S22	30	pTB10-163	10q21.1	Y	Y.Nakamura/White
D10S24	10	p7A9	10p13-12.2	Y	W.Cavenee
D10Z1	1	pA10RP8	10cen	Y	H.Willard
D11S12	5	pADJ762, pADJ765	11p15.5	Y	R.White
D11S16	0	pLambda32-1	11p13	Y	L.Cavalli-Sforza
D11S29	35	L7	11q23-qter	E,Y	A.E.Retief/E.Dietzsch

Table 1. (Continued)

Symbol	Excluded cM	Probe	Chromosomal band	Lab	Source
D11S36	5	phi2-14	11q	Y	M.Litt
D11S83	0	phi2-25	11q23-qter	Y	M.Litt
D11S84	45	p2-7-1D6	11q22	E,Y	M.Litt
D11S144	18	MCT128.1	11q22.3-q23.3	E,Y	R.White/Y.Nakamura
D11S146	5	pHBI59	11q12-q13.2	Y	Y.Nakamura
D11S147	0	pHBI-18p2	11q23-qter	E	R.White/Y.Nakamura
D11S347	0	pLambda19-2	11q13	Y	L.Cavalli-Sforza
D12S6	0	p1-7	12q14	E	M.Litt
D12S7	10	pDL32B	12q14-q24.1	E	L-C.Tsui
D12S8	25	p7G11	12q14-qter	E	R.White
D12S17	5	pYNH15	12q	E	R.White/Y.Nakamura
D13S2	10	p9D11	13q22	E	W.Cavenee
D13S3	5	p9A7	13q21-q34	E,Y	W.Cavenee;R.White
D13S5	10	pHUB8	13q22-q34	Y	T.Dryja
D13S6	1	pHU10	13q12-q14	Y	T.Dryja
D13S7	0	pHU26	13q22	Y	T.Dryja
D13S10	0	p7D2	13q14.1-q14.2	Y	R.White
D14S1	15	pAW101	14q32.32-q32.33	E	R.White
D14S16	20	pTHH37	14q32	E	R.White/Y.Nakamura
D15S1	20	pMS1-14	15q14-q21	E	R.White
D17S5	20	pYNZ22.1	17p13.3	E	R.White/Y.Nakamura
D17S34	18	p144-D6	17p13	E,Y	M.Litt
D17S58	0.1	EW301	17p11.2-cen	Y	D.F.Barker
D17S71	1	puc10-41	17p12-p11.2	Y	D.F.Barker
D17S73	15	EW207	17cen-q12	Y	D.F.Barker
D17S250	5	Mfd15	17	E	J.Weber
D17Z1	0	p17H5,pDL27B	17cen	Y	H.Willard;L-C.Tsui
D18S1	10	pHF12-62	18	E	R.White
D18S3	20	B74	18p11.3	E	J-L.Mandel
D18S5	15	OS4	18q21.3-qter	E	T.Miki
D18S7	10	OLVIIA8	18q11.1-q11.2	E	G.Thomas
D18S8	15	OLVIIE10	18q21.3	E	G.Thomas
D18S11	15	pERT25	18q23	E	M.Lalande
D18S34	15	Mfd26	18	E	J.Weber
D18S35	5	Mfd32	18	E	J.Weber

Table 1. (Continued)

Symbol	Excluded cM	Probe	Chromosomal band	Lab	Source
D19S7	35	p4.1	19cen-q12	E,Y	ATCC/Shaw
D19S8	18	p17.1	19q13.2	E,Y	ATCC/Shaw
D19S20	10	pJC23.1	19	E	R.White/Y.Nakamura
D19S30	5	p20B18	19p13.1-q12	E	B.Wieringa
D19S41	10	Mfd11	19cen-q13.2	E	J.Weber
D20S4	18	pMS1-27	20q13.2	E,Y	R.White
D20S5	15	pRI2.21	20p12	Y	D.J.Shaw
D20S6	18	pD3H12	20p12	E,Y	D.J.Shaw
D20S13	10	pPhi64	20p	Y	R.W.Deed
D20S14	35	p4.8	20p	Y	N.K.Spurr
D20S27	20	Mfd25	20	E	J.Weber
no symbol	5	Mfd23	unassigned	E	J.Weber
no symbol	5	Phi106	unassigned	Y	R.W.Deed
no symbol	0.1	CL149	unassigned	Y	A.D.Roses
no symbol	0	LDR92	unassigned	Y	A.D.Roses
no symbol	1	LDR111	unassigned	Y	A.D.Roses
no symbol	10	Phage565	unassigned	Y	J.Wasmuth
no symbol	10	LDR93	unassigned	Y	A.D.Roses
no symbol	0	Bam41	unassigned	Y	A.D.Roses

[a] One- sided interval; Haldane function is used.

[b] Y=Yale University; E=Erasmus University

LAB: Group testing for linkage. E = Erasmus U., Y = Yale U. EXCLUDED: One-sided interval shown in cM using Haldane function.

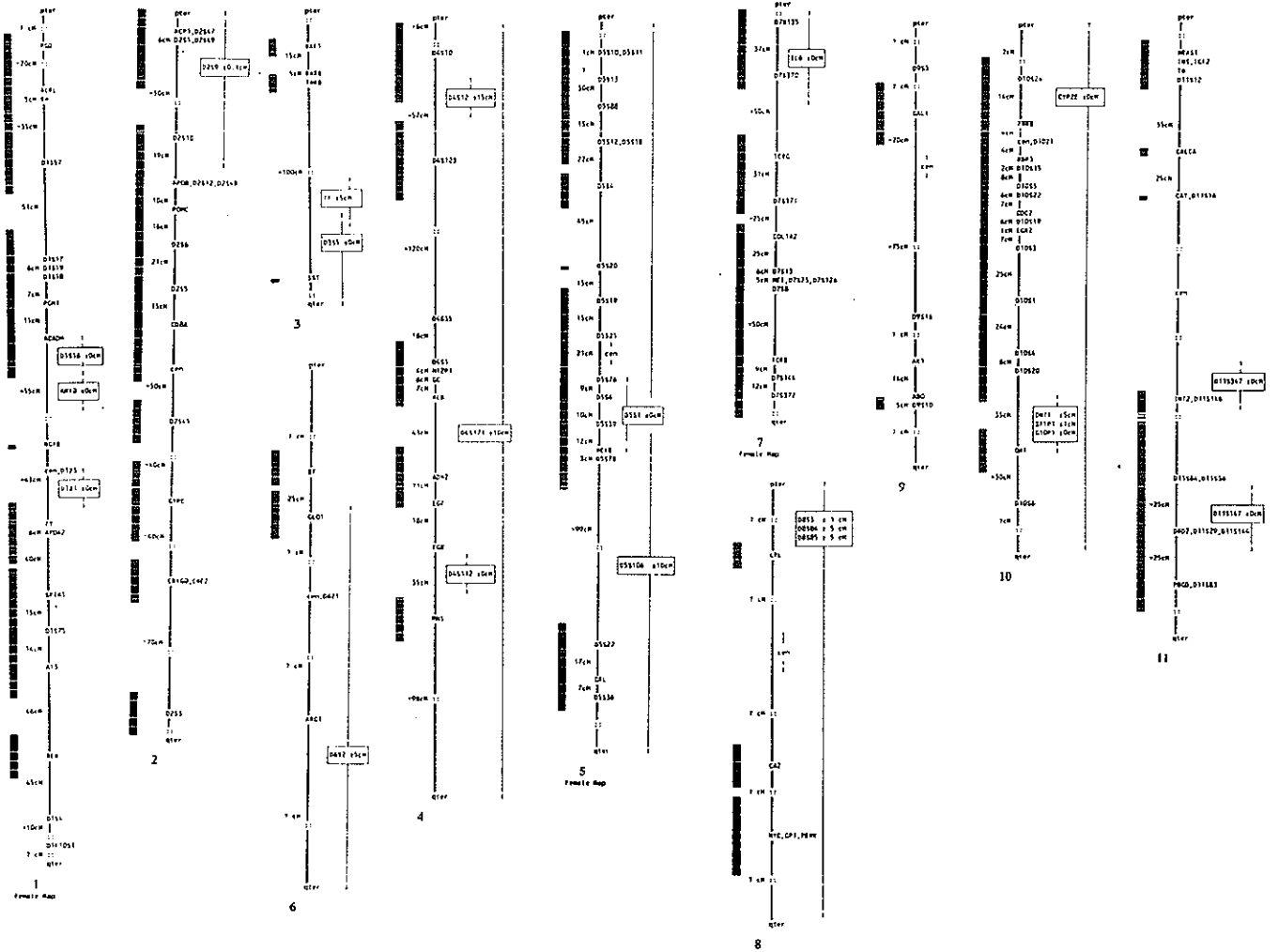
Table 2. Genetic Model Parameters

Parameter	New Haven		Rotterdam
Single Autosomal Dominant	Yes		Yes
Gene Frequency	0.003		0.003
Age of Onset Correction	Yes. Linear function increasing from age 2 until maximum at 21.		No. Before age 15 children included only if affected.
	Minimum	Maximum	
Penetrance, Male	.048	.999	.999
Penetrance, Female	.027	.561	.560
Phenocopies, Male	.0002	.0050	.0002
Phenocopies, Female	.0000	.0001	.0001

earlier decision to prefer the larger sex-specific recombination map in each region so as not to overestimate the excluded region, the total length is roughly 4860 cM, considerably in excess of the conventional estimate of approximately 3300 cM for the female map. The second difficulty is that, in tallying the total non-overlapping exclusion zone across chromosomes, some of the genetic markers tested for linkage have not been localized precisely. Thus, a minimum and maximum exclusion zone are calculable. The minimum assumes that all exclusions from non-localized markers occur in regions already excluded by loci on the map so the contribution of non-localized markers is ignored. The maximum allows all the exclusions by non-localized markers to count fully.

The exclusion zones illustrated by chromosome in Figure 1 make it easy to pick out the obvious gaps which the search for the GTS locus can focus on next. Most of chromosomes 1, 2, 5, 7, 10, 12, 18, and 19 have been excluded and much progress has already been made on chromosomes 4, 8, 11, 17, and 20. There are large numbers of RFLPs already documented in the literature whose physical mapping locations overlap many of these gaps, e.g. Human Gene Mapping 10 (1989) and the on-line Human Gene Mapping Library database in New Haven, but the mapping relationships of these loci are equally necessary to know so we can assess our progress. Our ongoing efforts as well as those of the research community at large, to establish a basic genetic map spanning the human genome will be an important element in determining how efficiently we can advance in our efforts to find the

TS EXCLUSION MAPS



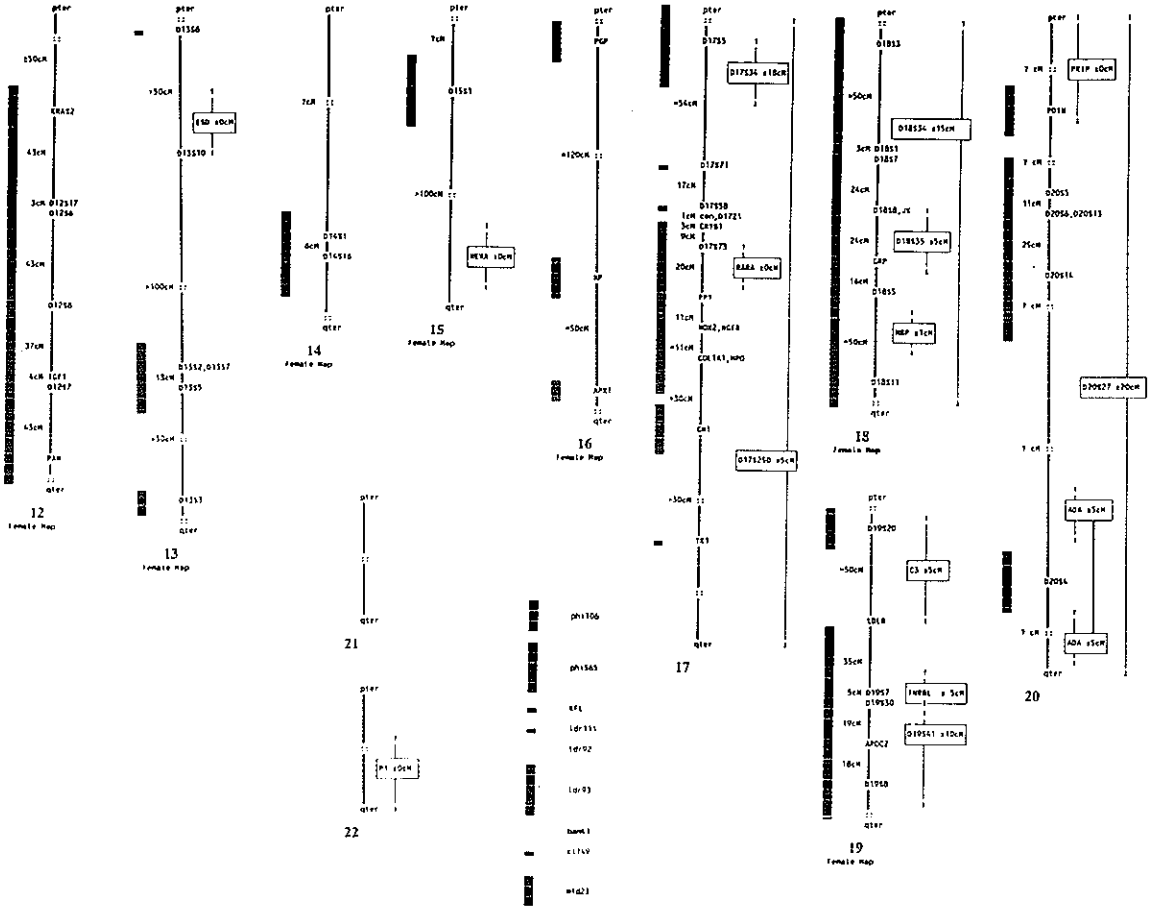


Figure 1 GTS exclusion zones along autosomal map. See text discussion of the conventions followed in constructing the exclusion maps

Table 3. Calculation of the Total Non-Overlapping Exclusion Distance by Chromosome.

Chromosome	Mapdistance excluded via linkage (cM)				Map size
	Mapped	Non-localized	Total		
	loci	loci	Min.	Max. (cM)	
1	310	0	310	310	450
2	310	0	310	310	410
3	22	10	32	32	140
4	187	0to20	187	207	350
5	262	0to20	262	282	360
6	47	0to10	47	57	150
7	200	0	200	200	220
8	85	0to22	85	107	185
9	42	0	42	42	170
10	220	0to12	220	232	250
11	160	0	160	160	260
12	195	0	195	195	250
13	50	0	50	50	250
14	42	0	42	42	150
15	35	0	35	35	135
16	55	0	55	55	180
17	157	10to36	167	193	280
18	190	0	190	190	190
19	115	10	125	125	130
20	145	10to50	155	195	250
21	0	0	0	0	50
22	0	0	0	0	50
Pseudoautosomal	0	0	0	0	?
Unassigned	0	0to122	0	122	...
Totals			2869	3161	4860
% of autosome			59%	65%	100%

GTS locus.

Since our concerted efforts have already excluded more than half of the relevant genome, we expect that the prize of finding the GTS locus lies in the near future. We (Yale group) ave already had one statistical fluke to tantalize us in the course of this search. ACP1, a locus on 2p, gave a false positive signal in one family (a peak lod just > 3). Both follow-up typing of nearby flanking markers and subsequent multipoint linkage analyses allowed us to identify this very clearly as a bogus signal. We are hopefull that the next interesting signal will produce a very strong positive lod score that replicates across pedigrees and the result is reinforced by converging evidence from flanking markers.

Several lines of evidence (for a review see Shapiro *et al* 1988) indicate that brain neurochemical systems, in particular the dopamine system, may be involved in the etiology of GTS. The genetic markers we have tested included a number of candidate genes, such as the dopamine D2-receptor (DRD2), pro-dynorphin (PDYN), proopiomelanocortin (POMC) and the gastrin releasing peptide (GRP). Genetic linkage to GTS was not obtained with any of these four candidate loci (Devor *et al.* 1990, Gelernter *et al.* 1990, Heutink *et al.*, 1990). Testing candidate genes is one approach to finding a disease locus but most such candidate loci are proposed rather casually, without a compelling case to support them. Those which have been cloned and are polymorphic are easily subsumed under the systematic screening strategy which seeks to test for linkage with a comprehensive series of polymorphic loci. Ultimately, a basic panel of polymorphisms forming a continuous map on each chromosome will exist and provide the means to test for linkage and locate the map positions of disease-related loci. Comings *et al.* (1986) postulated the GTS gene on chromosome 18. In the present study no evidence was found for linkage on chromosome 18. This is in agreement with the recent exclusion of chromosome 18 as a site for GTS with part of the results presented here (Heutink *et al.* 1990). Comings *et al.* (1989) suggested the tryptophan oxygenase gene (4q25-q31.3) is a candidate gene for GTS. Several markers in this region (ADH2, EGF, FGB, MNS, D4S112) were typed in this present study, none of them gave evidence for linkage.

Genetic linkage analysis for full blown GTS only and for a broad phenotype allowing for GTS or CMT or OCD gave results which are similar to those we have summarized here for the affected phenotype that includes GTS or CMT. We did not include here individuals presenting only OCD symptoms as affected since there is no general agreement on whether OCD alone can be regarded as a variant phenotype of GTS.

The possibility of heterogeneity needs to be considered. Genetic heterogeneity

could mask a positive result given the summation of lod scores across families. Both our groups
routinely look at the linkage results separately for each family. Thus far no heterogeneity has been observed in the linkage results. The very small positive lod scores that have occurred so far (with the exception of the false positive result on chromosome 2, mentioned earlier, which was clearly excluded by adjacent loci in the same family) are not very different from zero, so no formal heterogeneity test has been applied.

CONCLUSION

Our search for the GTS locus by means of a genetic linkage strategy has eliminated from consideration more then half -and perhaps as much as two-thirds- of the autosomal genome. This range of exclusionary values seems reasonable at this juncture. An important assumption underlying our assessment is that the major locus for GTS is the same in the different pedigrees pooled together for this report. Thus, if there are different loci underlying Tourette Syndrome in some of our families, we could have missed such a linkage if, for example, the nearby marker was not segregating sufficiently (or simply not typed) in the family with the linkage, while the marker was informative enough in the other non-linked families to give us exclusion for the region. The search also might not be as far along as we claim if the human autosomal map is substantially larger that present evidence suggests. Whatever the exact degree of progress the pace of the search is gaining momentum. New labs around the world are preparing to join the hunt for the GTS locus or have recently started. Our own labs are concentrating on regions unexamined thus far, as illustrated by the exclusion maps we have presented here. Perhaps very soon we shall obtain strong, positive evidence of linkage for GTS in our pedigrees so that the hunt for the DNA sequence of the GTS locus can begin.

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CHAPTER 5.4

PROGRESS IN GENE LOCALIZATION

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INTRODUCTION.

Despite years of intensive biochemical research, little is known about the biochemical basis of familial psychiatric disorders. Isolation of a gene causing such a disorder could lead to a better understanding of the biochemical features that it presents.

Until the discovery of restriction fragment length polymorphisms (RFLPs) and other polymorphic DNA markers, such as variable number of tandem repeats (VNTR's) and simple-sequence tandem repeats, such as CA repeats (1-3), the only way to unravel the genetic cause of an inherited disorder was to isolate the defective protein resulting from the genetic defect. Antibodies raised against this isolated protein can then be used to isolate complementary DNA (cDNA) clones from an expression library. The corresponding messenger RNA (mRNA) sequence can be used to localize and isolate the complete gene on genomic DNA. The use of polymorphic DNA markers provides a way to isolate the gene responsible for disorders without a known biochemical defect. This method is called "reverse genetics" (4). (Figure 1).

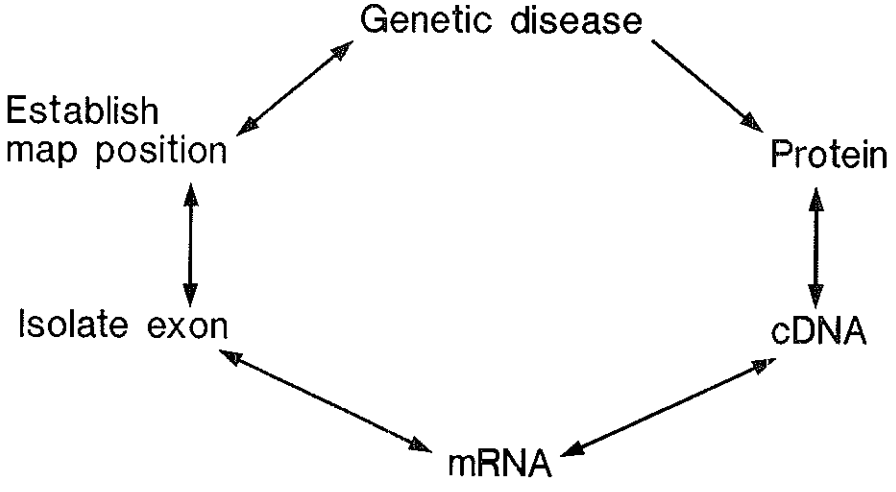


FIGURE 1 Reverse genetics.

The first step in reverse genetics is to establish the chromosomal localization of the gene and then to identify a specific gene within this region in which mutations are strictly correlated with the disease. By correlating the inheritance of a distinct, well-localized polymorphic marker with the inheritance of a disease, the gene localization of the disorder in the human genome can be determined. By using more of these polymorphic

markers in this region it will be possible to close in on the region in which the defective gene must be localized. When the region is small enough, partly overlapping clones from genomic DNA libraries can be used to zoom in on the gene. Finally, cDNA clones can be isolated from this region that are candidate genes for the disorder. By carrying out either expression studies with mRNA or mutation analysis of these genes in patients, the defective gene can be identified. Recent examples of genes cloned by "reverse genetics" are the genes causing cystic fibrosis, neurofibromatosis type I and the fragile X syndrome. (5-7).

GENETIC STUDIES OF GILLES DE LA TOURETTE SYNDROME.

There is no biochemical cause known for Gilles de la Tourette's syndrome (GTS). Reports have suggested involvement of dopaminergic, adrenergic and serotonergic systems in the etiology of the disorder (See Ref. 8 for review.), but at present no defective protein or precise structural or functional brain defect is known. In order to find the gene(s) involved in the etiology of GTS, a number of research groups perform "reverse genetics" studies.
Several strategies can be used in localizing the defective gene responsible for GTS. (Table 1.)

Table 1. Stategies to localize/isolate the GTS gene.

1.	Search for chromosomal abnormalities associated with GTS.
2.	Search for other diseases co-segregating with GTS.
3.	Candidate gene approach.
4.	Systematic screening for polymorphic markers linked to GTS.

Search for chromosomal abnormalities associated with GTS.
In general, the karyotypes of patients with GTS do not show chromosomal abnormalities. Some case studies describe patients in whom GTS and a chromosomal aberration co-occur. (See Ref. 8 for review.). Comings *et al.*(9) presented evidence for the localization of the GTS gene. They reported a 46,t(7:18)(q22;q22.1) reciprocal translocation in six relatives suffering from GTS. This suggested a localization of the GTS gene near the 18q22.1 breakpoint. Donnai (10) reported a GTS patient with a deletion of the long arm of chromosome at 18q22.1 providing further evidence for this location. These findings led to the tentative assignment of the GTS gene to chromosome 18q22.1 by the chromosome

18 committee at Human Gene Mapping 9 (11). In linkage analyses on Dutch and Norwegian caucasian families, however, strong evidence for nonlinkage of GTS with this localization was found (12).
A *de novo* deletion, del(9)(qter->p2304:) in a Latin-American male GTS patient was reported by Taylor *et al.*(13). Linkage analysis on chromosome 9p is hampered by a lack of polymorphic DNA markers that are available in this region. The markers that are available on chromosome 9p have been tested by the several groups but do not show evidence for linkage. However, since only one of the tested markers maps into the deletion (D.Krizman pers. com.), additional markers have to be generated before definite results can be obtained whether or not GTS is linked to this chromosomal region.

Co-segregation of other inherited disease within GTS families.
For a complex disorder like GTS the linkage analysis could be simplified if the disorder would cosegregate, with an disorder that has already been localized or inherits in an Mendelian way. In all the families currently being investigated for linkage analyses on GTS there are no known diseases cosegregating with GTS.

Candidate gene approach.
A number of candidate genes for GTS have been proposed and tested. For example, genes involved in the dopaminergic system. Linkage studies are carried out to test the hypothesis that the disease gene and the candidate gene are the same by demonstrating that an allele from the candidate gene is cosegregating with the disease. If this segregation is not completely correlated, the candidate gene cannot be the disease gene. None of the tested candidate genes showed linkage with GTS (12,14-16, unpublished results)(Table 2).
Other candidate genes for GTS are the dopamine receptors D3-D5 that have recently been cloned but remain to be tested. One could regard several other neurotransmitter receptor genes or neurotransmitter genes as candidate genes.
The candidate gene approach appears to be an attractive way for linkage analysis. The hypothesis of the involvement of a certain gene in the etiology of the disorder of interest can be directly tested. A number of candidate genes have been proposed without a very strong indication that they are involved in causing GTS. Those candidate genes that have been cloned, localized and are polymorphic, however, can easily be included in a systematic screening of the human genome.

Systematic screening for polymorphic markers linked to the disease locus.
For each autosomal genetic locus, an individual carries two alleles. Since two

Table 2. Candidate genes tested for linkage with GTS

Gene	Chromosomal location
Proopiomelanocortin	2p23
Dopamine receptor D1,	5q
Dopamine beta hydroxylase	9q34
Tyrosine Hydroxylase	11p15
Dopamine receptor D2.	11q22
Gastrin releasing peptide	18
Pro-dynorphin	20pter-p12

randomly chosen genetic loci are most likely on different chromosomes or far apart on a chromosome, the alleles of two loci are generally transmitted independently. However, if two loci are located close to each other on a chromosome the alleles from the two loci received from an individual tend to be transmitted together to offspring. The genetic distance between two loci can be expressed as the recombination rate. The closer two loci are to each other, the smaller the chances are for a recombination event to occur. Two loci at a distance of less then 50% recombination are considered genetically linked.

The basics of linkage analysis for human diseases is to test the hypothesis (H1) that a polymorphic locus is linked to a disease locus at a recombination fraction smaller then 50% against the hypothesis (H0) that these loci are unlinked, in other words, that there is a recombination fraction of 50%. The most widely used method of linkage analysis is the maximum likelyhood method. This method estimates the most probable recombination fraction between two loci and calculates the chance that an observed recombination fraction is significantly smaller than 50%. In other words, it compares the chance (P1) that the data have arisen if H1 is true, versus the chance (P2) that the data have arisen if H0 is true. The odds ratio of P1/P0 is a measure for the likelyhood that the data have arisen under linkage. This ratio is usualy converted into the lodscore.

For Mendelian disorders a LOD score of 3.0 (corresponding to an odds ratio of 1000:1) is considered as a treshold for detecting linkage and a lodscore of -2.0 as a treshold for non-linkage.

A more complex genetic transmission can be studied by formulating more complex hypotheses. An excellent overview of human linkage analysis is given by Ott (17,18). An overview of linkage analysis on complex traits is given by Lander (19).

In GTS families we have observed that some individuals in a pedigree have to be

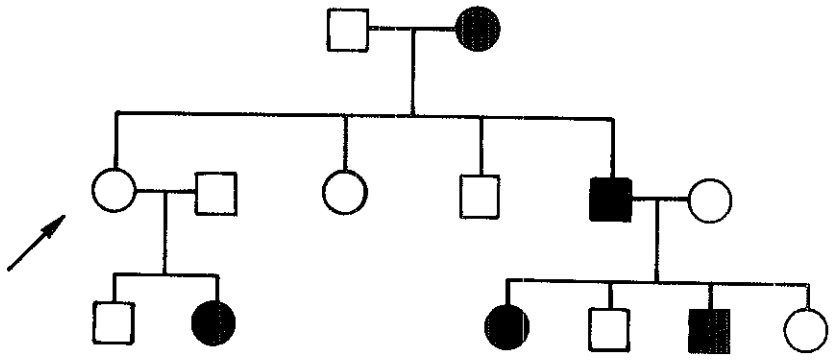


Figure 2. Segregation of a dominant disorder with incomplete penetrance. Filled symbols represent affected individuals. The arrow indicates an obligate gene carrier.

carriers of the disease genotype but fail to express the disease phenotype (Figure 2).

This phenomenon is reffered to as incomplete penetrance. In GTS the penetrance of the disease phenotype is associated with age and sex of the individual. The causes of incomplete penetrance are not known but plausible reasons might include: enviromental factors, differences in genetic background or the need for a second event for the disease to occur. Incomplete penetrance must be included in the genetic model used in the linkage analyses, because in complex disorders, unaffected children of affected parents are not completely informative. One can not be sure whether they have inherited the disease allele. Moreover, with the symptoms presented as GTS, it is likely that phenocopies will occur that will also require corrections in the linkage analysis. Including these genetic parameters reduces the power of the linkage analysis in comparison with straightforward Mendelian disorders.

Genetic analysis of family data of Tourette families has been extensive (See Ref. 8 for review). Most studies have been consistent with the hypothesis that susceptibility to the disorder is most likely due to a single genetic locus with an autosomal dominant mode of inheritance, reduced penetrance and variable expression. However, other models cannot always be excluded (8). There is general agreement that in families affected by GTS, the chronic multiple-tic syndrome (CMT) is a variant, being a milder phenotype of the disorder (20,21). Whether obsessive-compulsive symptoms (OCS) are etiologically related is still a matter of

discussion.
The estimates of the prevalance of GTS are not based on large population studies and should be looked at with caution (20,22,23). The number of phenocopies occuring in the population is also a rough estimation. Therefore, it is not possible to define the genetic parameters precisely. The clinical diversity of GTS makes it difficult to decide which clinical features should be regarded as being part of the phenotype of the disorder. We can divide the clinical features into at least four groups with broadening diagnostic criteria based on DSM-III-R criteria. The first group consists only of those individuals presenting the full blown GTS. The second group includes those individuals with the CMT syndrome. The third groups also includes individuals with OCS, and the fourth groups includes a number of other psychiatric symptoms: phobia, alcoholism, attention deficit disorder, etc.
By defining the phenotypes thus, there are four possible models to be tested in the linkage analysis. The four models will show a broadening of the diagnostic criteria. For each model, the penetrance values, phenocopy rates and gene frequences must be determined. In behavioural disorders, there is the possibility of misdiagnosis or misclassifications due to the variability in expression of the symptoms. All off the above mentioned features complicate the linkage analysis and will reduce its power.

FAMILY MATERIAL

The research groups working on linkage studies of GTS have sampled extended families affected with the disorder (12,20,21; unpublished results). Some groups work with one large pedigree, other groups work with several smaller three- or four-generation pedigrees. During genetic workshops organized by the Tourette Syndrome Association diagnostic criteria and diagnosis of family members are discussed and, if necessary, adjusted.
The advantages of large pedigrees are clear in the case of genetic heterogeneity. The chance that a disease will be heterogeneous (i.e., due to a mutation at more than one locus) within one family is small except when attempts to expand the pedigree by identifying as many affected members as possible may actually introduce heterogeneity. Large pedigrees are often found in more or less isolated communities. Finding a linkage would have to be followed by extensive studies in other families to determine whether the finding is general or very rare. A set of smaller families are clearly less powerfull then the large pedigrees in case of heterogeneity. If the families are sampled in the same isolated geographical region the chances of heterogeneity are smaller. However, if the smaller families are

powerfull enough to generate a significant lod score by themselves there is the advantage that it is possible to test disease loci that are only linked to part of the families.

Linkage studies of the collaborating groups.

Under the auspices of the Tourette Syndrome Association, six research groups (25) have been part of a collaborative effort to map the defective gene for GTS. For the sake of efficiency, all the results obtained from the linkage analyses are shared among the groups. As mentioned above, the groups work with different subsets of families affected by GTS. Under the assumption of genetic homogeneity, the data obtained in different subsets of families can be combined if the diagnostic criteria and the genetic parameters used in the linkage analysis are the same. The collaborating groups agreed to use the results of the segregation analyses of Pauls an colleages (23,24) as the basis for the genetic parameters used in the linkage analyses (Table 3).

Table 3. Genetic model parameters

Gilles de la Tourette Syndrome including Chronic Multiple-Tic Syndromes.[1]

Gene frequency		:	0.003- 0.005
Penetrance	male	:	0.900
	female	:	0.600
Phenocopies	male	:	0.0002-0.005
	female	:	0.0000-0.0001

[1] Single autosomal dominant gene

RESULTS AND DISCUSSION.

To date, the collaborating groups have tested 558 markers. No strong and definite evidence for linkage has been obtained yet, although several markers show positive lod scores in a subset of the families (unpublished results). These lod scores have not yet reached a significant level and need further attention if more conclusive

results are to be obtained. The results of the linkage analyses are combined in an exclusion map for the human autosomes (Figure 3).
An exact calculation of the proportion of the human autosomes that have been excluded, under the assumption of genetic homogeneity, cannot be carried out for two reasons. First, the exact length of the human autosomal map is not known. Second, a number of markers are not exactly localized and therefore it is unknown whether their exclusion zones overlap with those of well localized markers. A conservative estimate, based only on the well localized loci in the largest sex-specific maps, is that at least 80% of the human autosomes has been excluded as a possible site for the GTS gene. The remaining gaps in the map are mainly located on chromosomes 3,4,6,9,14 and 16. The different groups are now focusing on these chromosomal regions. The number of well-localized polymorphic markers on these chromosomes,is limited, and more markers have to be generated in order to get conclusive results for these chromosomal regions.

Clinical heterogeneity.
In recent years, promising findings in linkage analyses for several psychiatric disorders could not be confirmed (26,27,28,29). Some of the results had to bereanalysed because of changes in the affection (clinical diagnosis) status which resulted in a weakening of the evidence for linkage. In Mendelian disorders the level of a lod score of three is an acceptable minimum of significance for evidence of linkage. A reasonable question is whether the same criteria can be applied for more complex disorders such as psychiatric disorders. As discussed above, in GTS and other complex disorders the linkage analyses is complicated by a variety of factors, including incomplete penetrance, phenocopies, clinical heterogeneity and diagnostic uncertainties. Genetic studies on GTS are therefore highly dependant on the clinical definitions of the phenotype. Without a detailed description of the possible phenotypes the linkage studies have little chance to succeed. The clinical heterogeneity of GTS also raises the question whether such a broad range of symptoms can be the result of one defective gene. Although segregation analyses indicate that single gene transmission is the most likely genetic model, there exists the possibility of a multiple-gene model. Other models can not always be ruled out either (See Ref. 8 for review). The clinical heterogeneity might also depend on enviromental factors.

Genetic heterogeneity.
Now that at least 80% of the human genome has been excluded under the assumption of genetic homogeneity, the possibility of genetic heterogeneity should

be considered seriously. Genetic heterogeneity would undermine the collaborative exclusion data to a great extend. As in lower organisms such as yeast or bacteria, in which genetic heterogeneity is common, there are examples of genetic heterogeneity in human disorders (30,31,32). Because the clinical heterogeneity for GTS is also expressed within the families, it is not possible to divide the pedigrees according to the clinical phenotype. The chances that there is genetic heterogeneity within a family are small. Therefore, the collaborating groups working on GTS have selected two large families, each capable of generating a significant lod score. Aside from the set of families that the groups already use, the data for markers that are being tested will be expanded onto these two families. The exact genetic model, and whether the disorder is caused by a single gene or by multiple genes, can be determined only after a linkage has been found.

Guidelines for linkage studies.

The difficulties associated with the genetic study on GTS are very much the same as for other psychiatric diseases. In recent years some guidelines have evolved by which linkage studies on complex diseases should be setup (29), and these can be applied in the linkage studies for GTS. The most important guideline is that when a linkage finding is published it should describe in detail in what way the results were achieved. The source for the marker localization should be made clear, and the haplotypes for the markers should be made available to other investigators. This makes it possible for other investigators to verify and replicate the findings.

Before a linkage study is actually started, it is essential that the genetic model or models being used are specified clearly. These models should not be allowed to change. Adjustments for multiple testing have been proposed (33,34,35,36) but there is no general agreement. The power of the family material can be tested with simulation studies (37) including sensitivity testing. In other words, the analysis determines which individuals have the greatest influence on the lodscore should their diagnosis prove to be wrong. It is essential to be very conservative in the diagnostic asignment, and to go back to the families periodically to check the reliability of the diagnosis. The marker typing and the diagnostic assessment should be blinded with respect to each other and should be re-eavaluted by independent persons. Positive findings should be followed by retyping the markers that generate the positive score and by typing of more markers in the region to extract the full information of the family material. If possible the pedigree(s) should be extended, or independent family material should be tested for the same markers. Linkage results based on a narrowly defined genotype may be given greater weight than results based on a broader disease categorization. Within the

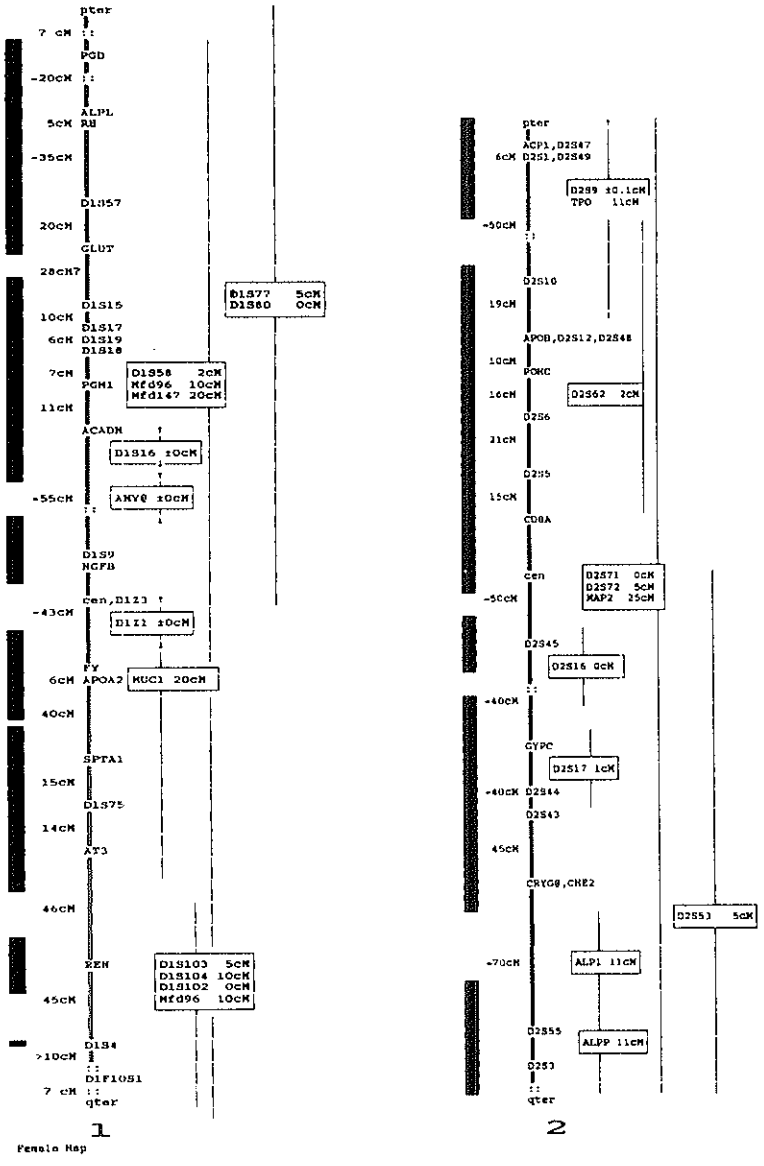
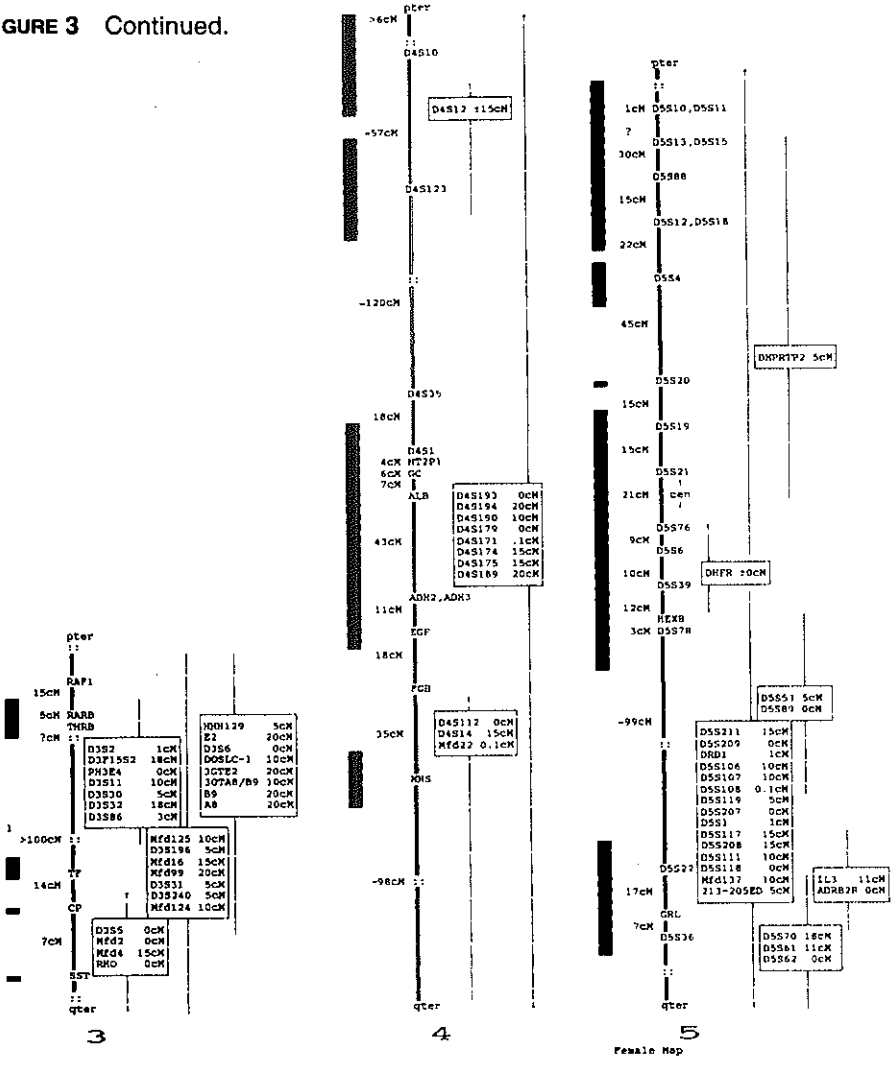


FIGURE 3 Exclusion maps for TS. For each human autosome a stick map was constructed in which the genetic loci that were tested for linkage with TS and CMT are ordered along the map. The map is based on information provided by the summaries of the individual chromosome committee reports published by Human Gene Mapping 10 or 10.5 (1989, 1990). Haldane's mapping function was used to convert recombination fractions to estimated map distances in centiMorgans

FIGURE 3 Continued.



(cM). The estimated map distance is given between the loci. Markers that lack precise localization are in boxes next to the chromosome map. Arrows indicate the approximate placement of these loci along the genetic map. Exclusion zones (i.e., a LOD score less than −2.0) assuming genetic homogeneity are shown at the left of each stick map by the dark blocks; each square block represents a region of about 5 cM. Within the boxes the numbers next to the marker symbols show the size of the exclusion zone around that locus. The results reflect pooling of results of both pairwise and multipoint linkage analyses.

FIGURE 3 Continued.

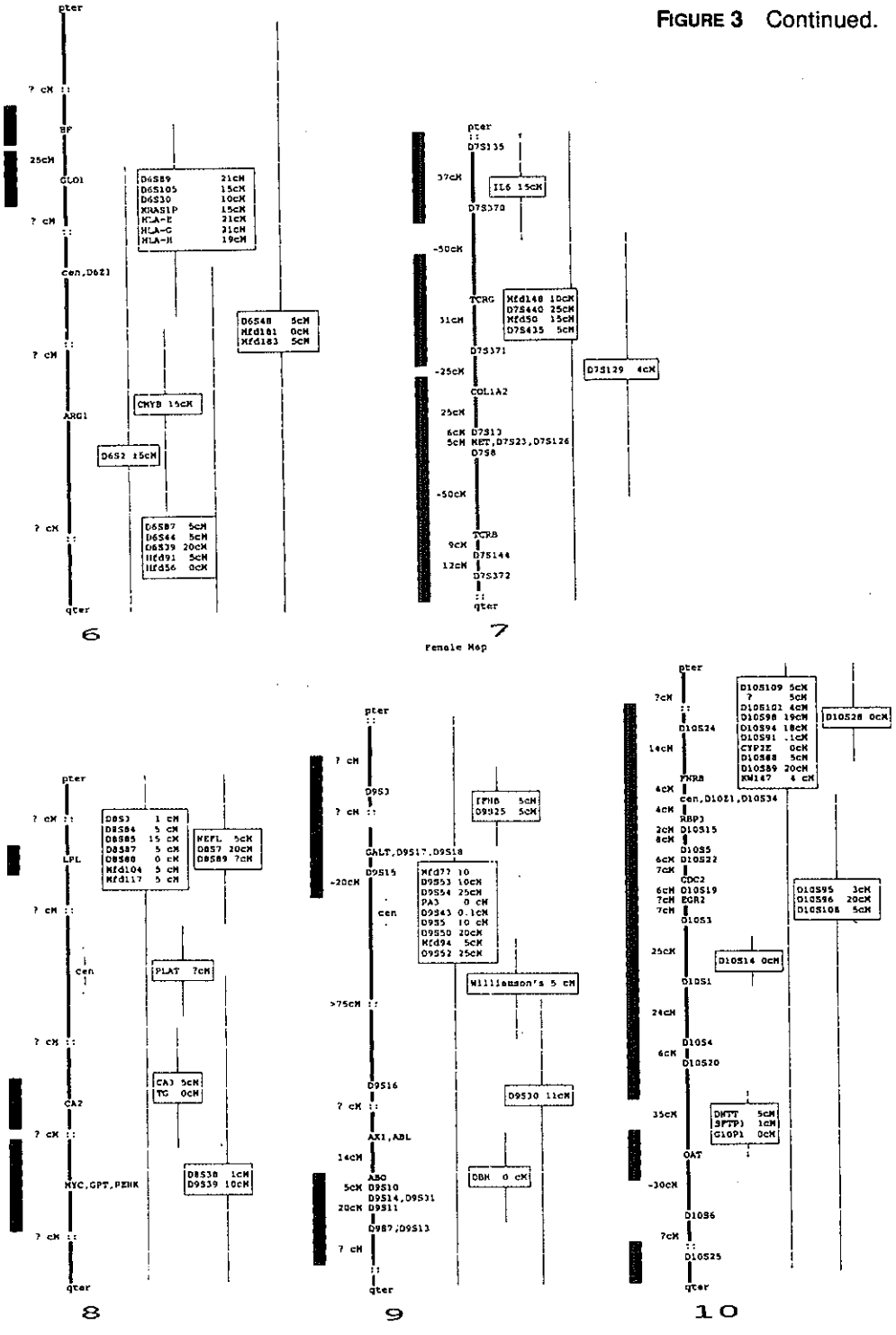
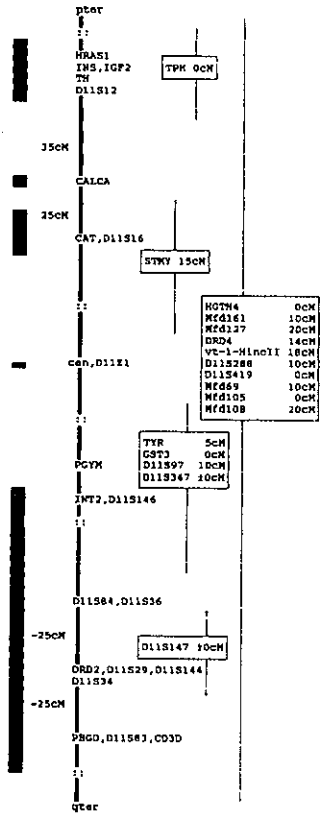


FIGURE 3 Continued.



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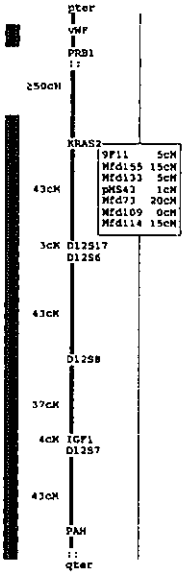

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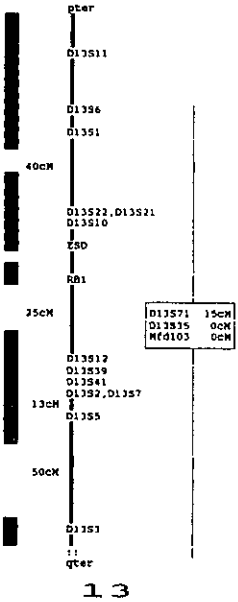
Female Map



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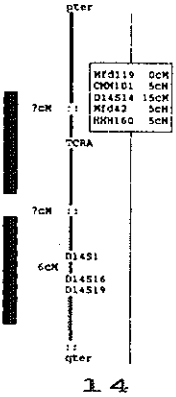
Female Map



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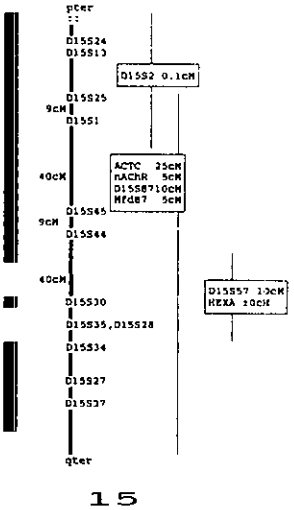
Female Map



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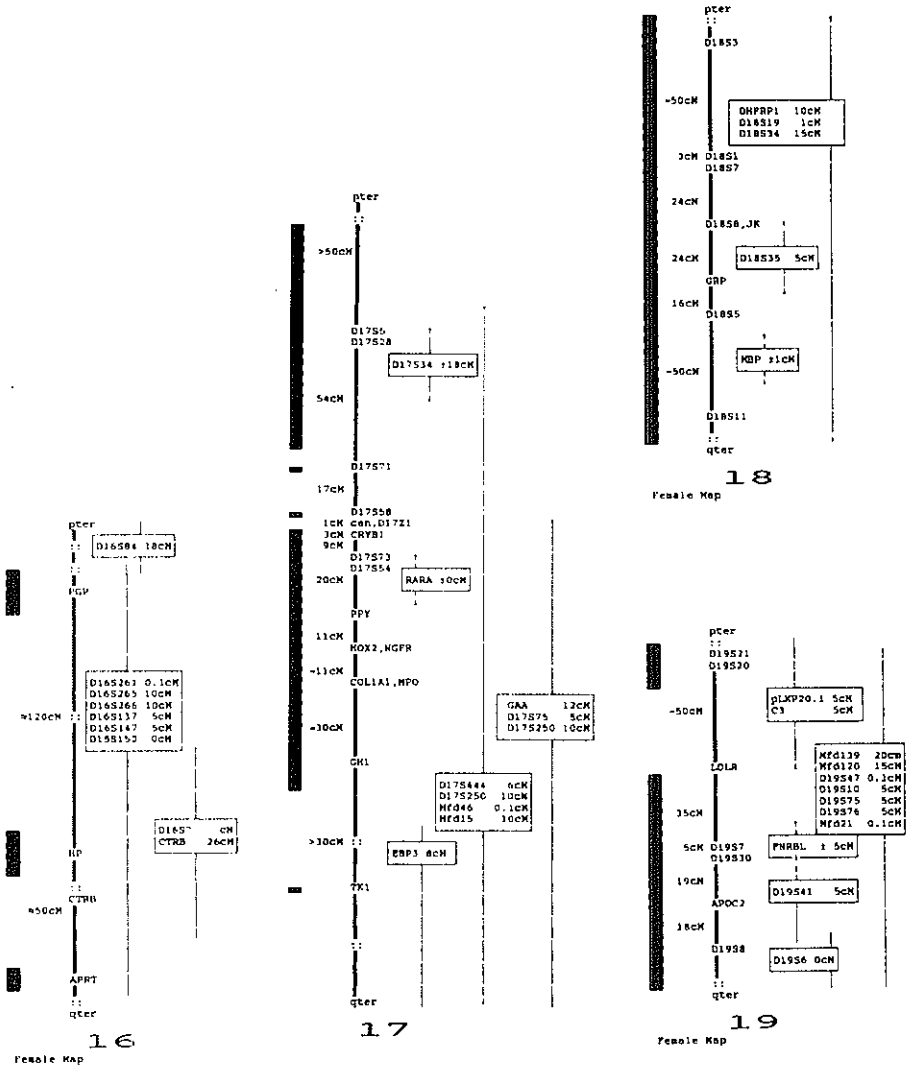
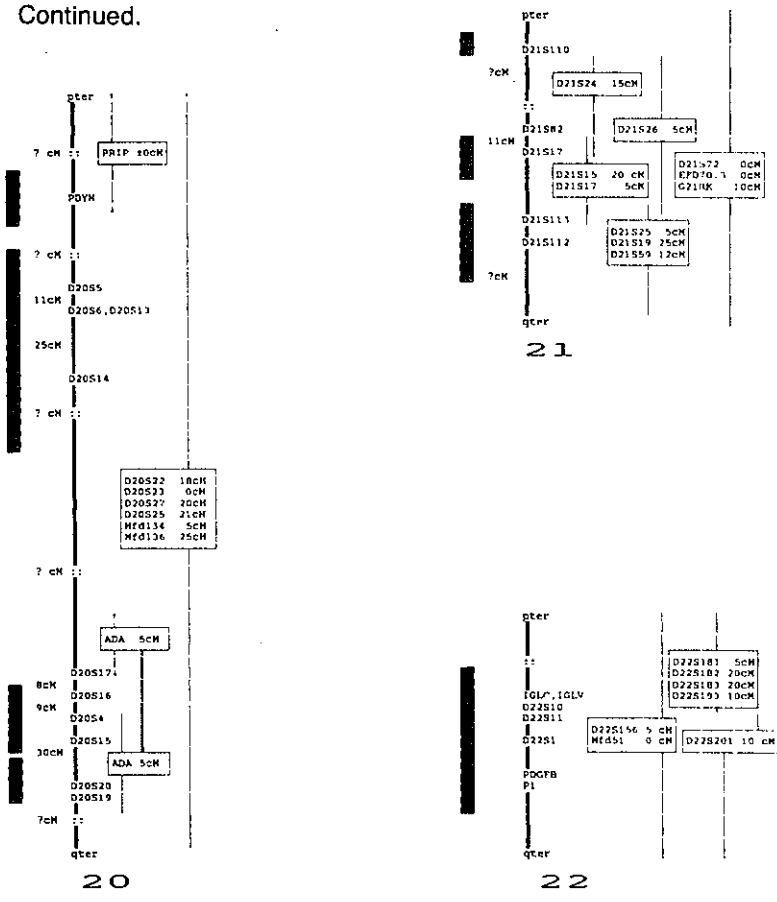


FIGURE 3 Continued.

collaborating groups there is agreement on these guidelines. Instead of publishing positive results immediately, the other groups in the consortium will get the the opportunity to replicate the findings in their own family material as well as in the shared family material.

To prevent false-negative findings in linkage analysis, other statistical approaches could be used. Several statistic methods, like the affected sib-pair, the extended sib-pair, or the affected pedigree member method (ASP, ESPA or APPM (38,39) do not need the specification of the genetic model but look only at the ratio of the

FIGURE 3 Continued.



inherited alleles. There are differences between the different approaches that are beyond the scope of this chapter. Each method having its advantages and disadvantages. These methods can saveguard against false negative findings due to a misspecified genetic model. As a result of the close collaboration among the research groups working on the genetics of GTS, a large part of the human genome has been investigated for genetic linkage under the assumption of genetic homogeneity. Screening of the remaining part of the genome is in full progress. We now have to seriously consider the possibility of genetic heterogeneity. By sharing our family material and data, the collaborating groups should be able to detect linkage even if there is genetic heterogeneity. By following the discussed guidelines and arrangements, we hope to use an efficient way localizate the GTS gene. Linkage will open the door to isolating the the disease gene allow us to define the genetic transmission model in more detail. Eventually, characterization of the gene

and its product will help us to understand the basic mechanisms causing GTS.

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CHAPTER 6

CLINICAL OBSERVATIONS ON HEREDITARY PARAGANGLIOMAS

6.1 CLINICAL OBSERVATIONS

Paragangliomas of the head and neck, also known as glomus tumors or chemodectomas, originate from the paraganglion system. In general these rare, slow growing tumors are benign, less then ten percent develops into metastases (1,2). The clinically most relevant tumor sites are the carotid body tumor, the vagal and jugular body tumor, located in the neck, middle-ear or skull base, respectively. Paragangliomas closely resemble the tissue from which they arise. Carotid- and jugulotympanic paraganglia are branchiomeric paraganglia, the vagal body is an intravagal paraganglion (Fig 1). All these organs are part of the parasympathetic extra-adrenal paraganglion system. This system functions as a series of afferent receptor organs.

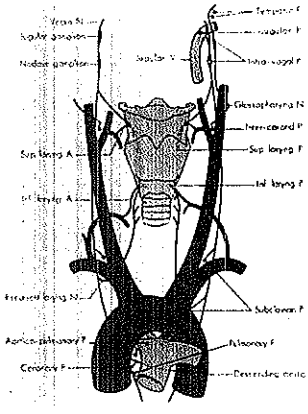

Fig 1. Sites and anatomic relationship of branchiomeric and intravagal paraganglia.

Paragangliomas are neuroendocrine tumors and produce catecholamine and in most cases nor-epinephrine. The tumors are usually chromaffin negative and somatostasin positive. A normal carotid body is present as a small grain shaped structure which is largest around the age of 20. At a later stage in life the bodies become sclerotic. The other organs of the paraganglion system are small microscopical structures, composed of aggregates of only a few cells. A branchiometric paraganglion is a ball like structure of vascular tissue including arteries, capillaries and veins. Two types of cells are predominant. Type I or chief cells contain large cytoplasms with poorly defined margins and a large round or oval nucleus. Type II cells are sustentacular elongated cells that ensheathe nerve

axons. They resemble Schwann cells and may even merge with these cells. In the tumors, these two types of cells are arranged in clusters in which the sustentacular cells are surrounding a central core of chief cells. Paraganglia show extensive vascularity and innervation by nerve fibers from the carotic branch of the glossopharngael, vagus nervus and by non-myelinated sympathetic fibers from the superial cervical ganglion (3,4). The glomus tissue arises from the neural crest (neuroectoderm), while the fibrous stroma and vessels of the carotid body have an mesodermal origin (5).

Non-chromaffin paragangliomas may be clinically aggressive, producing disabling symptoms and even death by, slow, progressive enlargement with encroachment on vital organs, or by infiltrative growth into surrounding structures. Patients usually complain about pulsatile tinnitus and hearing loss. As the lesion expands involvement of lower cranial nerves (VII-XII will significantly influence morbidity.

The treatment of paragangliomas usually consists of total surgical excision. Surgical treatment carries the risk of postoperative complications and morbidity. Surgery is not always radical and this can lead to partial facial- or cranial nerve palsy. For elderly people and those in poor health, radiotherapy might be preferable with the aim to slow down tumor growth.

6.2 GENETICS OF HEREDITARY PARAGANGLIOMAS

6.2.1 Initial studies

The incidence of hereditary paragangliomas is difficult to asses as these tumors are very rare. The only large population study to date was performed on 600.000 surgical patients from the Sloan-Kettering Memorial Cancer Center between 1937-1975 (1). In total 69 paragangliomas were found, resulting in a minimum incidence of 0.012%.

Familial occurrence of carotid body tumors and glomus jugulare tumors was described in the first halve of this century by Chase, Goekoop and Bartels (6-8). In these early studies the transmittance of the phenotype was consistent with an autosomal dominant mode of inheritance. In the study by Bartels (8) the association between carotid body tumors and glomus jugulare tumors was noticed. Grufferman (9) reviewed 88 familial cases and 835 non-familial cases with glomus body tumors and found that familial glomus tumors are much more likely to be multicentric than sporadic tumors. Bilateral disease occurred in 31.8% of familial cases and only in 4.4% of sporadic cases. In 6% of cases, second primary tumors, mostly other paragangliomas, occurred.

Wilson (10) reviewed the reports of familial cases and described a family with male-to-male transmission and a 'skipped' generation. Van Baars (11) studied a large family with hereditary paragangliomas with 26 affected individuals. The trait was traced through 6 generations. Penetrance of the tumors appeared to be almost complete at the age of 60. Although all reports concluded that the inheritance pattern was consistent with an autosomal mode of inheritance both Herrmann (12) and Van Baars (11) noted that affected males were more likely to have affected offspring then affected females. To explain these atypical findings Herrmann postulated a mechanism of 'delayed mutation' as an explanation for the incomplete penetrance (12). This implies transmission of a premutant allele of an autosomal gene through unaffected carriers and forward mutation in affected persons which then transmit the condition to their offspring in a regular fashion. Knudson (13) put forward a two-step mutation theory to explain the inheritance of a number of cancer syndromes: in hereditary cases the first mutation is pre-zygotically present and is transmitted in a Mendelian fashion. Tumors develop only after a second somatic mutation event. Non-hereditary cases occur only if two somatic mutations occur in one cell. Both models could explain the observed incomplete penetrance but not the specific transmission of hereditary paragangliomas through the paternal line. Van der Mey *et al.* (14) concluded that the transmission pattern of hereditary paragangliomas could be explained in terms of genomic imprinting.

6.2.2 Imprinting in hereditary paragangliomas

Familial occurrence of hereditary paragangliomas is consistent with an autosomal dominant mode of inheritance with incomplete penetrance, and the penetrance seems to be influenced by genomic imprinting. Penetrance of the disease phenotype is strongly related to the sex of the transmitting parent, since clinical manifestations of the tumors are exclusively expressed through the paternal line. Affected females never transmit the disease phenotype to their children and re-appearance of the phenotype is observed only after passage of the disease gene through a male gene carrier.

Van der Mey *et al.* (14) proposed that paragangliomas are caused by an autosomal gene that is inactivated by the imprinting process during female oogenesis resulting in an unaffected offspring. Gene expression is reactivated during male spermatogenesis by removal of the imprint leading to affected offspring in the following generation.

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CHAPTER 7.1

A GENE SUBJECT TO GENOMIC IMPRINTING AND RESPONSIBLE FOR HEREDITARY PARAGANGLIOMAS MAPS TO CHROMOSOME 11Q23-QTER

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ABSTRACT

Paragangliomas of the head and neck are slow growing tumors which rarely show malignant progression. Familial transmission has been described consistent with an autosomal dominant mode of inheritance. Clinical manifestations of hereditary paragangliomas are determined by the sex of the transmitting parent. All affected individuals have inherited the disease gene from their father, expression of the phenotype is not observed in the offspring of an affected female until subsequent transmittance of the gene through a male carrier. This finding strongly suggests that genomic imprinting is involved. We report the results of a linkage study on a large Dutch pedigree with hereditary paragangliomas. Highly significant evidence for genetic linkage to chromosome 11q23-qter with the anonymous DNA marker D11S147 was detected with a peak lod score of 6.0 at a recombination fraction Θ = 0.0. Likelihood calculations yielded an odds ratio of $2.7 \cdot 10^6$ in favor of genomic imprinting versus the absence of genomic imprinting.

INTRODUCTION

Paragangliomas of the head and neck, also known as glomus tumors or chemodectomas, arise from the paraganglionic system formed by neuroepithelial cells that are derived from the neural crest during embryogenesis. The tumors are mostly benign and slow growing. The clinically most relevant tumor sites in the head and neck are the carotid body tumor, the vagal and jugular body tumor located in the

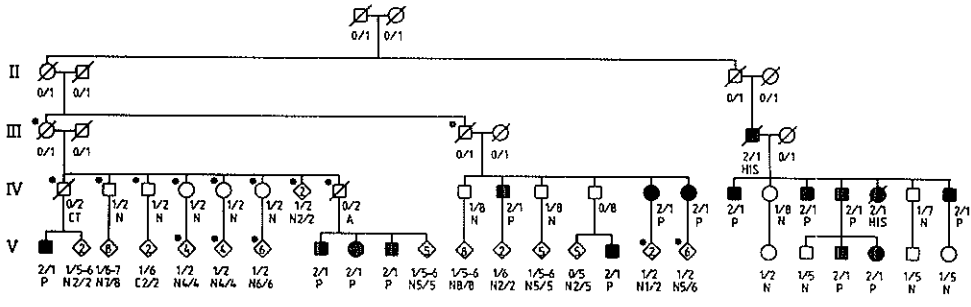

Fig. 1. Pedigree of family with hereditary paragangliomas. The affection status of individuals was classified as 1 for unaffected individuals, 2 for affected individuals and 0 for individuals that were not diagnosed. The following symbols are used: Solid symbols: affected; Open symbols: unaffected or unknown affection status; 1/2:affection status /liability class; N: MRI-negative; P: MRI-positive (N 5/5: MRI negative in 5 out of 5 examined sibs); HIS: Status histologically verified. C: Clinically investigated; CT: CT-scanned. A: Anamnestic. * : Individual is potentially subject to genomic imprinting.

neck, middle-ear or skull base, respectively (1). Familial occurrence has been reported in several studies and is consistent with an autosomal dominant mode of inheritance with incomplete penetrance. X-linked inheritance in these families could be excluded because of the extensive male to male transmission of the disorder (2-5). Penetrance of the disease phenotype is age-dependent, with onset rarely occurring before the age of 18 (4). Penetrance also appeared to be strongly related to the sex of the transmitting parent, since clinical manifestations of the tumors were exclusively expressed through the paternal line. Affected females never transmitted the disease phenotype to their children and re-appearance of the phenotype was observed only after passage of the disease gene through a male gene carrier. These findings were confirmed in a retrospective analysis of medical records of fifteen pedigrees with hereditary paragangliomas (6). As a possible explanation for these atypical findings Van der Mey, *et al.*(6) proposed that the maternal and paternal alleles of the gene associated with hereditary paragangliomas must be subject to genomic imprinting. Genomic imprinting is a process which confers functional differences on the maternal and paternal alleles. The imprintable gene is transmitted in a Mendelian manner, but expression of the phenotype will be determined by the sex of the transmitting parent (7).

We now report the results of a linkage study on a large Dutch five generation pedigree with hereditary paragangliomas in which the inheritance pattern could very well be explained by genomic imprinting.

Table 1. Results of simulation analysis on all available family members and on 29 key individuals. Results were based on 200 replicates and a four allele marker.

	Number of replicates	
Lod score	All available individuals(n=95)	29 key individuals
>1.0	180	177
>1.5	159	160
>2.0	140	130
>2.5	105	100
>3.0	83	72
>3.5	57	49
>4.0	41	36
Max. lod score	6.47	6.44
Mean lod score	2.74	2.63

RESULTS

Transmission of the disease phenotype in the family that was studied is fully consistent with the hypothesis of genomic imprinting: all affected persons have inherited the disease gene from their father (fig. 1). Individuals marked with an asterisk are possible non-penetrant gene carriers subject to genomic imprinting.
Simulation studies with the SLINK program package (9,10) under the assumption of a single autosomal gene subject to genomic imprinting, were performed to determine the power of this family to detect linkage. In total 200 replicates were generated with a 4 allele marker at 5cM of the disease locus. A maximum lod score of 6.74 and a mean lod score of 2.74 were reached (Table 1). In following simulation studies those individuals that contribute considerably to the lod score were determined. After several rounds of reduction in the number of individuals, 29 key individuals were selected for the initial genome search. With this core family a maximum lod score of 6.44 and a mean lod score of 2.63 at 5 cM of the disease locus was reached in the simulation study; 96 % of the lod score is derived from 29 samples.
We performed a random genome search on this core family with polymorphic DNA markers to localize the gene responsible for hereditary paragangliomas. After finding a positive lod score with the core family of 29 samples with marker D11S836 (Mfd108), additional markers in the region were tested. Pairwise lod scores of all the markers tested in the region are given in Table 2. With the anonymous DNA marker D11S147 (HBI18P2) significant evidence for linkage was obtained. To extract all possible information from the family all 95 individuals

Table 2. Pairwise lod scores between chromosome 11q23-qter markers and hereditary paragangliomas at various recombination fractions.*

	Lod of recombination fraction						
Probe	0.00	0.01	0.05	0.10	0.20	0.30	0.40
D11S29	0.82 (0.64)	0.87	0.88 (0.69)	0.78 (0.66)	0.51 (0.51)	0.27 (0.31)	0.09
PBGD	2.36 (1.95)	2.32	2.15 (1.8)	1.90 (1.6)	1.33 (1.11)	0.76 (0.61)	0.26
CD3D	4.51 (1.35)	4.39	3.92 (1.35)	3.36 (1.25)	2.33 (0.93)	1.42 (0.55)	0.59
D11S147	6.09 (4.67)	5.95	5.39 (4.32)	4.70 (3.88)	3.35 (2.88)	2.04 (1.76)	0.78
D11S836	-∞ (-3.83)	1.01	1.94 (0.98)	1.99 (1.36)	1.54 (1.28)	0.94 (0.83)	0.36

* Lod scores are based on genotypic data from 95 individuals, numbers between brackets are lod scores derived with the core family of 29 individuals.

were tested with markers in the region (Table 2). Highly significant evidence for genetic linkage under the assumption of genomic imprinting was obtained on chromosome 11q23-qter. With the anonymous DNA marker D11S147 (HBI18p2) a lod score of 6.0 was reached, a marker for the nearby locus CD3D (Mfd69) gave a lod score of 4.3. Both results were obtained at a recombination fraction of $\Theta = 0.0$. Other markers that were tested on other autosomes generated no evidence for linkage.

We constructed a linkage map of chromosome 11q23-qter including markers D11S29, DRD2, CD3D, PBGD and D11S147. (Figure 2). Sex specific recombination frequencies between the markers were determined. Female recombination fractions were kept at 2.45 times that in males. Marker D11S836 was tested on 33 CEPH families and incorporated in the determined linkage map. D11S836 was mapped on the telomere site of D11S147 with odds >1000:1. (unpublished data).

Figure 2. Most likely order and female recombination rates between gene markers on chromosome 11q23-qter. The odds against inversion were higher the 1000:1. Sex specific recombination frequencies between the markers were determined. Female recombination fractions were kept at 2.45 times that in males. Exact recombination frequencies of D11S386 with the other markers was not determined (nd).

Female map:

Locus:	DRD2	D11S29	CD3D	PBGD	D11S147	D11S386
cen	———	———	———	———	———	——— qter
Theta:	0.12	0.08	0.0	0.02	nd	

Multi-point analysis for the localization of the disease locus, including data from D11S29, D11S147, CD3D and PBGD, yielded a peak lod score of 7.49 for a localization at D11S147. Construction of haplotypes for the markers in the region revealed no recombination events between the disease phenotype and the markers CD3D, PBGD and D11S147. We observed three recombination events for the most telomeric marker D11S386. This places the locus for paragangliomas on chromosome 11q23-qter proximal of D11S836.

Although genomic imprinting accounts very well for the observed segregation pattern, an autosomal dominant mode of inheritance with an incomplete penetrance remains a plausible alternative explanation. Multi-point analysis of the disease locus with the same marker data but assuming no genomic imprinting

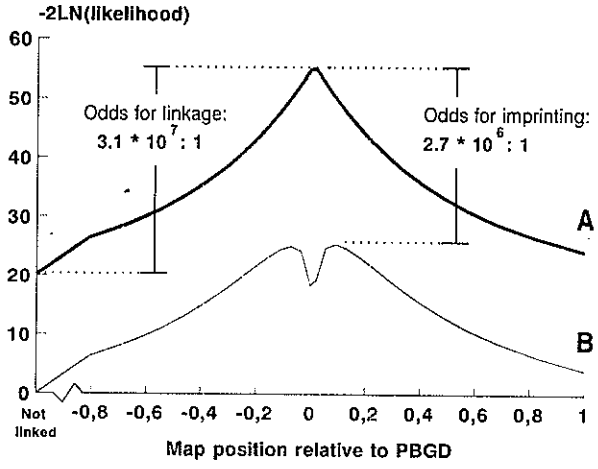

Fig. 3. Location map summarizing likelihoods calculated for hereditary paragangliomas at various map positions on chromosome 11q23-qter in a fixed marker map. A: with genomic imprinting; B: without genomic imprinting.

generated a maximum lod score of 5.43. Although considerably lower than the lod score obtained for the imprinting model it is nevertheless significant. We therefore made a statistical comparison of the two genetic models via the likelihood ratio method. In standard linkage analysis of a disease locus with a genetic marker, the likelihood is maximized over a varying recombination fraction. In order to determine the odds for genomic imprinting we performed a likelihood comparison between two four point linkage analyses to determine the best position of the disease locus in respect to markers CD3D, PBGD and D11S147. One analysis assumed complete genomic imprinting of the maternal allele and the alternative analysis assumed no genomic imprinting. The ratio between the maximum likelihoods of the two models corresponds to the odds for genomic imprinting. An odds ratio as high as $2.7 \cdot 10^6$ in favor of genomic imprinting was calculated (figure 3) providing strong evidence for the hypothesis that this gene, that is associated with hereditary paragangliomas is subject to genomic imprinting.

DISCUSSION

In this study we report the localisation on chromosome 11q23-qter of a gene that is responsible for hereditary paragangliomas and subject to genomic imprinting.
Before starting a random genome search with polymorphic DNA markers we performed simulation studies in order to determine the power of the family to

detect linkage. The maximum lod score obtained in the simulation studies is well above the accepted significance level of 3.0 (Table 1), the mean lod score is very close to the significance level. We concluded that the large family is powerful enough to detect linkage. The workload that is involved with testing microsatelite markers quickly increases with sample size. Additional simulation studies enabled a two third reduction of the family size without losing power to detect a linkage. After finding a positive lod score full information from the family was extracted by testing all available samples. Although the results from the simulation studies and the actual genotypic data are difficult to compare, because the position of the disease locus still has to be determined, it seems that they are in reasonable agreement with each other. The results presented here indicate that simulation studies can effectively decrease the workload associated with a random genome search.

By using the likelihood ratio method we obtained an odds ratio as high as $2.7 \cdot 10^6$ in favor of an autosomal dominant gene subject to genomic imprinting versus an autosomal dominant gene with reduced penetrance (figure 3). This method provides strong statistical evidence for the hypothesis that this gene, that is associated with hereditary paragangliomas is subject to genomic imprinting.

Genomic imprinting has been operationally defined as differential expression of genetic material depending on the parental origin of the gene (7). For hereditary paragangliomas Van der Mey *et al.*(6) proposed an autosomal dominant (onco) gene that is inactivated by the imprinting process during female oogenesis resulting in unaffected offspring. Gene expression is proposed to be reactivated during male spermatogenesis by removal of the imprint and this would lead to affected offspring in the following generation. An alternative explanation assumes the functional inactivation of both alleles of a tumor suppressor gene, whose normal activity is required for the proper development of carotid body tissue (8). In affected individuals the maternal might be inactivated by the imprinting process the paternal by a physical disruption of the gene sequence such as a point mutation or a deletion. A comparable imprinting mechanism has been described in mice for the gene encoding Insulin-like Growth Factor 2 (IGF2)(12). Mice carrying a targeted mutation in IGF2, resulting in a non-functional disrupted gene, produce growth deficient progeny when the disrupted gene was transmitted through the male germ line. In these mice the maternal allele is not expressed, presumably because of the imprint. Transmittance of the disrupted gene through the female germ line resulted wild type offspring, indicating proper functioning of the normal male allele in these mice. The imprinting process could act directly on the gene responsible for the phenotype or alternatively influence the expression of a

modifier gene *in trans* (7,13).

For a number of human genetic disorders the influence of genomic imprinting on the expression of disease phenotypes has been described (13). Uniparental disomy as well as deletions of chromosome 15q11-q13 have been associated with both Prader-Willi (PWS) and Angelman (AS) syndromes. These disorders are examples were either absence of paternal (PWS) or maternal (AS) chromosome contributions leads to the disease phenotype (13,14). For Huntington disease and Myotonic dystrophy, genomic imprinting dependent on the sex of the transmitting parent has been suggested for a subset of the cases, to explain the differences in the disease phenotype and/or age of onset (13). In human cancer syndromes like Wilms' tumor, loss of heterozygosity resulting in expression of the mutant allele gives rise to the disease phenotype. The loss of heterozygosity preferentially involves the maternal allele, implicating that the paternal allele is imprinted (15). Interestingly, the parallel between Wilms' tumor and paraganglioma may extend beyond the involvement of genomic imprinting in its genetic aetiology, in that both are likely to be a derangement of normal tissue development. Wilms' tumors develop from metanephric blastemic tissues that have failed to undergo the normal maturation process (16). Since the distribution of paraganglion cells in human fetus and newborn infant is considerably wider than in the adult (17), paragangliomas may arise from cells that have not responded properly to signals for programmed tissue regression. Therefore we suspect that genes involved in signal transduction or cell-cell communication, such as growth factors, growth factor receptors, or cell adhesion molecules (N-CAM), are possible candidate genes for hereditary paragangliomas, rather than genes involved in later stages of malignant tumor progression.

The clinical significance of the localization of the gene responsible for hereditary paragangliomas lies in the screening for carriers of the disease gene. Efficient screening could lead to an early detection of the tumors improving the possibilities for treatment of patients. Genetic screening will also reveal the non-carriers who have the population risk. This would dismiss non-carriers from undergoing extensive medical examinations and from the psychological stress of being at high risk of developing the disease.

Hereditary paragangliomas is one of the few examples of clinically important effects of imprinting at a single gene (locus) level where the genomic imprinting appears to be absolute. The penetrance of the disease phenotype in the direct offspring of a male gene carrier is complete by age 50 in contrast to the "imprinted" offspring of a female gene carrier where the disease phenotype is absolutely non-penetrant. The localization of the gene for paragangliomas opens the way for

isolating the responsible gene. The fact that genomic imprinting of paragangliomas can be studied in large pedigrees makes hereditary paragangliomas a very attractive model to study the molecular mechanisms of imprinting. Isolation and characterization of this gene will provide important genetic and biochemical clues about the process of germline specific modification of gene expression.

Table 3. Liability classes used in the linkage analyses.

Liability class	Penetrance	
Class 1	1.00	< Married in or affected.
Class 2	0.00	< Children of affected mother
Class 3	0.00	< Unaffected, at risk, <15 years
Class 4	0.10	< Unaffected, at risk, 15-20 years
Class 5	0.35	< Unaffected, at risk, 20-30 years
Class 6	0.65	< Unaffected, at risk, 30-40 years
Class 7	0.90	< Unaffected, at risk, 40-50 years
Class 8	0.95	< Unaffected, at risk, >50 years

MATERIAL AND METHODS

Family study

We examined all available members (n=95 out of 113) of one large Dutch five generation pedigree with 16 ascertained patients with head and neck paragangliomas (Figure 1).

Diagnoses of family members were based on medical history, physical and otolaryngological examination, whole body Magnetic Resonance Imaging and determination of free urinary catecholamine excretion. For confirmation of paragangliomas, contrast enhanced computed tomography or angiography was performed. When a hormonal active lesion was suspected [123I]MIBG scintigraphy was applied (18).

DNA studies

Genomic DNA was isolated from peripheral blood as described by Miller *et al.* (19). Restriction digestion was carried out according to the manufacturer's recommendations. Gel electrophoresis of 5µg DNA samples on 0.7 % agarose gels,

and DNA immobilization by alkaline blotting onto nylon membranes (Hybond +, Amersham), were performed according to standard procedures (20). Hybridization conditions were as described by Maniatis *et al.*(20) and washing was performed at 65 °C on 0.1 x SSC final stringency. DNA was labelled by primed synthesis according to the protocol of Feinberg and Vogelstein (21). Marker PBGD was kindly provided by Dr. M. Goossens. Markers D11S29 and D11S147 were obtained from ATCC. Microsatelite markers were analyzed as described by Weber and May (11).

Linkage analyses

Linkage analyses were performed using the LINKAGE program package version 5.03 (21,22). Simulation studies were performed with 200 replicates of a 4 allele marker (allele frequencies 0.25 each) at 5 cM of the disease locus. Simulated data were generated using the SLINK program package (9,10) under the assumption of an autosomal dominant gene subject to genomic imprinting. To determine which individuals contribute considerably to the lod score a peeling procedure of the pedigree was performed. In subsequent rounds of simulation analysis each time marker data from one individual were omitted. In the final simulation analysis only those individuals that contributed considerably to the lod score were included. These results were compared with the results from the simulation analysis on all available members of the family.

In the statistical analyses of linkage, autosomal dominant inheritance was assumed. We allowed only for a single copy of the abnormal gene to segregate in this family. The analyses were carried out under two models. In the first model genomic imprinting was implicated in that complete absence of penetrance of the phenotype was assumed when the gene was inherited from the mother. In the second model the penetrance of the gene was incomplete and age dependent, regardless of the sex of the transmitting parent. To facilitate the analyses, eight liability classes were defined to account for age of onset and the two different inheritance patterns (Table 3). For likelihood calculations under genomic imprinting the penetrance in children of female gene carriers was assumed to be 0.0 (liability class 2). For calculations under the assumption of reduced penetrance, unaffected children of female gene carrier were assigned to their age class . The gene frequency of the disease gene was fixed at 0.001.

In multi-point analyses we used Haldane's mapping function for interference. A linkage map of chromosome 11q23-qter was constructed based on genotypic data from 40 CEPH families, using the CRI-map program package (24).

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CHAPTER 7.2

A MICROSATELLITE-BASED INDEX MAP OF HUMAN CHROMOSOME 11.*

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*Adapted from: Science 258:67-86 (1992)

The map consists of 72 loci with 41 uniquely placed along a sex-average map length of 245 cM. Seventeen of the loci have heterozygosities of at least 70%, 69 are RFLP markers, and 12 are microsatellites assayed by PCR. There are no major inconsistencies between marker order in the current map and those in previous maps (1-4). The largest gap between markers occurs at the terminus of the long arm, between D11S129 and D11S387 (19.3 cM on the sex-average map). Other gaps of substantial size occur in p13 between D11S151 and D11S467 (16.7 cM) and p15 between HBB and D11S419 (15.9 cM). The female map is 276 cM in length and the male map is 225 cM. In general, female distances are as much as two to three time greater than male distances in the central region of the chromosome. However, toward the telomeres, male and female distances tend to equalize somewhat. In the most distal region, male distances show a marked and significant increase over female distances. This pattern is especially notable on the long arm. In constructing this map, the CEPH database was used directly without attempting to identify and remove probable typing errors. This may account for the 1.5-fold increase in map length over that predicted from chiasmata counts (5). It may also account for the discrepancy between the physical and genetic localizations of the marker D11Z1, which maps physically to the centromere and genetically distal to D11S429, a marker localized to 11q13.1 by FISH.

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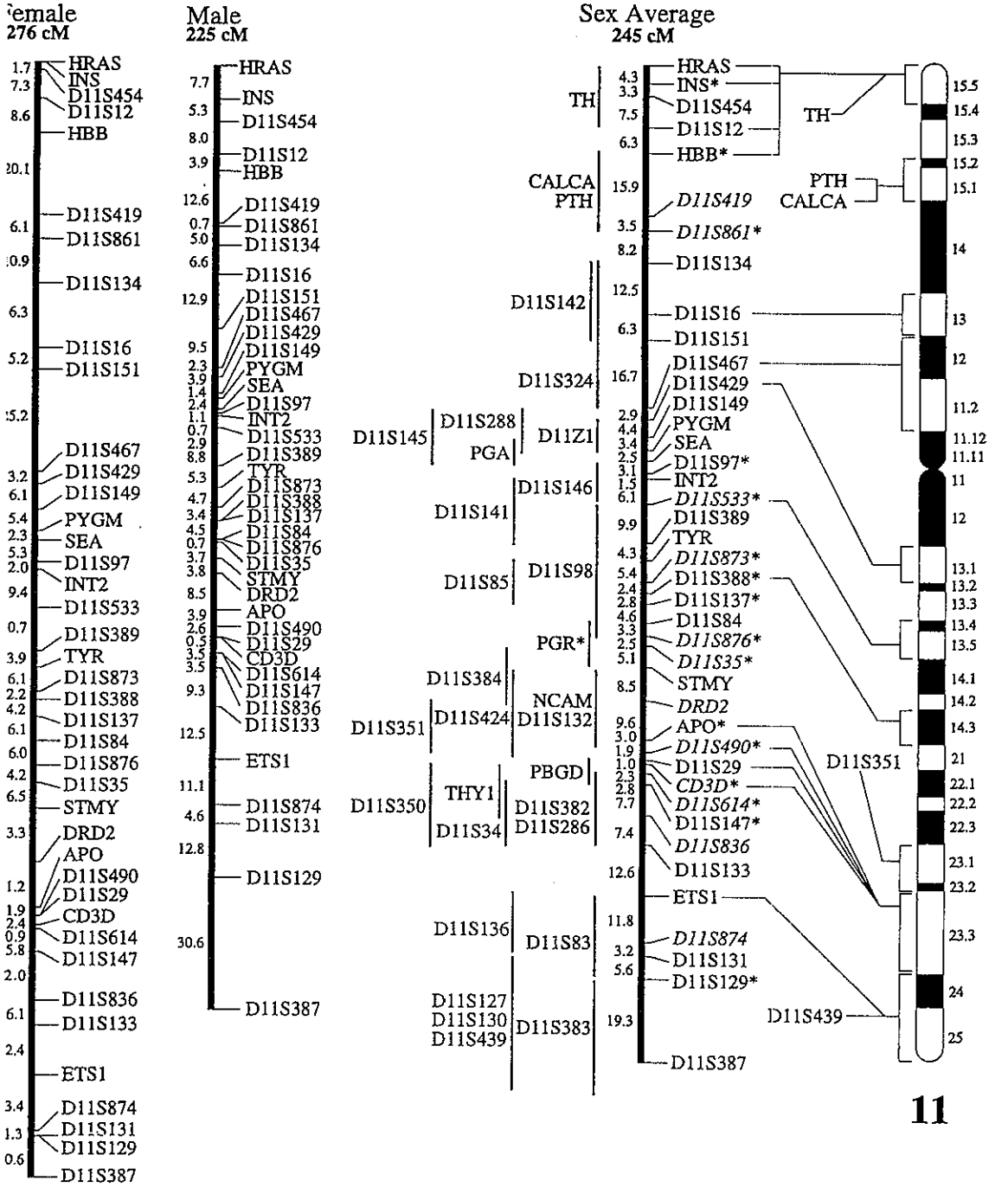
Fig. 1 Genetic linkage map for human chromosome 11. Graphical representations is shown for the autosome. The cumulative length for the female, male and sex-average genetic map are indicated at the top (in centimorgans; recombination fractions were converted to centimorgans by means of the Kosambi mapping function). In order to best illustrate the map intervals and display the marker names, the chromosomes are not strictly represented according to their physical and genetic size although the female map is represented as larger than the male map, and the sex-average map is shown as intermediate between the male and female maps. Loci uniquely placed with odds for order of at least 1000:1 are tethered to the map (to the right of the vertical map line) and are represented by HGM gene names and D segment numbers where available. A "*" indicates loci with heterozygosities of at least 70%. Microsatellite markers assayed by PCR are shown in italics. The intermarker spacing in centimorgans is indicated to the left of the map line. The sex-average map also shows markers that are not uniquely ordered. The genetic intervals for these markers are indicated by solid vertical lines to the left of the corresponding intervals on the map line. These intervals represent the placement of the markers at 1000:1 odds. The marker names are located to the left of the interval lines, and when more than one marker maps to the same interval, the markers are listed (the order for these groups of markers with respect to each other has not been determined). Representative tie points between markers included in the genetic maps that have also been cytogenetically mapped are drawn between the sex-average map and the chromosomal idiogram (to the right of the sex-average map). Brackets indicate the physical map position of markers based on cytogenetic mapping data (5). Arrows at the end points of tie lines between the idiogram and the genetic maps indicate a mapped polymorphism from a cloned telomere segment. The idiograms depict the Giemsa staining pattern at 550-band resolution for metaphase chromosomes [ISCN 550 G-bands (6)].


Female
276 cM
Male
225 cM
Sex Average
245 cM
HRAS
INS
D11S454
D11S12
HBB
D11S419
D11S861
D11S134
D11S16
D11S151
D11S467
D11S429
D11S149
PYGM
SEA
D11S97
INT2
D11S533
D11S389
TYR
D11S873
D11S388
D11S137
D11S84
D11S876
D11S35
STMY
DRD2
APO
D11S490
D11S29
CD3D
D11S614
D11S147
D11S836
D11S133
ETS1
D11S874
D11S131
D11S129
D11S387
INS*
HBB*
D11S419
D11S861*
D11S134
D11S16
D11S151
D11S467
D11S429
D11S149
PYGM
SEA
D11S97*
INT2
D11S533*
D11S389
TYR
D11S873*
D11S388*
D11S137*
D11S84
D11S876*
D11S35*
STMY
DRD2
APO*
D11S490*
D11S29
CD3D*
D11S614*
D11S147*
D11S836
D11S133
ETS1
D11S874
D11S131
D11S129*
D11S387
TH
CALCA
PTH
D11S142
D11S324
D11S145
D11S288
PGA
D11Z1
D11S146
D11S141
D11S85
D11S98
PGR*
D11S384
D11S351
D11S424
NCAM
D11S132
PBGD
D11S350
THY1
D11S34
D11S382
D11S286
D11S136
D11S83
D11S127
D11S130
D11S439
D11S383
D11S439
D11S351
11

CHAPTER 7.3

FURTHER LOCALIZATION OF THE GENE FOR HEREDITARY PARAGANGLIOMAS (PGL), AND ADDITIONAL EVIDENCE FOR LINKAGE IN UNRELATED FAMILIES.

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Submitted

ABSTRACT

Paragangliomas of the head and neck are slow growing tumors which rarely show malignant progression. Familial transmission has been described consistent with an autosomal dominant gene which is maternally imprinted. Clinical manifestations of hereditary paraganglioma are determined by the sex of the transmitting parent. All affected individuals have inherited the disease gene from their father, expression of the phenotype is not observed in the offspring of an affected female until subsequent transmittance of the gene through a male carrier. Recently we assigned the gene responsible for paragangliomas (PGL) to chromosome 11q23-qter by linkage in a single large Dutch kindred. We now report confirmation of this localization in five unrelated Dutch families with hereditary paragangliomas. On the basis of segregation of haplotypes in the available family material we localize the PGL locus between markers STMY and CD3D on chromosome 11q22.3-q23.

INTRODUCTION

Paragangliomas of the head and neck, also known as glomus tumors or chemodectomas, arise from the extra-adrenal paraganglionic system. This system is formed by neuroepithelial cells which are derived from the neural crest during embryogenesis. In the Netherlands, with a population of approximately 15 million, about 20 cases are reported each year (PALGA, kanker registratie). Paragangliomas are mostly benign, less than 10% develop into proven metastases (1). Familial occurrence has been reported and is consistent with an autosomal dominant mode of inheritance. Penetrance of the clinical manifestations of hereditary paragangliomas is not only age-dependent but was also found to be related to the sex of the transmitting parent. Affected individuals have inherited the disease gene from their father while expression of the phenotype is not observed in the offspring of an affected female until subsequent transmission of the gene through a male gene carrier. This atypical segregation pattern of expression of the phenotype is consistent with genomic imprinting (2-4), a process which confers functional differences on the maternal and paternal alleles. The mechanism that causes these functional differences is largely unknown but involves modifications of nuclear DNA that affect gene expression (5).

To explain the segregation of hereditary paragangliomas Van der Mey *et al.*(3) proposed an autosomal dominant (onco) gene which is inactivated by the imprinting process during female oogenesis resulting in unaffected offspring. Gene expression is presumed to be reactivated during male spermatogenesis by removal of the imprint and this would lead to affected offspring in the following

generation. In female oogenesis a new imprint is gained that leads to silencing of the gene. An alternative explanation is based on the functional inactivation of both alleles of a tumor suppressor gene, whose normal activity is required for the proper development of carotid body tissue (6). In affected individuals the maternal allele might be silenced by the imprinting process while the paternal allele must be inactivated by a physical disruption of the gene sequence such as a point mutation or a deletion. Furthermore, in this model the imprinting process could either act directly on the gene responsible for the phenotype or alternatively influence the expression of a modifier gene *in trans* (5,7).

For a growing number of human genetic disorders genomic imprinting appears to be involved in the expression of disease phenotypes (7). However, hereditary paragangliomas is one of the rare examples where the clinically important effect of genomic imprinting at a single locus is absolute and can be studied in large pedigrees.

Recently we reported evidence for linkage of the gene responsible for hereditary paragangliomas to markers for chromosome 11q23-qter in a large five generation pedigree (4). In this study we report a more detailed localization of the disease locus. In addition we report evidence for linkage in five independently ascertained families from the Netherlands. Our findings confirm the localization of PGL to chromosome 11q22.3-q23.

MATERIAL AND METHODS

Family studies.

We examined all available family members from six extended Dutch families with head and neck paragangliomas. Family FGT1 has been described previously (4). Families FGT3, FGT4, FGT9, FGT10 and FGT18 were recently ascertained (fig1-2). Clinical procedures were described elsewhere (4) but briefly for FGT1: diagnoses of family members were based on medical history, physical and otolaryngological examination, and determination of free urinary catecholamine excretion. In a number of cases whole body Magnetic Resonance Imaging (MRI) was performed. For confirmation of paragangliomas, contrast enhanced computed tomography or angiography was performed. When a hormonal active lesion was suspected [123I]MIBG scintigraphy was applied (8). Diagnoses of family members of the other families were based on medical history, physical and otolaryngological examination, and determination of free urinary catecholamine excretion.

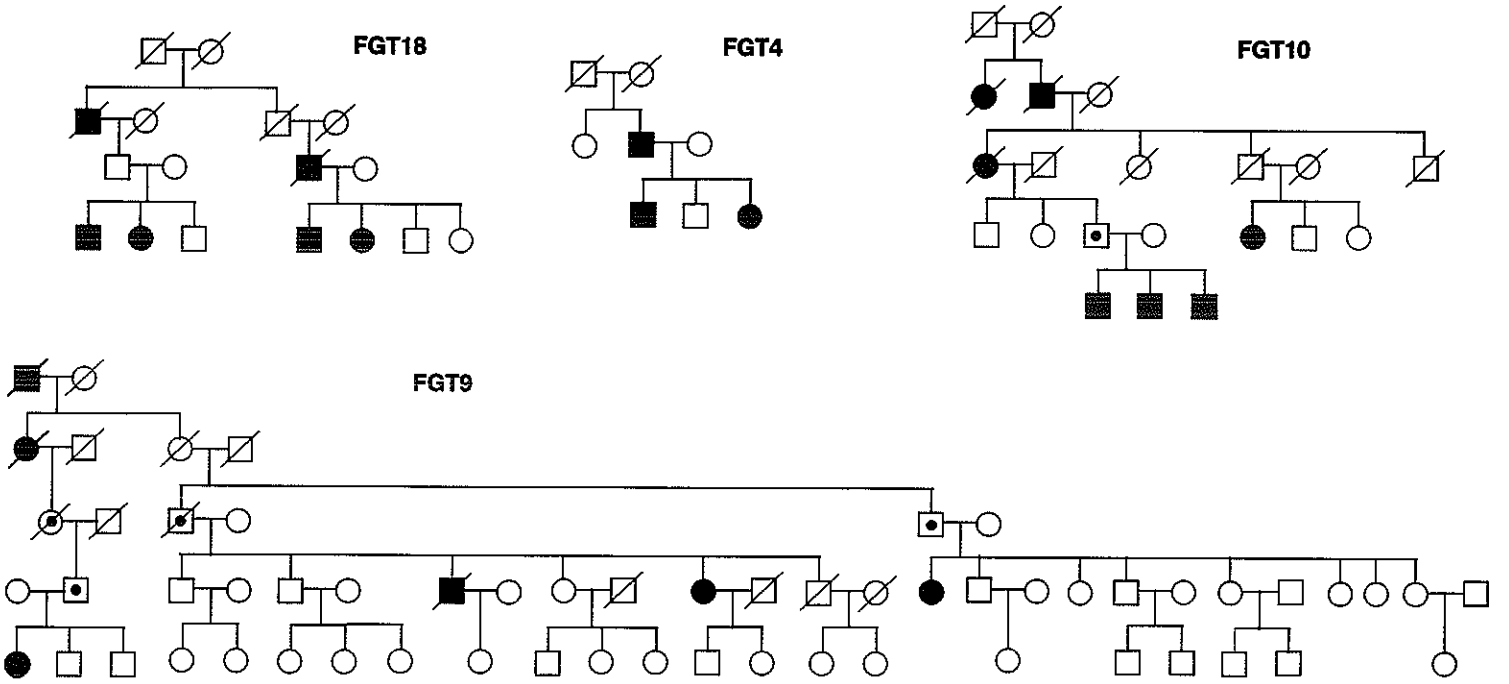

Fig 1. Pedigrees of four Dutch families with hereditary paragangliomas. Filled boxes indicate affected individuals. Blank symbols indicate individuals that did not show tumor growth or from whom the disease phenotype could not be established. Dotted symbols indicate individuals subject to genomic imprinting.

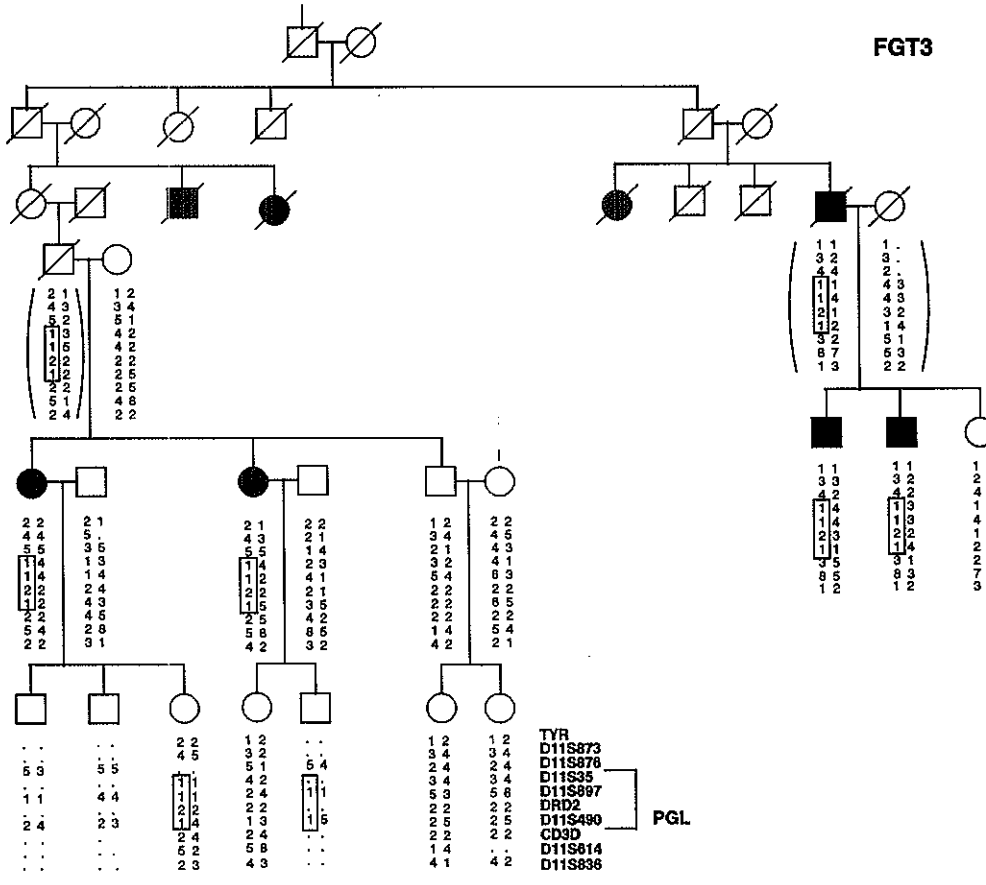

Fig 2. Haplotype analysis of family FGT3. Markers of chromosome 11q are ordered according to the NIH/CEPH collaborative linkage map (13) from centromere to telomere. X= recombination events observed.

DNA studies

Genomic DNA was isolated from peripheral blood as described by Miller *et al.* (9). Restriction digestion was carried out according to the manufacturer's recommendations. Gel electrophoresis of 5µg DNA samples on 0.7 % agarose gels, and DNA immobilization by alkaline blotting onto nylon membranes (Hybond +, Amersham), were performed according to standard procedures (10). Hybridization conditions were as described by Maniatis *et al.*(10) and washing was performed at 65 °C to 0.1 x SSC final stringency. DNA was labelled by primed synthesis according to the protocol of Feinberg and Vogelstein (11). Information and sources of all polymorphic markers used are described in the Human Genome Database (GDB)(12) and the NIH/CEPH collaborative linkage map (13). Microsatelite markers were tested in multiplex reactions essentially as described by Weber and May (14) using a Perkin-Elmer-Cetus 9600 Thermocycler. Initial denaturation was 10′ at 94°C followed by 25 cycles of 30″ denaturation at 94°C, 30″ annealing at 55°C and 90″ extension at 72°C. After 25 cycli a final extension time of 5′ at 72°C was used. Gel electrophoresis on polyacrylamide gels was performed as described by Weber and May (13).

Linkage analyses

Linkage analyses were performed using the LINKAGE program package version 5.1 (15,16). In the statistical analyses of linkage autosomal dominant inheritance was assumed. We allowed for a single copy of the abnormal gene to segregate in this family. In the statistical analyses genomic imprinting was implicated in that complete absence of penetrance of the phenotype was assumed when the gene was inherited from the mother. To facilitate the analyses, eight liability classes were defined to account for age of onset and the absence of penetrance in children of female gene carriers (4). The gene frequency of the disease gene was fixed at 0.001. Multi-point analysis was performed by subsequent three-point analyses on markers from chromosome 11q13-qter. Fifteen markers spanning the region from INT2 and D11S836 from the NIH/CEPH Collaborative Linkage map (13) were analysed. D11S527 was added to this map based on a microsatellite index map for the long arm of chromosome 11 (17).

RESULTS

Twenty polymorphic markers localized on chromosome 11q13-qter were typed in family FGT1 in addition to the five markers that were previously reported (4). Two-point analyses were performed between all markers and the disease locus

Table 1. Pairwise lod scores between chromosome 11q13-qter markers and FGT1.

	Recombination fraction (Θ)						
Locus[1]	0.000	0.010	0.050	0.100	0.200	0.300	0.400
INT2	-4.383	-1.827	-0.611	-0.184	0.091	0.117	0.064
D11S527	-∞	-7.618	-2.910	-1.309	-0.042	0.325	0.299
TYR	-2.102	-0.932	-0.288	-0.056	0.069	0.058	0.025
D11S873	-9.746	-5.053	-2.130	-0.987	-0.166	0.044	0.052
D11S84	-2.881	-0.907	-0.090	0.196	0.300	0.205	0.078
D11S35	-4.007	-2.138	-0.752	-0.143	0.267	0.285	0.167
D11S385	-0.686	-0.676	-0.602	-0.478	-0.224	-0.057	-0.000
D11S897	4.757	4.686	4.368	3.922	2.927	1.849	0.868
D11S424	1.004	0.989	0.911	0.789	0.517	0.270	0.108
STMY	-1.644	-1.351	-0.381	0.068	0.330	0.281	0.109
DRD2	0.862	0.841	0.761	0.661	0.458	0.264	0.105
D11S938	3.512	3.435	3.122	2.723	1.912	1.121	0.437
D11S144	0.756	0.752	0.703	0.639	0.474	0.277	0.093
APOC3	6.527	6.408	5.925	5.303	4.002	2.614	1.157
D11S490	2.866	2.920	2.928	2.756	2.180	1.445	0.660
D11S939	3.072	2.994	2.687	2.314	1.603	0.945	0.367
D11S29	2.777	2.831	2.838	2.665	2.093	1.372	0.612
D11S614	2.073	2.074	2.018	1.869	1.446	0.947	0.442
D11S874	1.428	1.413	1.327	1.179	0.826	0.464	0.166
D11S528	1.152	1.150	1.111	1.013	0.733	0.412	0.155

[1] Order of the loci is presented from centromer to telomer.

(Table 1). Several markers generate significant evidence for linkage with PGL. With marker APOC3 a maximum lod score of $Z=6.527$ at $\Theta=0.0$ was obtained. These findings strengthen our earlier reported evidence for linkage which placed PGL on chromosome 11q23-qter (4). From the multi-point analysis the most likely position of PGL is between markers STMY and D11S836 (fig 3).

A subset of the markers typed in family FGT1 has been typed in five additional families. The segregation pattern of paragangliomas in these families is consistent with genomic imprinting; the disorder is never transmitted by an affected female

Table 2. Cumulative pairwise lod scores between chromosome 11q13-qter markers in five families with hereditary paragangliomas.

	Recombination fraction (Θ)						
Locus[1]	0.000	0.010	0.050	0.100	0.200	0.300	0.400
D11S527	1.025	1.037	1.042	0.976	0.733	0.495	0.160
TYR	-2.857	-1.034	-0.414	-0.201	-0.061	-0.020	-0.004
D11S873	-2.040	-0.174	0.438	0.584	0.508	0.298	0.100
D11S876	0.267	1.924	2.359	2.256	1.632	0.879	0.259
D11S35	2.368	2.350	2.138	1.858	1.248	0.683	0.248
D11S897	1.858	3.394	3.686	3.401	2.416	1.331	0.438
DRD2	3.230	3.150	2.825	2.419	1.632	0.912	0.396
D11S490	-0.706	0.836	1.271	1.282	1.021	0.635	0.298
CD3D	-3.853	-0.578	0.582	1.239	0.773	0.414	0.107
D11S614	-0.700	0.991	1.470	1.456	1.073	0.592	0.180
D11S836	-∞	-1.075	0.148	0.820	0.566	0.373	0.161
D11S528	-2.265	1.468	2.368	2.369	1.913	1.567	0.447

[1] Order of the loci is presented from centromer to telomer.

(fig 1-2). Two-point analysis revealed positive lod scores with markers for 11q13-qter in each of these families. Although none of these families by itself is informative enough for detecting linkage summation of the results yielded significant evidence for linkage with a lod score of Z=3.686 at Θ=0.05 with marker D11S897 (Table 2). This marker maps to the candidate region of PGL defined in family FGT1. Multi-point analysis raised the lod score in these five families to Z=5.4 at marker DRD2. Combined multi-point analysis of all six pedigrees determines the most likely position of PGL between STMY and CD3D (fig 3). Haplotype analysis of marker data revealed two recombination events in family FGT1 (fig 4) between the markers D11S147 and D11S836, and two recombination events between the markers D11S614 and D11S836 in family FGT4 (data not shown). These events define D11S836 as the distal boundary of the candidate region for PGL. Two recombination events between STMY and APOC3 in family FGT1 define STMY as the proximal boundary of the candidate region (fig 4).
In family FGT3 the haplotype, linked with the disease locus in one part of the

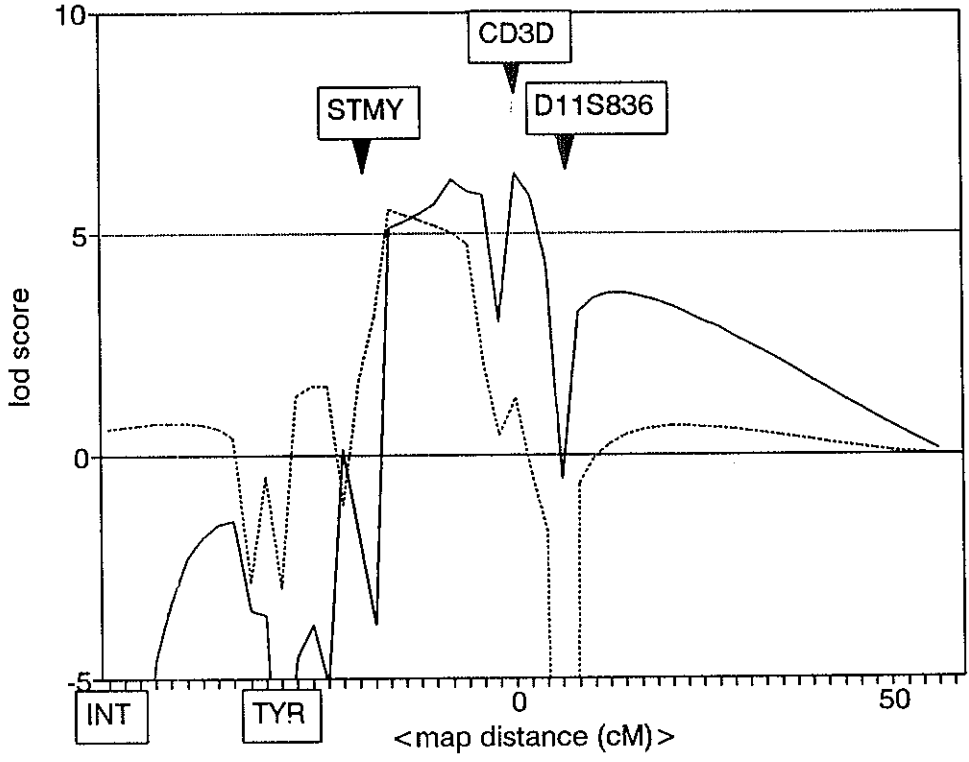

Fig 3. a) Multi-point analysis of 16 markers from chromosome 11q on family FGT1. Inter marker distances and marker order were based on refs 13 and 17.
b) Multi-point analysis of 9 markers from chromosome 11q on families FGT3, FGT4, FGT9, FGT10, and FGT18. Inter marker distances and marker order were based on refs 13 and 17.

family only segregates in part to the other segment of the family (fig2). Recombinations between D11S876 and D11S35 on the proximal side and recombinations between CD3D and D11S490 on the distal side are the simplest explanation for this finding. These results place the PGL locus between markers STMY and CD3D, narrowing the candidate region for PGL to 26 cM on the sex average linkage map (16).

DISCUSSION

We recently reported linkage of hereditary paragangliomas to markers on chromosome 11q23-qter in a large five generation pedigree (FGT1). In this study we report a more detailed localisation of the disorder between markers STMY and CD3D on chromosome 11q22.3-q23 based on linkage analysis and haplotype analysis of six families with hereditary paraganglioma.

The additional families described here are not informative enough to detect linkage by themselves but the cumulative lod scores are above the accepted level of significance for linkage (Z_{max}=3.686 at Θ=0.05 with marker D11S897). In all families segregation of hereditary paragangliomas is consistent with genomic imprinting. Although in a number of obligate carrier males, the disease phenotype could not be established, due to either non-penetrance or unretrievable anamnesis.

Haplotype analysis revealed only a small number of recombination events in the available family material informative for gene mapping purposes. An explanation for this low number of recombinations is that PGL is maternally imprinted. Hence, obligate recombinants can only be detected in affected offspring of male gene carriers. Children of affected females are not affected and will therefore not be informative. Clinical manifestation of hereditary paragangliomas is age dependent and this implies that unaffected individuals neither will be informative because they are still at risk of developing the disorder.

In family FGT1 three persons inherit the complete haplotype that is linked with the disease locus from their father, who is a gene carrier, but they have until very recently not shown signs of tumor growth on MRI scans. Two individuals are currently 24 years of age and one individual is 36 years of age and these individuals are at risk of developing the disease phenotype during the years to come. Two individuals which have inherited the complete disease-associated haplotype will not develop the disorder because it was inherited from their affected mother. Six individuals have inherited only part of the haplotype associated with the disease locus between D11S873 and APOC3 from their father.

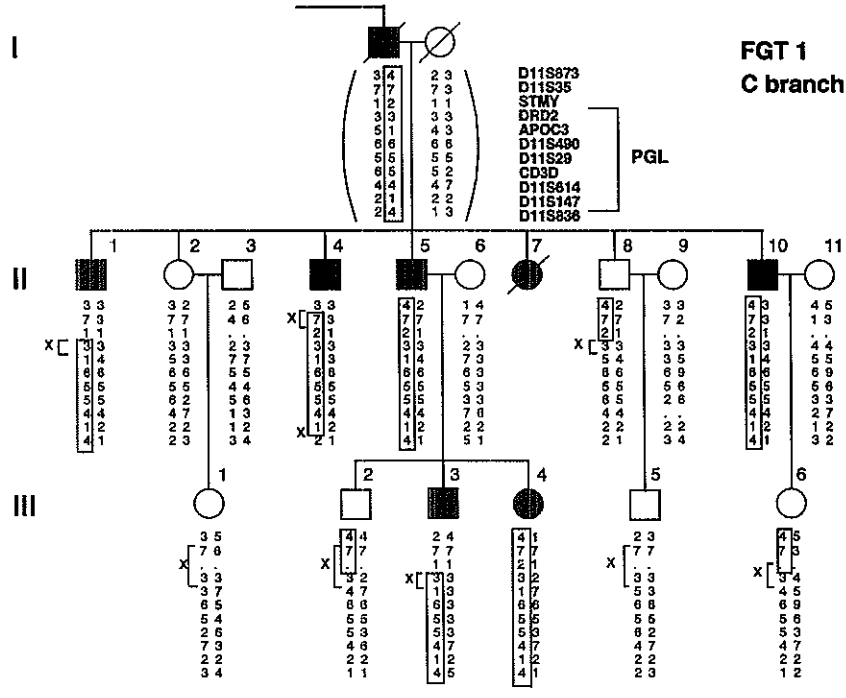

Fig 4. Haplotype analysis of family FGT1. Markers of chromosome 11q are ordered according to the NIH/CEPH collaborative linkage map (13) from centromere to telomere. X= recombination events observed.

These individuals have until recently shown no signs of tumor growth in MRI scans. They are 23-46 years of age and have 10%-65% chance still to develop tumor growth if the disease gene is located proximal from APOC3 (4). In the future these individuals will become fully informative in the haplotype analysis.

Recently linkage was detected in a family with hereditary paragangliomas with markers INT2 and TYR at chromosome 11q13-q14 (18). We have tested markers from this region, including the markers INT2 and TYR in family FGT1 and have obtained strong evidence against linkage in this region. The five additional families ascertained by us supported linkage to chromosome 11q22.3-q23 but not to markers on chromosome 11q13-q14. Linkage data from both research groups will need to be compared if either there is an overlap in the candidate regions for the locus as defined by the different families, or locus heterogeneity.

Currently we are in the process of expanding the material from the available families and are ascertaining new families. Hereditary paragangliomas is a rare disorder and the number of families that can be ascertained could become a limiting factor in the reduction of the candidate region for PGL. On the other hand, preliminary results suggest that allelic imbalance on 11q can be observed in a number of tumors, both from sporadic as well as from familial cases (P. Devilee and C.J. Cornelisse, unpubl. res.). Additional information for the location of PGL may be obtained through detailed mapping of the regions on 11q undergoing these genetic changes.

On the basis of the determined haplotype that segregates with the disease, a number of individuals in the described pedigrees should either have recombinations between markers and the disease gene or will become affected in the future.

Genomic imprinting appears to be responsible for irregular patterns of inheritance and variable expressions in human disorders (7). A growing number of human disorders show differences in phenotypes, age of onset and severity that seem to be related to the sex of the parent transmitting the gene. In a number of cancer syndromes genomic imprinting seems to be involved in disease onset. Sporadic Wilms tumor, rhabdomyosarcoma and osteosarcoma show preferential loss of maternal alleles (19-21). Uniparental disomy is reported in Beckwith-Wiedemann syndrome (22,23) and a recent example of genomic imprinting is the t(9;22) translocation associated Philadelphia chromosome positive leukemia (24). The translocated chromosome 9 is preferentially of paternal origin and the translocated chromosome 22 preferentially of maternal origin. These translocations occur somatically during the life of the individual. Genomic imprinting must be involved either in formation of the translocation or, the parentally inverse counterpart may

not cause leukemia because one of the genes involve is silenced. In retinoblastoma and hereditary paraganglioma 'skipped' generations are observed in families. Usually the 'skipped' individuals are children of affected females (3,25).

Paragangliomas of the head and neck are usually benign and slow growing, therefore candidate genes can be proposed that are involved in cellular signalling, such as growth factors, growth factor receptors, or cell adhesion molecules (N-CAM), rather than genes involved in later stages of malignant tumor progression (26). In this study N-CAM is localized in the candidate region but it lacks a polymorphism that is informative enough to determine its possible role in paraganglioma development.

The mechanism responsible for genomic imprinting is largely unknown but must involve modifications of the nuclear DNA in order to produce these phenotypic differences. The repression of heterochromatin is often associated with hypermethylation of CpG dinucleotides (27). Allele specific differences in methylation pattern have been detected in a number of tissues and site specific changes in DNA methylation pattern are known to influence gene expression (28,29). However, methylation does not seem to play a role in the silencing of the maternal allele of Igf2 (30). Epigenetic modifications outside the promotor region or other unidentified modifications at the promotor were proposed to be responsible for the imprinting. Methylation differences were found several kilobases upstream from the first exon. Possibly there are transcriptional elements for pairs of alleles which have a role in determining the parent of origin specific expression.

Now that flanking markers have been found for hereditary paragangliomas the candidate region can be reduced by testing all available polymorphic markers. Additional family material may be needed to reduce the candidate region of PGL before the next step in the 'positional cloning' of the PGL gene can be undertaken. When a resolution of only a few cM is reached the actual physical cloning of the candidate region that may lead us to identification of the responsible gene can be undertaken. Identification of this gene will not only help us to understand the molecular development of paraganglioma development but in addition offers an ideal model system to study the phenomenon of genomic imprinting.

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CHAPTER 8

GENERAL DISCUSSION

The successes of the positional cloning strategy during the last decade in the study of the gene defect in disorders with simple Mendelian inheritance has tempted investigators to apply the same strategy for disorders with a more complex segregation pattern. Although linkage analysis in complex disorders is complicated by reduced penetrance, uncertainty of diagnosis, genetic heterogeneity, genomic imprinting and polygenic inheritance, the recent establishment of the localization of genes responsible for complex disorders as Alzheimer disease, diabetes mellitus type I and a hereditary form of hypertension showed the potential of the linkage approach. Initial successes reported for psychiatric and behavioral disorders could not be supported but the mapping of these disorders might benefit from the experience obtained from the studies mentioned above.
The work described in this thesis focused on two human disorders that show a complex segregation pattern. Gilles de la Tourette syndrome and hereditary paragangliomas are clinically and genetically unrelated, but similar approaches could be used in an attempt to localize the responsible genes. Before a mapping study can be started the mode of inheritance for the disorder has to be determined. Segregation studies are a way to test the likelihood of different modes of inheritance on clinical data. Subsequent simulation studies can be used to determine the statistical power of the family material in the planning of a linkage study. In addition these studies can also be used to investigate the strategy that will give the best chances to detect linkage and that will minimize the workload.

8.1 GILLES DE LA TOURETTE SYNDROME

From segregation studies it was concluded that the pattern of inheritance of GTS is best explained by an autosomal dominant trait with reduced penetrance (Chapter 4). Associated behavioral problems as CMT and OCS are likely to be milder expressions of the same genetic defect. On the basis of these results three models with a broadening in the diagnostic criteria were defined (Chapter 3, Table 1).
To determine the ability of the family material to detect linkage with these models, simulation studies were performed. Under the assumption of genetic homogeneity the available family material was found to be powerful enough to detect a linkage under all three models.
In the ongoing studies to localize the gene(s) responsible for GTS three strategies are being used. Firstly, cytogenetic abnormalities in patients could give an indication for the localization of a disease gene. We have not observed chromosomal abnormalities in patients with GTS (Dr. J. Van Hemel, unpublished results). However, GTS patients with chromosomal aberrations have been

described. Comings *et al.*(1) and Donnai (2) presented evidence for the localization of the GTS gene on the long arm of chromosome 18q22.1. In our linkage studies strong evidence for non-linkage of GTS on chromosome 18 is described (Chapter 5.2).

A *de novo* deletion, del(9)(qter->p2304:) in a Latin-American male GTS patient was reported by Taylor *et al.*(3). Linkage analysis on chromosome 9p has been hampered by a lack of polymorphic DNA markers available in this region. Only one of the available markers did map into the region of the deletion (Dr. D. Krizman, pers. comm.). No evidence for linkage was found with this marker (Dr. J.L. Weber, pers. comm.). Recently large numbers of well localized STR markers have become available. With these markers more information from the region of interest can be obtained and more definite results can be reached. In addition to the published markers, we are in the process of generating additional STRs that are specifically localized in the region of the deletion. A flow sorted chromosome 9 genomic library is used for this purpose (4). In collaboration with Dr. P. de Jong we plan to construct a microdissection library for chromosome 9p22-pter that can be used for the generation of new markers.

A second approach for mapping disease genes is the candidate gene approach. So far this approach has not been successful for GTS after testing a number of candidate genes (Chapter 5.4, the most recent list is presented in Table 1).

Table 1. Candidate genes that have been tested for GTS

Gene	Chromosomal Location
Dopamine receptor D1	5q
Dopamine receptor D2	11q22
Dopamine receptor D3	3q13.1
Dopamine receptor D4	11p15.5
Dopamine receptor D5	4p15.1-p15.3
Prodynorphin	20pter-p12
Pro-opiomelanocortin	2p23
Gastrin Releasing peptide	18q21
Tyrosine hydroxylase	11p15
Dopamine beta hydroxylase	9q34

New candidate genes can be proposed as our knowledge on biochemical features associated with GTS increases. The candidate gene approach will gain importance in the future as large numbers genes will be identified as part of the Human

Genome Project.

Association studies are an alternative for the candidate gene approach, in which the co-segregation of a specific allele of a genetic marker and the disease is studied in patients. Association could exist with the gene primarily responsible for the trait under study. Alternatively an association could be found with a modifier gene which makes behavioral features in GTS more striking; this would increase the likelihood of diagnosis. Recently a possible association between the dopamine D2 receptor and GTS was reported (5). Replication of the study on large groups of well defined samples of GTS patients, and patients with associated behaviors is necessary to determine the value of the published finding. Linkage studies with the dopamine D2 receptor showed no evidence for linkage with GTS (6,7). The linkage findings indicate that the dopamine D2 receptor is not the primarily responsible gene for GTS, but it could act as a modifier gene on the expression of the disease phenotype.

Association studies often suffer from small sample sizes, heterogeneous clinical phenotypes and ill defined control groups. We are currently ascertaining a set of GTS patients large enough to perform association studies with sufficient power to detect a possible association with a high level of significance.

The mapping studies on GTS have focused on the third possible strategy; the systematic screening of the genome with well localized polymorphic markers (Chapter 5.2-5.4). Until now the collaborating groups have tested more then 600 polymorphic markers. No strong and definite evidence for linkage has been obtained yet, although several markers produced positive lod scores in a subset of the families. These lod scores have not yet reached a significant level and need further attention in order to obtain more conclusive results. Recent positive findings in the Dutch family set on chromosomes 3q and 1q are currently under investigation (unpublished results). For both localizations a subset of the families gave positive results. Heterogeneity analysis is in progress to determine whether these findings indicate true linkage.

The results of the linkage analyses of the consortium have been combined in exclusion maps for the human autosomes. These exclusion maps are based on the assumption that CMT is a variant expression of the GTS phenotype. A conservative estimate, based only on the well localized loci is that at least 80% of the human autosomes has been excluded as a site for the GTS gene (Chapter 5.4).

As indicated before, promising findings in linkage analyses for psychiatric disorders could not be confirmed (8-12). Some of the claims for linkage changed as a result of changes of diagnosis over time, expansion of the family material or re-typing of marker data resulting in a weakening of the evidence for linkage. Genetic

studies on psychiatric and behavioral disorders including GTS are highly dependant on the clinical diagnosis of the disorder. Without a precise etiologically oriented diagnostic classification of the phenotypes any linkage study will have little chance to succeed. The impact of diagnostic instability in one family presented in this thesis clearly indicates the importance of well defined clinical diagnosis and the necessity to check the reliability of diagnosis periodically.

The clinical heterogeneity of GTS raises the question whether the broad range of characteristics can be the result of a single defective gene. Segregation analyses indicate that a single gene model is the most likely genetic model, however, other genetic models could not be ruled out completely (Chapter 4). Currently we are performing segregation analysis on all available families within the consortium in order to obtain more information on the mode of inheritance. The clinical heterogeneity can also be the result of environmental factors. As at least 80% of the human genome has been excluded under the assumption of genetic homogeneity the possibility of genetic heterogeneity should be considered seriously. Genetic heterogeneity would undermine the collaborative exclusion data to a great extent. Construction of exclusion maps per family is in progress but awaits completion as the genotyping of markers has shifted towards highly polymorphic markers (STRs) and mapping of these markers is still in progress.

Clinical heterogeneity for GTS is also expressed within families and therefore it is not possible to divide the family material based on the clinical phenotype. The probability that genetic heterogeneity exists within a single family is small. Based on the results of the simulation studies the collaborating groups working on GTS have selected four large families, each capable of generating a significant lod score. Apart from the families the groups already use, the data for markers that are being tested will be expanded onto these four families. The exact genetic mode of inheritance can only be determined after linkage has been found.

A 'possible linkage' of GTS was reported for chromosome 3p (13). This claim was premature because the linkage data were insufficient to achieve significance and the analysis was inappropriate. The finding could not be replicated in the families available to the consortium (unpublished results). The conflicting linkage findings in psychiatric disorders have prompted guidelines by which a linkage study on complex diseases should be set up (12). These guidelines can be applied in mapping studies on GTS. Appendix A summarizes these objectives for the GTS consortium in a reaction on the premature report of a possible linkage finding. In publishing a linkage finding the most important factor is a detailed description of the way the results were achieved. The source of the marker localization should be made clear, and the haplotypes for the markers should be made available for other

investigators. This may enable others to verify the findings. Both the marker typing and the diagnostic assessment should be blinded with respect to each other and should be re-evaluated by independent investigators. Positive findings should be followed by retyping the markers generating the positive score and by the typing of more markers in the region to extract the full information of the family material. If possible the pedigree(s) should be extended or independent family material should be tested for the same markers.

Before actually starting a systematic genome search it is essential that the genetic model or models being used are spelled out clearly. These models should not be allowed to change. By testing several genetic models corrections for multiple testing should be made. Some adjustments for multiple testing have been proposed (14). Others suggested that linkage results based on a narrowly defined genotype may be given greater weight than results based on a broader disease category (12,15). The simulation study presented in this thesis indicates that for GTS the testing of three different diagnostic models will give the best possibility to detect linkage.

In addition to these guidelines arrangements have been made to prevent premature publishing of positive linkage findings. Prior to publishing positive results the other groups in the consortium will have the opportunity to replicate the findings using their own or shared family material.

To prevent false negative findings in linkage analysis other statistical approaches can be applied. The affected sib-pair analysis (ASP) the extended sib-pair analysis (ESPA) or the affected pedigree member method (APPM) do not require the specification of the genetic model but only look at the ratio of the inherited alleles (16-18). These methods may safeguard against false negative findings due to the use of a mis-specified genetic model. Large numbers of highly polymorphic markers have become available and these methods can be used as a powerful instrument in linkage analysis. Within the consortium we are ascertaining affected sib-pairs. Once the number of these pairs is sufficient to detect linkage even in the case of genetic heterogeneity, a set of approximately 200 well localized highly polymorphic markers will be tested.

With the discussed guidelines and arrangements we hope to lay the foundation for an efficient way to find the localization of the GTS gene.

It might be essential to integrate all clinical and genetic data into a central database. Such a database can be used for studies on subsets of the family material defined by clinical phenotype or by associations with certain genes. Also alternative mapping approaches could be applied; Lander and Botstein (19) have advocated interval mapping and simultaneous search procedures to surmount

heterogeneity. Interval mapping uses likelihood ratios comparing the likelihood for a disease gene being located in an interval between two markers with the likelihood of it being located outside this interval. The expected contribution to the lod score is largest when no recombination is found in the interval containing the disease locus. Recombination between the disease locus and flanking markers contributes little. Therefore, families are linked to a locus either tightly or not at all. Simultaneous searches will help to dissect out heterogeneity by studying many loci together. Multiple hypotheses can be studied to analyze even more complex multi locus models. In such a way, advanced methodology of linkage studies will open the search for the isolation of the gene that is causing the Gilles de la Tourette syndrome and will lead to a more defined genetic model. Eventually the identification of the gene(s) will help us to understand the pathogenesis of the Gilles de la Tourette syndrome.

8.2 HEREDITARY PARAGANGLIOMAS

Hereditary paraganglioma has been described as an autosomal dominant disorder with reduced penetrance. Expression of the phenotype is influenced by age of onset and the sex of the transmitting parent (20). In the linkage study described in Chapter 7.1, two genetic models were defined. The first assumes an autosomal gene with reduced penetrance. In the second model genomic imprinting was implicated in that children of an affected mother will not show expression of the disease phenotype.

A simulation study was performed based on the imprinting model in order to reduce the workload of the systematic genome search. To determine which individuals in the family contribute considerably to the power of the family to detect linkage a 'peeling' procedure of the pedigree was performed. In subsequent rounds of simulation analysis each time marker data from an additional individual were omitted. In the final simulation analysis only those individuals that contributed considerably to the lod score were included. These results were compared with the results from the simulation analysis on all available members of the family. With 29 out of 96 individuals, 96% of the statistical power of the family was maintained, reducing the workload to approximately 30%.

After linkage was found within this core family at chromosome 11q23-qter, the results were confirmed with all available family members. By using the likelihood ratio method strong evidence was obtained that expression of the gene for hereditary paragangliomas is influenced by genomic imprinting (Chapter 7.1).

During recent years highly polymorphic STR markers have become available. The

value of these markers is increased by incorporating them in the available linkage maps (Chapter 7.2). By testing additional polymorphic markers, from this map, on six families with hereditary paragangliomas the candidate region was be reduced to 26 cM (on the sex average map) between markers STMY and CD3D. Within the family material very few recombination events were observed. An explanation for this finding is the fact that only meioses of affected offspring are fully informative. Offspring of affected females never show expression of the phenotype and meioses of unaffected offspring of male gene carriers are nor fully informative as a result of the late age of onset. Additional family material is currently being ascertained and new highly polymorphic markers have become available (21). In addition we are isolating new markers that will be incorporated in the existing linkage maps in collaboration with Dr. P. Devilee and Dr. J.L. Weber (Leiden University and Marhsfield Medical Foundation, respectively).

Hereditary paragangliomas is a rare disorder and only a restricted number of families is available for linkage mapping. The minimum interval that can be obtained by fine mapping may be as large as 5-10 cM. Physical maps are being developed by participants in the Human Genome Project and identifying the expressed sequences in the region will become possible but identification of the disease gene will remain a huge task. Additional methods to further reduce the candidate region might be necessary.

In cancer syndromes allelic loss of the region harboring the responsible gene is often observed. Breakpoint mapping in tumors can accordingly be another tool in determining the candidate region of the gene responsible for hereditary paragangliomas. Collection of tumor material of patients for breakpoint mapping is in progress (Dr. P. Devilee, pers. comm.).

In diabetes mellitus type I and a hereditary form of hypertension the use of animal models resulted in the localization of susceptibility genes (22,23). Several examples of familial paragangliomas in different breeds of dogs have been described (24). Among bull-dog related breeds an elevated risk was found for the development of aortic body tumors for male dogs. It is not known whether genomic imprinting influences this risk. Comparative gene mapping between human and dog is in an early stage but in the future dogs might become an animal model to study expression of PGL.

Genomic imprinting is a process which confers functional difference on the maternal and paternal alleles; the mechanism for these functional differences on maternal and paternal alleles is unknown but must involve modifications of nuclear DNA. Methylation of cystidine residues and heterochromatin are often associated with gene silencing but seem to be a secondary effect in at least two

examples of genomic imprinting (25,26). Recently, parental methylation differences upstream of regulatory sequences of the imprinted Igf2 gene in the mouse have been observed indicating a regional effect of imprinting (26). Regulatory elements specifically involved in imprinting could be localized outside the normal regulatory elements of a gene and might influence the expression of several genes simultaneously. This situation resembles the X-chromosome inactivation (27,28). Evidence for an imprintor gene influencing the expression of the Igf2 gene in inbred mice (29) shows that this imprintor gene could encode a specific methylase that directly imprints a set of imprintable genes or alternatively it could modify the primary imprinting mechanism in the germline or shift the time of inactivation of a specific region resulting in normal cell functioning.
Comparative mapping of the candidate region for human PGL with the mouse genomic map reveals that PGL might be localized in a region homologous to mouse chromosome 9 (30). In this region no evidence for imprinting has been found. However, the size of the candidate region for PGL between markers STMY and CD3D leaves open that PGL is localized on a different syntenic group that is homologous to mouse chromosome 7, a chromosome were several imprinting events are known (31).

8.3 CONCLUDING REMARKS

Simulation studies can contribute to the effectiveness of the actual linkage studies. For hereditary paragangliomas the 'peeling' of a pedigree resulted in a 70% reduction in workload and cost of the systematic genome search. The peeling strategy is generally applicable for other disorders and could be useful for disorders with very complex inheritance patterns and highly variable phenotypes. With the availability of high numbers of PCR markers quick genome screening for linkage is possible. As participants in the primer project of the Netherlands Organization for Scientific Research (N.W.O) headed by Dr. R. Frants we obtained more then 250 well localized CA repeats. These markers can be tested in multiplex reactions with an average of four markers in a single experiment which greatly increases the genotyping efficiency. As much as 2500 genotypings per week per person have been reported (32), but this number is difficult to be achieved if the results have to be analyzed manually. Automated DNA sequencers have become available with software to analyze polymorphic PCR markers. Theoretically an efficiency of 2500-5000 genotypings per week per person will be possible. The marker data can be incorporated into the database program PRIMITIV, written by Dr. L.A. Sandkuijl, that is coupled to the LINKAGE programs. Analyses of a high

number of markers in a single batch can be performed, and different genetic models can easily be implicated. Automating the work on systematic genome searches will reduce the workload and its associated costs considerably. However, improvement of the probabilities of success for mapping studies on complex disorders will largely remain dependent on the collaboration between clinicians, statisticians, molecular biologists and especially the collaboration of patients and their relatives. For GTS, under the auspices of the American Tourette Syndrome Association, a consortium of research groups, including clinicians and molecular biologists, started a collaboration to map and identify the responsible gene(s) for GTS. In this project a tremendous amount of clinical and genetic data have been collected. That information can be used for future research especially when data are becoming accessible for the consortium in a structured database. Such a combined knowledge of clinical, biochemical and genetical characteristics of GTS will become the stepping stone for answering the specific questions on the gene(s) involved in the pathogenesis the Giles de la Tourette syndrome.

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APPENDIX A

LINKAGE AND TOURETTE SYNDROME

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Lancet 337:123-122-123 (1991).

SIR,- Dr Brett and colleagues (Oct 27,p 1076) claim a "possible linkage" for Gilles de la Tourette syndrome with genetic markers at C3p21-14. This claim is premature.

A lod score of 3, properly arrived at, is an acceptable minimum level of significance for evidence of linkage in mendelian disorders but it provides no safeguard for complex disorders. Promising linkage findings for psychiatric disorders have not been confirmed, (1-3) and one proposed remedy has been to raise the threshold lod score arbitrarily to 6 or to apply statistical correction for multiple tests (4-7).

The analysis reported is inappropriate. The maximum lod score of 2.998 at RAF1 was obtained by omitting the negative lod scores at that point contributed by the BglI polymorphism at RAF1. The discrepancy between results at the RAF1 locus for the two polymorphisms (z=0.951 at 0% recombination and 0.373 at 20% recombination) probably arises from different subsets of meiotic events in the family that are informative with the two different enzyme systems. Such results are common for polymorphisms at the same locus and should be combined into a haplotype system for the locus. It is unjustified to use one of these genetic systems selectively. The same can be said about the two different multipoint analyses; only two of the four possible multipoints are presented, and even these gave conflicting results. An even more serious error is that the order of the markers used does not agree with the known physical mapping evidence (8). An incorrect ordering of markers will in itself distort a multipoint linkage analysis and produce spurious positive or negative results. On what data was the linkage map used based? Because of the inconsistency in the multipoint analyses and the controversial linkage map only data from the two-point analyses can be used. A lod score of 2.09 is therefore the final finding.

A finding of a positive linkage should be followed up by replication in independent families (4) but the testing of five additional families for the same markers gave no further evidence for linkage indeed, the pedigrees gave negative results except for a very small positive score with RAF1. A false-positive finding of linkage or genetic heterogeneity is, therefore, very likely. This report does a disservice to patients with Tourette syndrome and their families by raising the false hope of important progress in this disorder.

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SUMMARY

During the last decade 'positional cloning' has developed into a successful strategy to identify genes involved in human disorders. As a first step in positional cloning, linkage studies are used to determine the chromosomal localization of a gene. The work described in this thesis focused on the localization of the genes responsible for two disorders; Gilles de la Tourette syndrome (GTS) and hereditary paragangliomas (PGL). The segregation of both disorders follows a complex pattern. The penetrance of GTS is influenced by sex and age. The expression of the phenotype follows a waxing and waning course over time and patients are often able to suppress symptoms for a limited time. Associated behavioural problems as the chronic multiple tic syndromes (CMT) and obsessive compulsive symptoms (OCS) are possibly variant expressions of the disorder.
The penetrance in hereditary paragangliomas is influenced by age of the patient and the sex of the transmitting parent. An explanation for the observed segregation pattern is that the maternal copy of the gene is inactivated by genomic imprinting.
GTS and hereditary paragangliomas are clinically and genetically two unrelated disorders, but very simular approaches can be used to localize the responsible genes. Linkage analysis on complex disorders is complicated by incomplete penetrance, diagnostic uncertainties, locus heterogeneity and polygenic inheritance. With incorrect definitions of the mode of inheritance and the genetic parameters, mapping studies will have a reduced probability to succeed.

Simulation studies on a large set of available families revealed that they are informative enough to detect linkage under the assumption of locus homogeneity. When locus heterogeneity is assumed, four of the available families are informative enough to detect linkage.
The diagnostic uncertainties for GTS could lead to misclassification of individuals in the linkage analysis. It has been proposed that for complex disorders with diagnostic uncertainties a very narrow phenotype would reduce the probabilities on false positive linkage findings. However, this method would result in a loss of information. An alternative strategy is to apply several diagnostic models that show a broadening in diagnostic criteria. Corrections for multiple testing procedures should be applied. Simulation studies revealed that for the available GTS family set the testing of three different models will give the best probabilities for detecting linkage.
No evidence for linkage was obtained by studying genomic regions for which cytogenetic abnormalities in patients have been described, and by testing of

candidate genes. In an ongoing systematic genome search of a consortium under the auspices of the American Tourette Syndrome Association, more the 80% of the genome can be excluded as a site for the GTS gene when locus homogeneity is assumed. If locus heterogeneity is assumed not more than 40%-50% of the genome can be excluded.
The consortium will concentrate on the four most informative families in the ongoing collaborative effort to localize the responsible gene for GTS. In addition alternative statistical methods like the sib-pair analysis and association studies will be applied.

Linkage studies on hereditary paragangliomas were performed on a single large kindred. The segregation of paragangliomas in this kindred is consistant with an autosomal gene whose expression is influenced by genomic imprinting. Genomic imprinting is a process that confers functional differences on maternal and paternal alleles; the mechanism that causes these differences is unknown but must involve epigenetic modifications of nuclear DNA.
By using simulation studies, 29 key individuals were determined of the family that contributed considerably to the statistical power of the kindred. As a result of these simulations the workload was reduced by 70%. With these key individuals a systematic genome search was performed, resulting in highly significant evidence for linkage for hereditary paragangliomas to chromosome 11q23-qter. This finding was confirmed by using all available family members. By performing a likelihood ratio test, strong evidence for a genetic model of an autosomal gene whose expression is influenced by genomic imprinting was obtained against the alternative hypothesis of an autosomal gene with reduced penetrance. By testing more polymorphic markers, that were incorporated in a collaborative linkage map, a more detailed localization for the disease locus was obtained. Evidence for linkage of PGL to the same chromosomal region was obtained in five additional families. Haplotype analysis limited the candidate region to a region of 26 cM between markers STMY and CD3D.
In order to reduce the candidate region further, new families are being ascertained on which polymorphic markers will be tested. In addition new polymorphic markers are being developed within the candidate region. As an alternative method breakpoint mapping in tumor material is in progress. Prelimenary results indicate that this method will be useful in reducing the candidate region to a size that will enable the cloning of the candiate region from genomic DNA, the next step that will lead to the identification of the responsible gene for hereditary paragangliomas.

SAMENVATTING

De afgelopen jaren heeft 'positional cloning' zich ontwikkeld tot een succesvolle strategie om genen te isoleren, die betrokken zijn bij humane aandoeningen. Een eerste stap in 'positional cloning' is het vinden van de chromosomale positie van een ziekte gen met behulp van koppelings studies. In dit proefschrift worden koppelings studies beschreven voor twee aandoeningen met een complex segregatie patroon: het Gilles de la Tourette syndroom (GTS) en erfelijke paragangliomen (PGL). De penetrantie van GTS wordt beïnvloed door het geslacht en de leeftijd van de gen drager. De expressie van het fenotype volgt een wisselend patroon en patiënten zijn vaak in staat de symptomen tijdelijk te onderdrukken. Gedragsstoornissen die vaak met GTS geassocieerd zijn, zoals chronische multiple tics (CMT) en obsessief compulsieve symptomen (OCS) zijn mogelijk andere uitingen van hetzelfde gen defect.

De penetrantie van erfelijke paragangliomen wordt beïnvloed door leeftijd van de patiënt en het geslacht van de ouder die het ziekte gen doorgeeft. Het overervings patroon van erfelijke paragangliomen kan verklaard worden door aan te nemen dat de maternale bijdrage geïnactiveerd wordt door 'genomic imprinting'.

GTS en erfelijke paragangliomen zijn twee aandoeningen die klinisch en genetisch niet aan elkaar gerelateerd zijn. Echter om de verantwoordelijke genen te lokaliseren kan eenzelfde aanpak worden gebruikt. Koppelings onderzoek aan complexe aandoeningen wordt bemoeilijkt door incomplete penetrantie, diagnostische onzekerheden, locus heterogeniteit en polygene overerving. Wanneer het overervings model en de genetische parameters niet juist worden gedefinieerd zullen de kansen op het lokaliseren van de verantwoordelijke genen afnemen.

Uit simulatie studies met een groot aantal beschikbare families bleek dat deze families informatief genoeg zijn om koppeling te detecteren onder de aanname van locus homogeniteit. In het geval van locus heterogeniteit zijn vier van de beschikbare families informatief genoeg om koppeling te detecteren.

De onzekerheden in het diagnostiseren van GTS kunnen leiden tot misclassificatie van individuen in de koppelings analyse. Als remedie hiertegen is het gebruik van een zeer strikte definitie van het fenotype voorgesteld. Een voordeel van deze methode is dat het aantal vals-positieve uitkomsten wordt verminderd, het gaat echter ten koste van informatie. Een alternatieve methode is het gebruik van verschillende diagnostische modellen, mits er een correctie voor het gebruik van meerdere testen wordt toegepast. Uit simulatie studies bleek dat voor het beschikbare familie materiaal het gebruik van drie diagnostische modellen de beste kansen op het vinden van koppeling geeft.

Bij de koppelings studies die zijn beschreven in dit proefschrift werd gebruik gemaakt van drie strategieën. i.) Chromosomale afwijkingen in patiënten kunnen een indicatie geven voor de ligging van een ziekte gen. ii.) Het testen van kandidaat genen. iii.) Het systematisch screenen van het genoom op zoek naar polymorfe markers die gekoppeld zijn aan GTS. Tot nog toe werd met geen van deze strategieën koppeling gevonden. Onder de auspiciën van de Amerikaanse Tourette Syndroom Associatie wordt binnen een consortium van samenwerkende laboratoria de screening van het genoom voortgezet. Wanneer van locus homogeniteit wordt uitgegaan kan reeds meer dan 80% van het humane genoom worden uitgesloten als plaats voor het GTS gen. Echter bij locus heterogeniteit is dit getal niet hoger dan 40%-50%.
Het consortium zal zich concentreren op het gebruik van de vier meest informatieve families om gezamenlijk te komen tot het vinden van een lokalisatie van het gen dat verantwoordelijk is voor GTS. Naast het gewone koppelings onderzoek zullen alternatieve statistische methodieken worden toegepast zoals de 'sib-pair' analyse en associatie studies.
Het koppelingsonderzoek voor erfelijke paragangliomen werd toegepast op één, zeer uitgebreide familie. Het overervings patroon van paragangliomen kan goed verklaard worden door een gen dat autosomaal overerft en waarvan de expressie beïnvloed wordt door 'genomic imprinting'. 'Genomic imprinting' is een proces waarbij functionele verschillen ontstaan tussen maternale en paternale allelen. Het mechanisme dat hieraan ten grondslag ligt is onbekend, maar wel is duidelijk dat epigenetische veranderingen van nucleair DNA betrokken zijn bij het proces.
Door het gebruik van simulatie studies werden 29 individuen in de familie geïdentificeerd die een aanzienlijke bijdrage leverden aan de statistische kracht van de familie. De werklast kon hiermee met 70% worden verminderd. Met deze sleutelfiguren werd een systematische screening van het genoom uitgevoerd. Dit resulteerde in bewijs voor koppeling van erfelijke paragangliomen op chromosoom 11q23-qter. Dit resultaat kon bevestigd worden door alle beschikbare familieleden te testen. Met behulp van een 'likelihood ratio' test werd bewijs geleverd dat het overervingspatroon beter verklaard kan worden door een autosomaal gen waarvan de expressie wordt beïnvloed door 'genomic imprinting' dan door een model van een autosomaal gen met incomplete penetrantie. Een aantal polymorfe markers werd in kaart gebracht in een gezamenlijke 'linkage map' en deze markers werden op het beschikbare familie materiaal getest, zodat PGL nauwkeuriger kon worden gelokaliseerd. In vijf onafhankelijke families werd aanvullend bewijs voor koppeling op chromosoom 11q23 gevonden. Met behulp van haplotype analyses van al het beschikbare familie materiaal konden de grenzen van de kandidaat regio voor

PGL bepaald worden tot de markers STMY en CD3D, een afstand van 26 cM.
Om de kandidaat regio verder te verkleinen worden momenteel nieuwe families onderzocht. In aanvulling hierop worden ook nieuwe polymorfe markers ontwikkeld in de kandidaat regio. Een alternatieve methode om de kandidaat regio te verkleinen is het karteren van chromosomale breekpunten in tumor materiaal. Voorlopige resultaten duiden erop dat deze methode inderdaad een bijdrage kan leveren aan het lokaliseren van PGL. Wanneer de kandidaat regio voldoende is verkleind, kan worden over gegaan tot klonering van het gebied vanuit genomisch DNA. Uiteindelijk zal deze 'positional cloning' strategie leiden tot de identificatie van het verantwoordelijke gen voor erfelijke paragangliomen.

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Stellingen behorende bij het proefschrift

GENE MAPPING OF COMPLEX DISORDERS

Gilles de la Tourette syndrome and Hereditary Paragangliomas

Peter Heutink

Rotterdam, 15 juni 1993

1. De grootste aandacht in de humane genetica gaat niet langer uit naar de wetten van Mendel, maar juist naar de uitzonderingen hierop.

2. Het prematuur publiceren van koppelingsresultaten voor psychiatrische aandoeningen brengt grote schade toe aan de geloofwaardigheid van het onderzoeksveld.

   Heutink et al. The Lancet 337:122-123 (1991).

3. Cruciaal voor het lokaliseren van genen betrokken bij psychiatrische aandoeningen is het onderlinge begrip tussen clinicus en geneticus.

   Pauls DL. Nature Genet 3;4-5, 1993.

   Dit proefschrift

4. De recente klonering van het gen verantwoordelijk voor de Chorea van Huntington illustreert dat wetenschappelijk onderzoek soms een kwestie is van stug volhouden.

   The Huntington's disease Collabortive Research Group, Cell 72; 971-983, 1993.

5. De enorme omvang en complexiteit van onderzoek in de moderne genetica heeft als positief gevolg dat samenwerking tussen research groepen en uitwisseling van data noodzakelijk is geworden.

   The Huntington's disease Collabortive Research Group, Cell 72; 971-983, 1993.

   Pakstis et al. Am J Hum Genet 48;281-294, 1991.

6. De differentiële methylering van parentale allelen is geen oorzaak maar slechts een gevolg van het mechanisme dat genomische imprinting veroorzaakt.

   Sasaki et al. Genes Develop 6:1843-1856, 1992.

   Stöger et al. Cell 73;61-71, 1993.

7. Het voorkomen van patienten met het Angelman syndroom zonder aanwijsbare chromosomale afwijking op chromosoom 15, kan verklaard worden door een mutatie in een transcriptie inactiverend *imprintor* gen dat niet in de kritische regio voor Angelman syndroom is gelegen.

Forejt J, Gregorova S. Cell 70;443-450, 1992.

8. Representational Difference Analysis (RDA) zal zich de komende jaren ontwikkelen tot een belangerijk instrument voor het identificeren van ziekte genen.

Lisitsyn et al. Science 259:946-951, 1993.

9. Bij het in kaart brengen van het humane genoom zijn experimenten met de computer zeker zo belangrijk als die met een pipet.

10. Het is opmerkelijk dat het enthousiasme van wetenschappelijk onderzoekers stijgt wanneer na het oplossen van een probleem het aantal vragen alleen maar toeneemt.

11. Zolang de structuur van de diverse afdelingen binnen de Verenigde Naties geen recht doet aan de samenstelling van de totale wereldbevolking zullen de Noord-Zuid tegenstellingen toe- in plaats van afnemen.

12. Het onmachtig toezien van de EEG in de kwestie Joegoslavië heeft deze organisatie gereduceerd tot een vrijhandels zone.

13. Wetenschap is als klimmen; voordat het doel bereikt is vraag je je af waarom je het eigenlijk allemaal doet; zodra het doel is bereikt is er behalve voldoening slechts de wil tot meer.