
Genetics of hereditary head and neck paragangliomas (glomus tumours)
Evert M. van Schothorst
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Genetics of hereditary head and neck paragangliomas (glomus tumours)

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(met een samenvatting in het Nederlands)

Genetics of hereditary head and neck paragangliomas (glomus tumours)

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I've conquered my past
the future is here at last
I stand at the entrance
to a new world...
U2

Voor mijn ouders

CONTENTS

1

INTRODUCTION

1.1 Tumours of the paraganglion system.

Paragangliomas are tumours of the extra-adrenal paraganglion system, which belongs to the parasympathic nerve system.
In general, the distribution of paraganglion cells in the human fetus and newborn infant is considerably more extensive and prominent than in the adult (Glenner and Grimley, 1974). This might explain the occurrence of well differentiated paragangliomas at sites where no conspicuous or constant paraganglion has been described as a normal occurrence in the adult.
The exact function of the paraganglion system is unknown, except for the carotid bodies and the aortic bodies which play a role in the oxygen- and pH-sensing system. These tiny glomus bodies, which are about 0.5 x 0.5 x 0.25 cm in size (rice grain shaped), function as chemoreceptors and the tumours are therefore sometimes called "chemodectomas".

"Swellings" or "ganglions" along the tympanic nerve (glomus tympanicum tumour) were first described in 1840 and 1878, by Valentin and Krause, respectively (Gulya, 1993). The definitive description of the "glomus jugularis" is credited to Guild (1941, 1953). The "glomus jugularis", now referred to as a jugulotympanic paraganglion (Glenner and Grimley, 1974), is a normal structure occurring laterally in the temporal bone. Rosenwasser (1945) realized that its neoplastic counterpart manifested clinically as a vascular tumour (glomus jugulare tumour).

Nests of paraganglion cells were discovered in the ganglion nodosum of birds by Muratori in 1932. Similar cell clusters were observed in the perineurium of the human vagus nerve in 1935 and in the same year the first description of a tumour (a paraganglioma) arising from this vagal paraganglionic tissue was published (glomus vagale tumour; Cantrell et al., 1984; Fernandez et al., 1975).

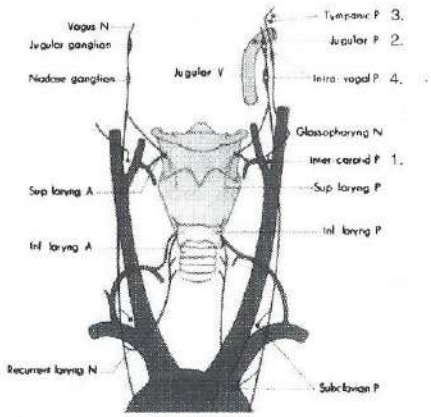

Figure 1.1. Localization of paragangliomas in the head and neck region.
1) carotid body tumour at the carotid bifurcation, which is the most common site of origin, 2) the glomus jugulare tumour at the jugular bulb, 3) the glomus tympanicum tumour at the tympanicum plexus of Jacobson's nerve and 4) the vagal body tumour at perineurium of the vagal nerve.

Tumours arising from chromaffin cells in the adrenal medulla or peripheral sympathetic ganglia are designated as phaeochromocytomas or chromaffinomas. These neuro-endocrine tumours, both adrenal and extra-adrenal, may produce catecholamines, such as serotonin, dopamine, acetylcholine and (nor)epinephrine. Elevated catecholamine production should be monitored in patients before surgery, since it may have catastrophic results if unnoticed. More than 99% of paragangliomas in the head and neck region are non-functional (Zak and Lawson, 1982). They are therefore also termed "non-chromaffin paraganglioma" (Lattes and Waltner, 1949). Due to this discordance in (non-) functionality for tumours sharing the same origin -e.g. in the head and neck region-, the separation in chromaffin and non-chromaffin tumours no longer satisfies.

The tumours are mainly located in the head and neck region at 1) the carotid bifurcation, which is the most common site of origin, the so called carotid body tumour, 2) the jugular bulb, the glomus jugulare tumour, 3) the tympanicum plexus of Jacobson's nerve, the glomus tympanicum tumour or 4) the perineurium of the vagal nerve, the vagal body tumour (Figure 1.1). If the glomus jugulare and tympanicum tumours are grouped together they are called glomus jugulo-tympanicum tumour (Gulya, 1993).

This thesis will primarily concentrate on the paragangliomas of the head and neck region or HN-paragangliomas.

1.1.2 Histology

A glomus body consist of round, large chief (type I) cells of neuroectodermal origin, which may contain numerous neuro-secretory granules containing catecholamine (Heath, 1991). These cells are localised in clusters and surrounded by the elongated sustentacular (type II) cells in an organised way (called Zellballen), thus creating the organoid appearance characteristic of the paraganglion and paragangliomas. Sustentacular cells are similar to the satellite cells of autonomic ganglia and they can be exclusively stained immunohistochemically for S100 (a calcium-binding protein of unknown function). The chief cells stain extensively for PGP9.5 (a non-granule-dependent protein of unknown function), NSE (neuron-specific enolase), Chromogranin, Serotonin, and the neuropeptides Leu-Enkephalin and Met-Enkephalin (van der Mey, 1992).

1.1.3 Detection and treatment

HN-paragangliomas can be detected with diagnostic (non-invasive) imaging techniques as computed tomographic (CT)-scanning and magnetic resonance imaging (MRI). Contrast-enhanced high resolution CT made it possible to classify all temporal-bone glomus tumours according to a surgical classification system (Valavanis, 1983), and the combination of these two imaging procedures could provide all of the necessary information with respect to the diagnosis, localization, and extent of the majority of glomus jugularis tumours (Phelps and Cheesman, 1990). Other techniques are Iodine-123

metaiodobenzylguanidine (^{123}I MIBG) scintigraphy (for functional paraganglioma imaging), ultrasound examination, angiography, and Dopler Sonography (Jansen et al., 1997). Urinary catecholamine screening is recommended for all patients with functioning paragangliomas, and in case of elevated catecholamine secretion, MIBG scintigraphy is indicated (van Gils et al., 1990a).

Treatment of HN-paragangliomas depends largely on the age of diagnosis and localization of the tumour. Actively growing tumours in young patients should be removed, while a "wait and see" policy could be adopted for the in general slow growing tumours as well as asymptomatic neoplasms in the elderly, since they represent little threat to the patients survival (van der Mey et al., 1992). Most morbidity is, in vagal body tumour cases, the result of neurologic rather than vascular damage (Green, 1988); mortality, mainly by stroke, is an important aspect surgeons should consider before starting with the resection of a HN-paraganglioma, although several other neurological deficits and vascular injuries can also occur. Besides surgical intervention, radiotherapy has been applied in several cases. However, usage has been heavily debated (Brackmann et al., 1987) and it has been suggested to be reserved for the elderly and those in poor health with the aim of slowing down local tumour growth (Hawthorne et al., 1988).

1.1.4 Clinical aspects

HN-paragangliomas are uncommon, mostly benign tumours with an estimated incidence ranging from 1 in 10,000 (Lack et al., 1979) to 1 in 100,000 - 1,000,000 (Oosterwijk et al., 1996). Between 2% and 20% metastasize, depending on the site of origin of the primary tumour; in general, less than 10% of all paragangliomas are malignant (Batsakis, 1979).
The age of onset is roughly between 18 and 60 years, with a peak incidence between 30 and 50 years. In a very small number of cases (30/2000) tumours were reported before the age of 20 (Zak and Lawson, 1982). The majority of reported cases concern Caucasians, although no studies are available to substantiate this clinical impression of uneven racial prevalence.

1.1.5 Genetic aspects

The disease presents as an autosomal dominant trait, although after maternal transmission, tumours never develop until subsequently a paternal transmission takes place (van der Mey et al., 1989). This non-Mendelian inheritance pattern has been explained by genomic imprinting, a process which establishes a functional difference between the paternal and maternal gene copy (see section 1.3; van der Mey et al., 1989; Heutink et al., 1992; McCaffrey et al., 1994). The percentage of patients with HN-paragangliomas with a positive family history ranges between 10% (Grufferman et al., 1980) and 50% (van der Mey et al., 1989). The actual percentage might be substantially higher because skipping of generations via maternal transmission of the gene obscures the hereditary pattern markedly.
The age-dependant penetrance rises from 10% (age 15-20) to a maximum of 95% (age >50 years) (Heutink et al., 1992), but a maximum of 100% at an age of 50 years has been reported for another large

family (van Baars et al., 1982). Multicentricity, either uni- or bilateral, is seen in at least 30% of hereditary cases (van Gils et al., 1990a).

The disease causing locus PGL1 has been mapped by linkage analysis (see section 1.4) to chromosome 11q22-q23 in a single, large Dutch pedigree (Heutink et al., 1992; this thesis). A second locus, PGL2, has been assigned to chromosome 11q13 by analysis of another, single Dutch family in which the PGL1 locus was excluded (Mariman et al., 1993, 1995). Linkage to 11q22-q23 has been confirmed in 3 North-American families and 3 families from unknown geographical origin, and the PGL2 locus could be excluded in a total of 8 families (Baysal et al., 1997a; Milunsky et al., 1997).

1.1.6 Syndromes in association with paraganglioma

Paragangliomas, including those of the adrenal medulla (phaeochromocytomas), can be part of (inherited) cancer syndromes such as von Hippel-Lindau disease (VHL), multiple endocrine neoplasia (MEN) type 2, and rarely in type 1 neurofibromatosis, Goldenhar syndrome and the Carney triad.

The frequency of phaeochromocytomas in affected subjects within a particular VHL family can range from 0 to >90%, with a mean of 14% (Eng et al., 1995). At the moment, only 3 cases are known where HN-paraganglioma is involved in von Hippel-Lindau disease: one glomus jugulare (Choyke et al., 1995), and two carotid body tumours (Hull et al., 1982; Zanelli and van der Walt, 1996).

In MEN2 families (A and B), affected individuals develop phaeochromocytomas, besides medullary thyroid carcinoma, parathyroid hyperplasia (A) or mucosal neuromas of the lips and tongue and a marfanoid habitus (B) (Vasen et al., 1987). Whereas both MEN2A and MEN2B carriers have germline mutations in the RET proto-oncogene, MEN2A mutations are always located in the 5'-terminal part (5' extracellular domain rich in cysteine residues and the transmembrane region), while MEN2B mutations are located in the 3'-terminal part (Tyrosine Kinase domain) of the gene, with >95% of the mutations causing a 918Met→Thr change (Morrison et al., 1996). Mechanistically, it is still unclear how this difference in mutation spectrum accounts for the observed difference in tumour spectrum.
In a study by Eng et al. (1995), no somatic mutations could be detected in both the RET and VHL genes in phaeochromocytomas from either MEN2 or VHL cases. In 10% of sporadic phaeochromocytomas somatic RET mutations were found and in only 1 case out of 48 (~2%) a somatic VHL mutation could be detected.

In type I neurofibromatosis, phaeochromocytomas occur at a frequency of only about 1% (Riccardi, 1981). The rare Goldenhar syndrome involves paragangliomas, although not all patients suffer from HN-paragangliomas. This developmental defect is characterized by hemifacial microsomia, ocular dermoids, ear appendages, and fistulas (Boles et al., 1987; Avon and Shively, 1988). The majority of cases are sporadic, but familial cases can be found. Extra-adrenal paraganglioma can be found together with leiomyosarcoma and pulmonary chondroma as a syndrome occurring mainly in young women which is known as the Carney triad (Carney, 1983). So far, no familial cases have been observed (Margulies and Sheps, 1988).

The occurrence of HN-paraganglioma and hypothyroidism has been observed in one family (father and daughter), but has never been observed in other families with probably the same founder mutation at the PGL1 locus. This makes it unlikely that it is another hereditary syndrome as suggested (Hart and Maartense, 1992; chapter 5).

In summary, paragangliomas can be found in association with other (inherited) cancer syndromes, although these paragangliomas are mostly located in the adrenal medulla (phaeochromocytomas) and only sporadically in the head and neck region.

1.2 Tumour biology and genetics

The majority of HN-paragangliomas are benign tumours. Benign tumours proliferate locally, but unlike malignant tumours they do not invade surrounding tissues or disseminate to local regional lymph nodes or distant organs. Tumours are clonal neoplasms i.e. all tumour cells are supposed to originate from a single, antecedent cell. According to the clonal evolution hypothesis, tumorigenesis is considered to be a micro-evolutionary, genetic process in which selection forces favour the clonal expansion of mutant cells (Nowell, 1976). The vast majority of human tumours appears to be of monoclonal origin (reviewed by Wainscoat and Fey, 1990). Polyclonal growth, on the other hand, involves several distinct hits in separate cells, each proliferating and expanding if a growth advantage is present. The neoplastic nature of HN-paragangliomas has been debated in the literature, because of their slow growth rate and the histological appearance resembling that of the normal glomus body, suggesting hyperplasia (Stiller et al., 1975). The finding of DNA aneuploidy in 37% of histologically and clinically benign HN-paragangliomas supports the notion that these tumours represent true clonal proliferations (van der Mey et al., 1991). Since HN-paragangliomas consist of two cellular components, viz. chief cells and sustentacular cells, they are considered by some authors to be biphasic tumours (Lack et al., 1979, Heath, 1991). So far, proof for this hypothesis at the molecular level is lacking.

Tumours result from genetic alterations perturbing normal cellular growth regulatory and differentiation programmes. Three different classes of 'cancer' genes have been identified: proto-oncogenes, tumour suppressor genes and mismatch repair genes (reviewed by Brown and Solomon, 1997). The proteins encoded by these 'cancer' genes are characterized by a broad range of functional differences. Many of these gene products are components of growth stimulatory or inhibitory signal transduction pathways; others play crucial roles in the cell cycle "clock system" (Hunter, 1997) or in maintaining genomic integrity.

1.2.2 Proto-oncogenes

Proto-oncogenes are functional in normal cells and play a role in the cellular processes of proliferation and differentiation (reviewed by Hunter, 1997). They encode growth factors or growth factor receptors,

transcription factors, nuclear proteins or function in signal transduction or apoptosis. Their oncogenic potential can be induced by several mechanisms, affecting only one of the two alleles of the gene. These mechanisms include specific chromosomal rearrangements (e.g. translocations; reviewed by Rabbitts (1994)), amplification, and DNA mutations (e.g. the codon 12 missense mutations in HRAS1; Moul et al., 1992). At the cellular level, an activated oncogene functions as a dominant cancer gene. Except for the RET oncogene, in which germline missense mutations cause the MEN2 and FMTC cancer syndromes (Morrison et al., 1996) and MET causing hereditary papillary renal cancer (Schmidt et al., 1997), alterations in oncogenes are exclusively found at the somatic level and are not associated with inherited predisposition to cancer. So far, HN-paragangliomas (carotid body tumours) were shown to stain immunohistochemically positive for the oncoproteins c-myc (12 out of 13 tumours), the apoptosis-suppressing bcl2 oncoprotein (11/13) and c-jun (5/13) (Wang et al., 1996a, 1997).

1.2.3 Tumour suppressor genes

Most tumour suppressor genes known to date have been identified through work on inherited cancer syndromes. The modern molecular concepts of tumour suppressor genes stem from three lines of experimental evidence. Firstly, according to the classical model of Knudson (1971), two rate-limiting genetic hits are required to develop childhood retinoblastoma, which were later shown to represent mutations in both alleles of a tumour suppressor gene. Secondly, these genetic hits were shown to consist of loss-of-function mutations. Thirdly, experiments with somatic cell hybrids showed that fusion of cancer cells with non-transformed cells generally leads to suppression of the malignant phenotype in the resulting hybrid. Thus, unlike proto-oncogenes which are constitutively activated, the currently available data indicate that tumour suppressor genes need to be inactivated in order to contribute to the process of tumorigenesis. Similar to proto-oncogenes, however, tumour suppressor genes encode proteins which constitute key points in many complex cellular pathways that regulate proliferation, differentiation, or apoptosis.

1.2.3.1 Knudson's two-hit model

In 1971 Knudson proposed that the development of both inherited and sporadic forms of retinoblastoma required two rate-limiting events, on the basis of cancer incidence data in the population. In hereditary cases the first mutation is pre-zygotically present and is transmitted in a Mendelian fashion (inherited or germline mutation). Consequently, all somatic cells of a carrier individual will be heterozygous for the mutation and will therefore show a predisposition for tumorigenesis (Figure 1.2a). The second, somatic, mutation inactivates the remaining wild-type copy usually by loss via a chromosomal mechanism like non-disjunction or mitotic recombination (Figure 1.2b). Inactivation by other mechanisms, like gene conversion, epigenetic silencing or a pathological point mutation, will also result in tumorigenesis, but without gross DNA rearrangements. In non-hereditary (sporadic) cases both mutations occur at the somatic level in one cell.

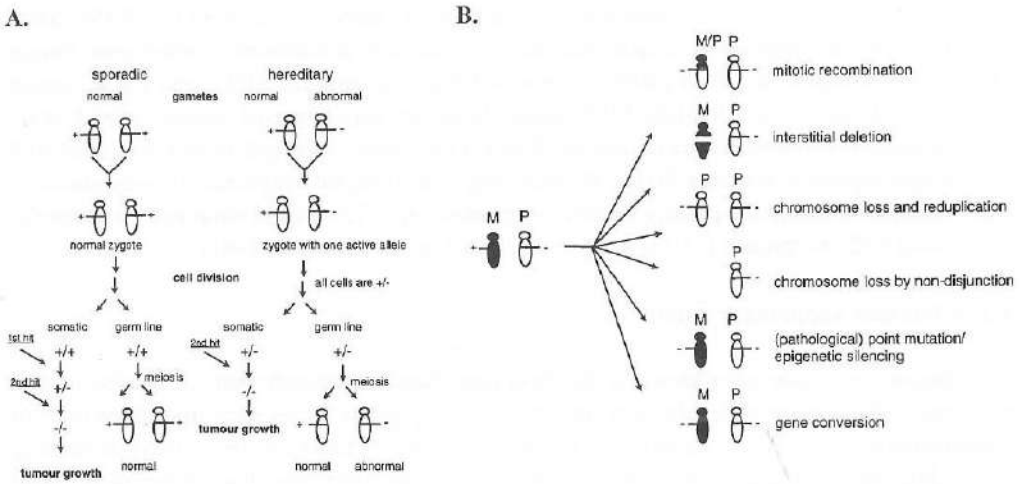

Figure 1.2. Inactivation and mapping of tumour suppressor genes.
1.2A: both the origin of sporadic and hereditary tumours is depicted. An inactive, mutated, tumour suppressor gene is depicted by -, and the wildtype as +.
1.2B: mechanisms to inactivate the remaining wildtype tumour suppressor gene (second hit). A - indicate both inactivation of the gene by mutation or epigenetic silencing.

1.2.3.2 LOH-analysis

Loss of the wildtype allele through non-disjunction or mitotic recombination in general affects quite large regions of the chromosome, and is therefore detectable by polymorphism analysis at loci flanking the gene-defect (see also section 1.4). This is then revealed as loss of heterozygosity (LOH), when DNA from normal cells of the patient is compared to that from the tumour. The combination of germline inactivating mutations and LOH of the wildtype allele has been shown to be true for virtually all tumour suppressor genes known to be associated with inherited cancer syndromes (Table 1.1). However, LOH is seen at many more chromosome regions than those harbouring cancer predisposing genes, both in inherited tumours as well as in sporadic tumours. This has been taken as evidence that these events are also targeted at tumour suppressor loci, and these may not necessarily be associated with inherited cancer syndromes. Indeed, some of those have been identified, including ECAD in lobular breast tumours (Berx et al., 1996), DPC4 in pancreatic cancer (Hahn et al., 1996), and DMBT1 in brain tumours (Mollenhauer et al., 1997). Thus the genetic two-step model has provided the foundation for

Table 1.1. Cloned genes responsible for inherited cancers.

Familial cancer	chromosome	gene	function
Adenomatous polyposis	5q21	APC	cell adhesion and signalling
Ataxia tel.	11q23	ATM	DNA repair
Breast-ovarian cancer	17q21	BRCA1	?
	13q12	BRCA2	?
Cowden disease	10q23	PTEN/MMAC1	protein tyrosine phosphatase/ focal adhesion
Cylindromatosis	16q12	CYLD1	?
Fanconi's anemia	9q22.3	FACC	?DNA repair
	16q24.3	FACA	?DNA repair
Hereditary nonpolyposis			
colorectal cancer	2p22	MSH2	DNA mismatch repair
	3p21-23	MLH1	DNA mismatch repair
	2q31-33	PMS1	DNA mismatch repair
	7p22	PMS2	DNA mismatch repair
	2p22	MSH6	DNA mismatch repair
Hereditary papillar			
renal cancer	7q31	MET	receptor for human growth factor (HGF)
Li-Fraumeni syndrome	17p13	TP53	cell cycle transcription factor
Melanoma	9p21	CDKN2A (p16)	cell cycle control
MEN type 1	11q13	MEN1	?
MEN type 2A, 2B, and MTC	10q11	RET	receptor tyrosine kinase
Neuroblastoma	1p36	p73	cell cycle transcription factor
Neurofibromatosis			
type I	17q11	NF1	RAS-mediated signal transduction
type II	22q12	NF2	membrane signalling and cell morphology
Nevoid basal cell			
carcinoma syndrome	9q22.3	PTCH	receptor for human homolog of hedgehog
Peutz-Jeghers syndrome	19p	LKB1	serine/threonine kinase
Retinoblastoma	13q14	RB	cell cycle regulator
Tuberous sclerosis	9q34	TSC1	?
	16p13	TSC2	RAP1-mediated signal transduction
Von Hippel Lindau	3p25-26	VHL	transcription elongation
Wilms' tumour	11p13	WT1	RNA splicing
(WAGR, Denys-Drash)			
Wilms' tumour (BWS)	11p15	?H19	
		?KIP2	
		?IGF2	

? Unknown. (Brown and Solomon, 1997 (review); Chandrasekharappa et al., 1997; Kaghad et al., 1997; Li et al., 1997a; Miyaki et al., 1997; Nelen et al., 1997; Slegtenhorst et al., 1997; Fearon 1997 (review); and Hemminki et al., 1998).

most efforts at isolating tumour suppressor genes by positional cloning. In this concept, the smallest region of overlap (s.r.o.) of LOH within a set of tumour samples will point to the location of the tumour suppressor gene of interest. LOH-analysis can also help in distinguishing between monoclonal and multiclonal tumour growth: monoclonal tumour cells will show the same pattern of allelic losses (an allelotype) as the founder mutant cell. A multiclonal tumour will, however, most likely show very weak losses, since specific losses in a subset of cells are obscured by the presence of tumour cells from a different origin without LOH at these specific regions (Abeln et al., 1997).

1.2.4 DNA mismatch repair genes

More recently, a third category of tumour genes has been discovered *viz.* DNA mismatch repair (MMR) genes. For hereditary non-polyposis colorectal cancer (HNPCC), the great majority of mutations were shown in the MMR genes MSH2, MLH1 and PMS2. These genes are homologues of repair genes in bacteria (MutS and MutL) and yeast (yMSH2, yMLH1 and yPMS1). Mutations in either one of these genes allows for a cascade of mismatches (mutator phenotype), thus providing the possibility of knocking out necessary growth-regulating genes (reviewed by Kolodner, 1996; Kinzler and Vogelstein, 1996).

A characteristic for the involvement of these MMR genes in tumorigenesis is the presence of microsatellite instability, which generates new alleles in tumour samples not observed in the patient's normal cells. This has never been observed in HN-paragangliomas, making it unlikely that PGL1 functions as a DNA mismatch repair gene.

1.2.4.1 Multistep model of tumorigenesis

Recent years have witnessed ever increasing LOH-patterns in many different types of malignancies. This raises questions as to whether all these LOH-events are targeted at tumour suppressor genes. Some of them might be non-selected hitch-hiker events, possibly as a result of genetic instability. The total number of tumour suppressor genes in the human genome is not known, but the current tally is approximately 30. While the genetic definition of a tumour suppressor gene (i.e. Knudson's two-hit model) has allowed their positional cloning, an increasing number become identified through functional cloning methods. This has led to genes that are able to revert the malignant phenotype of cancer cell lines upon transfection, or are showing altered or down-regulated transcription in tumour cells, but for which no genomic alterations have been detected. Examples of the latter are DCC (Fearon et al., 1990), FHIT (Ohta et al., 1996), and TSG101 (Steiner et al., 1997). Thus the definition of tumour suppressor genes is expanding rapidly, and therefore Haber and Harlow have suggested to define tumour suppressor genes as those genes that sustain loss of function mutations anywhere in the development of cancer (Haber and Harlow, 1997), i.e. in the germline, precursor, carcinoma, or metastatic stages. This provides a clear criterion with which to evaluate novel genes, should they emerge from positional cloning efforts, genome wide scans or functional screens.

Based on the age-specific incidence distribution, the majority of human cancers probably arise by a multistep process involving 5-7 distinct genetic events (Weinberg, 1989). Molecular evidence for such a cascade of genetic events first has been obtained by the pioneering studies of Vogelstein et al. (1988) on colorectal cancer. According to their genetic evolution model for colorectal cancer, at least 5 proteins have to be dis- or non-functioning before normal colonic epithelium cells transform via the adenoma-carcinoma sequence into an invasive and metastasizing tumour. This multistep process involves both the inactivation of tumour suppressor genes as well as activation of onco-genes and requires destabilisation of genomic integrity. Mutations in MMR genes clearly provide the conditions for genomic destabilisation and rapid accumulation of oncogenic mutations. For cancers not associated with mutations in MMR genes, the molecular cause of increased genomic instability has been less well elucidated. Failure of cell cycle checkpoints is most likely one of the main causes (Hartwell and Kastan, 1994; Paulovich et al., 1997).

Recently, Kinzler and Vogelstein have presented the "gatekeeper-caretaker" concept to distinguish between genetic events unlocking molecular pathways to tumorigenesis and events decreasing genomic stability facilitating the generation of mutants (Kinzler and Vogelstein, 1996, 1997). The APC gene is a typical example of a gatekeeper gene which is an early target for mutations in colorectal carcinogenesis, whereas MMR genes are typical caretakers whose functional inactivation leads to a mutator phenotype (Kinzler and Vogelstein, 1996).

1.3 Genomic imprinting

Several human disorders show a parent-specific non-Mendelian inheritance pattern, due to either tri-nucleotide repeat expansions like in Huntington disease or Myotonic Dystrophy, mitochondrial linked diseases (exclusively maternal transmission) or genomic imprinting. As outlined in section 1.1.5, familial HN-paragangliomas show an inheritance pattern most consistent with a model of an autosomal dominant gene subject to genomic imprinting.

Genomic or parental imprinting can be defined as the monoallelic and reversible, parent-specific mode of expression of mammalian genes (Barlow, 1994). Since genomic imprinting is in most instances conserved between human and mice it is thought to have some biological rationale. So far, there are three different models to explain characteristics of imprinting: a) the host-defence model, b) the expression competition model and c) the imprinting centre functioning over a large chromosomal region.

1.3.1 Host-defence model

According to the host-defence model, imprinting is a by-product of an epigenetic host defence system, involving cytosine DNA methyltransferase, which has evolved to inactivate retroviruses and

retrotransposons (Barlow, 1993). These imprinted genes are thus expected to resemble retroviruses in that they are small in length and have small, few or absent introns, either contain or are bordered by repeat sequences, and are CpG rich (Neumann et al., 1995; Hurst et al., 1996).
Methylation-marks can discriminate between the two alleles of a gene, although it is not known yet whether this is the primary cause or a consequence in imprinting (Neumann and Barlow, 1996). In most instances, methylation of CpG-islands located at the 5' promoter region silencing the gene is involved via local chromatin structure changes (Cedar, 1988). Methylated CpG's are conserved upon cellular mitotic division, keeping the imprint consistently the same. Only during gametogenesis the necessary resetting (including both erasure and setting) of the imprint takes place.

Methylated CpG's are highly mutable, either to TpG or CpA (if the other strand is involved). Most mutations in the non-imprinted p53 gene are due to this phenomenon (Hollstein et al., 1991; Denissenko et al., 1996).

Differential methylation in imprinted genes is suggested to be achieved by a dynamic process that senses gene dosage and adjusts methylation (Shemer et al., 1996) in a way similar to X-chromosome inactivation (Migeon, 1994; Latham, 1996; reviewed by Moore et al., 1995). The differences in methylation of CpG's on both alleles has been employed by techniques like restriction landmark genomic screening (RLGS) and bisulphite sequencing (Frommer et al., 1992; Feil et al., 1994) to discover new imprinted genes. Examples of genes identified by RLGS are the murine genes *U2af1-rs1/SP2* (Hatada et al., 1993), and *Grf1* (Plass et al., 1996). The bisulphite sequencing can specifically identify methylated CpG's within a large region, like in the SNRPN gene on chromosome 15q13 (Zeschnigk et al., 1997a). This allelic difference can be employed for specific amplification of parental alleles of the SNRPN gene and thus for genetic testing in the majority of cases of PWS/AS (see section 1.3.3; Zeschnigk et al., 1997b).

The methylation markings of the murine maternally expressed Insulin-like growth factor type 2 receptor (*Igf2r*) gene are located in intron 2, called region 2 or the so-called imprint-box which contains a 2 kb CpG-island (Stöger et al., 1993; Barlow et al., 1991). It inherits a methylation mark from the female gamete which remains restricted to the maternal chromosome in diploid cells in the embryonic and adult stages, thus surviving the global demethylation and *de novo* methylation during preimplantation development. It has become clear that on the paternal, silenced allele an antisense RNA is produced whose transcription was dependent on region 2 (Wutz et al., 1997). This reciprocal expression may indicate that expression competition could play a role, reminiscent of the imprinting of the IGF2/H19 pair (section 1.3.2; Banerjee and Smallwood, 1995).

The imprinting element of the murine *H19* gene is located within a 1.2 kb region of the promoter, and has been shown to function in *cis* as a parent-of-origin independent silencing element in Drosophila. This emphasizes that a common regulator function is present in imprinted genes in mammals, which may function in insects as well. Interestingly, insects do not methylate their DNA and therefore gene regulation is controlled in other ways like chromatin structural modifications (Lyko et al., 1997). Another example of the assignment of an imprint-box within or near a gene is the murine

U2afbp-rs1 gene. Here, this region consists of 200 bp within the CpG-island in the promoter region. This is the site where the methylation imprint resides, marking methylation during preimplantation development on the maternal, inactive allele (Shibata et al., 1997).

1.3.2 Expression competition

Another model assumes that imprinting has been shaped by parental tug-of-war, or paternal-maternal competition for reproductive success. Paternally expressed/maternally repressed genes are expected to promote growth of the placenta and fetus while maternally expressed/paternally repressed genes are predicted to have the opposite, growth inhibitory function; in this way, proper development of the embryo might be controlled (Moore and Haig, 1991).
According to this model, paternal genes encode mostly growth-promoting factors (Moore and Haig, 1991). An example is reciprocal imprinting of a ligand and its receptor; e.g. the Insulin-like growth factor 2 (IGF2) gene is paternally expressed, while its receptor, the IGF2R/mannose-6-phosphate-receptor gene is maternally expressed, thereby ensuring the contribution of both parental chromosomes (Barlow et al., 1991; DeChiara et al., 1991; Stöger et al., 1993; Xu et al., 1993; Lighten et al., 1997).

Chromosomal region 11p15.5 contains the imprinted genes IPL, $p57^{KIP2}$, KvLQT1, HASH2/ASCL2, *INS*, IGF2 and H19 (Figure 1.3). The genes IGF2 and *Insulin (INS)* are both paternally expressed, all the others maternally. This chromosomal region is involved in the disorder Beckwith-Wiedemann syndrome (BWS) and Wilms' tumour. BWS is a fetal overgrowth disorder characterised by i.e. multiorgan hyperplasia and a predisposition to embryonal tumours in childhood (MIM 130650). BWS can be considered to be a multigenic disorder, since mutations in two genes have already been identified in some BWS patients (KvLQT1 and $p57^{KIP2}$). In addition, IGF2 has been implicated as a cause of BWS. Many patients show uniparental paternal disomy for 11p15.5 (Henry et al., 1991) and thus express a double dose of IGF2. While this provided only circumstantial evidence for the involvement of IGF2, more recently, a mouse model overexpressing the endogeneous *Igf2* gene shows several phenotypic features of BWS (Sun et al., 1997).

IGF2 and H19 are only 80 kb apart in the same orientation; the H19 gene is methylated on the inactive, imprinted paternal allele while the IGF2 gene is methylated on the paternally active allele (Figure 1.3). The H19-transcript does not encode a functional peptide, comparable to the XIST gene (Pfeifer and Tilghman, 1994). The XIST gene is expressed specifically from the inactive X-chromosome, and its expression is thought to induce the inactivation process (Ballabio and Willard, 1992; Ray et al., 1997). The role of H19 as a tumour suppressor gene, as suggested by Hao et al. (1993), is most likely secondary in tumorigenesis; this is supported by the results indicating that absence of H19 expression is not sufficient to induce tumours in 1 year old mice (Ripoche et al., 1997), but longer incubation times are needed to confirm this. In this experiment, the H19 transcription unit was replaced by a neo-cassette which also underwent paternal imprinting like the wildtype endogenous H19 gene (Figure 1.3). This indicated that the function of H19 has no involvement with its imprinting and the

authors concluded that a sequence within the 10 kb 5' upstream regulates the imprinting of the H19 locus (Ripoche et al., 1997). This region includes the 1.2 kb region showing a silencing effect in Drosophila (Lyko et al., 1997).

A model has been proposed for the opposite expression of the IGF2 and H19 genes, in which one promoter is used in competition between the two genes (Banerjee and Smallwood, 1995). Deletion

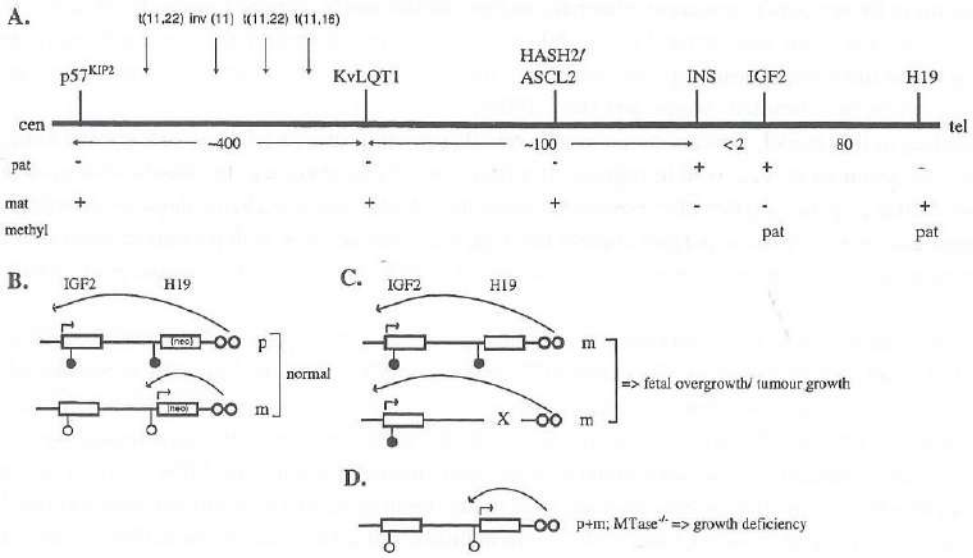

Figure 1.3. Chromosomal region 11p15.5 harbouring imprinted genes.
A: Genes are shown with the centromere to the left. Distances are given in kb (not on scale). Four translocations are depicted by arrows; all involve the KvLQT1 gene leading to either BWS or rhabdoid tumour. Parental (pat: paternal, mat: maternal) expression of genes is indicated for imprinted genes only, with the parental allele showing specific methylation below. **B-D**: Function of H19 and IGF2 methylation/expression. Boxes represent the two genes and the two circles represent downstream enhancers. In a normal situation (**B**), the paternal (p) allele is methylated (filled lollipops) resulting in IGF2 expression (arrow), while the maternal (m) allele is unmethylated and expresses H19. Replacement of H19 with a neo-cassette does not influence this process. Two methylated alleles or maternal H19 deletion results in bi-allelic IGF2 expression and fetal overgrowth or tumour growth (**C**). Methyltransferase deficient mice ($MTase^{-/-}$) have two unmethylated alleles and thus two active H19 genes and show growth-deficiency (**D**). For references: see text, supplemented with Eden and Cedar, 1995 and Reid et al., 1997.

of the murine maternal active H19 will lead to biallelic expression of IGF2 and fetal overgrowth (Leighton et al., 1995). This same phenomenon can be observed when the H19 gene is silenced by methylation of both copies, resulting in a paternal methylation pattern for IGF2 on the maternal

chromosome and thus two active copies (Figure 1.3). By contrast, deletion of the paternal active IGF2 allele results in growth deficiency with only the maternal H19 allele being active. This phenomenon is supported by investigation of knockout mice lacking the methyltransferase gene, in which both copies of H19 become non-methylated and thus expressed, while the IGF2 alleles become inactive upon loss of methylation (Figure 1.3; reviewed by Surani, 1993).

Recently, BWS patients with normal H19 methylation/expression and bi-allelic expression of IGF2 were reported, indicating that an alternative H19-independent pathway establishing or maintaining allele-specific IGF2 expression must exist (Joyce et al., 1997).

1.3.3 An Imprinting Centre

Chromosomal region 15q11-q13 is the other chromosomal region harbouring several imprinted genes: the small nuclear ribosomal protein N gene (SNRPN), PAR-5 (for Prader-Willi Angelman Region), PAR-1, a zinc-finger gene (ZnF127), the necdin gene NDN, IPW (Imprinted in Prader-Willi), the E6-AP ubiquitin-protein ligase gene UBE3A/E6-AP, and the $GABA_A$ receptor genes GABRB3, GABRA5 and GABRG3 (Figure 1.4). Thus far, only the UBE3A gene has been shown to be maternally expressed in certain tissues, while the others are paternally expressed.

Overall, loss of the paternal copy of this region will result in Prader-Willi Syndrome (PWS) and loss of the maternal copy will result in Angelman Syndrome (AS). PWS and AS are clinically distinct neurogenetic syndromes with mental, psychomotor, and growth retardation. In addition, PWS is characterized by infantile hypotonia, characteristic facies, small hands and feet, and hypothalamic dysfunction causing hypogonadism and hyperphagia with obesity (MIM 176270). The retardation in AS patients -"Happy puppet children"- normally is more severe and is paired with characteristic gait, microcephaly, ataxia, frequent laughter, and seizures (MIM 105830).

Both diseases can also occur through uniparental disomy (UPD), either maternal (PWS) or paternal (AS), indicating that a paternal expressed copy (PWS) or maternal expressed copy (AS) is present in this region. Non-overlapping microdeletions in the region have clearly indicated that PWS and AS are caused by distinct loci, and are not allelic (Nicholls, 1994).

In addition to genomic deletions, a specific point mutation in the SNRPN gene can result in familial AS (Dittrich et al., 1996). Mutations at the genomic level can give rise to a change in the usage of different alternative splicing products, e.g. mutations in 5' untranslated exons. Mutations within these exons of the SNRPN gene influence either the imprint switching or the imprint signal setting itself (Buiting et al., 1995; Dittrich et al., 1996; Figure 1.4). This region of the SNRPN gene has been mapped as an imprinting centre (IC; Saitoh et al., 1996) with both an imprintor function and a switch initiation site (Ferguson-Smith, 1996). The imprint centre functions *in cis* for the 2 Mb region around the SNRPN gene. It is suggested that mutations within exon 1 prevent the erasure of the imprint in the male germline leading to PWS due to a double maternal epigenotype. In the case of aberrant imprint switching of 15q13, a maternally transmitted grandpaternal chromosome will maintain the paternal epigenotype (as shown by methylation pattern). The resulting double paternal imprint pattern in this

region and loss of the maternally expressed copy will lead to the AS phenotype (Dittrich et al., 1996). Mutations in exons of the UBE3A/E6-AP gene, also mapping within this region, have been detected in a number of cases of AS not caused by UPDpat, cytogenetically visible deletions or imprinting mutations. Contrary to initial observations (Kishino et al., 1997; Matsuura et al., 1997; Greger et al., 1997), monoallelic expression from the maternal allele was recently observed specifically in brain tissue, both in human and mouse (Albrecht et al., 1997; Rougeulle et al., 1997; Vu and Hoffman, 1997). This tissue-specific maternal expression of UBE3A/E6-AP might thus be the cause for AS. Further investigations will need to confirm the involvement of UBE3A/E6-AP in other AS cases.

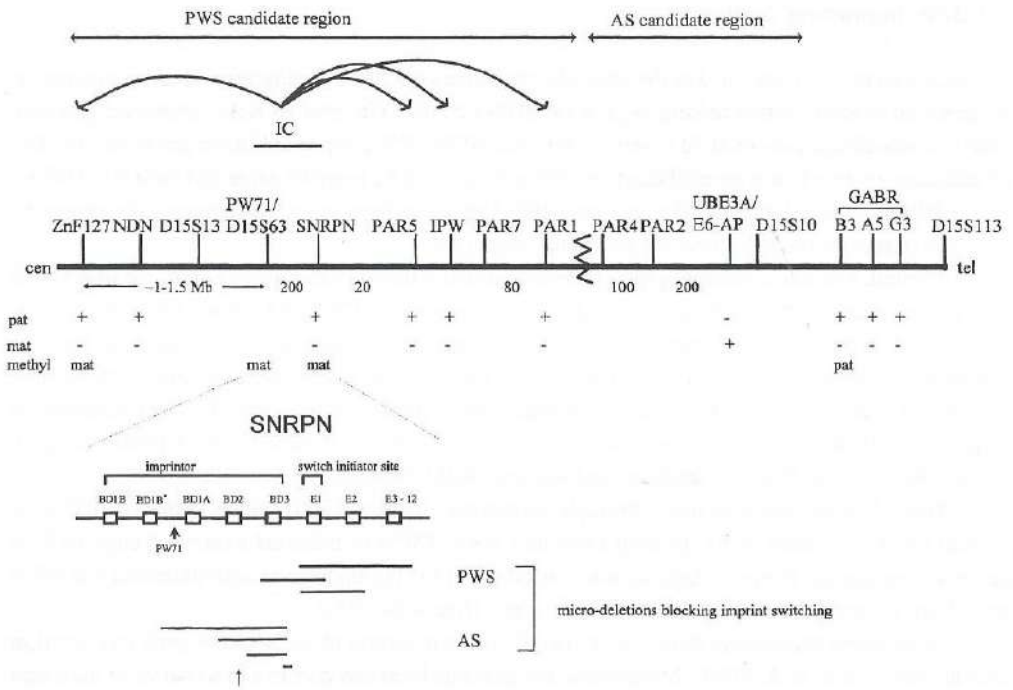

Figure 1.4. Chromosomal region 15q13 harbouring imprinted genes.
Top: Genes and markers (D15S) are shown with the centromere to the left. Distances are given in kb, unless otherwise stated (not on scale). Both PWS and AS candidate gene regions are shown based on translocation breakpoints with the imprinting centre (IC) within the SNRPN gene acting over a 2 Mb region. The jagged line represents the translocation breakpoint D15S174. Parental expression/methylation is given as in Figure 1.3.
Bottom: The SNRPN exons (not on scale) defining the imprintor region (BD exons) and the switch initiator site (exon 1). For simplicity, exons 3 to 12 are denoted by one exon (E3-12); translation starts in exon 4. Marker PW71/D15S63 is shown below the exon-structure. Microdeletions causing either PWS or AS are shown by bars, while the arrow indicates a point mutation within the splice-donor site of BD2 resulting in AS. For references: see text, supplemented with Buiting et al., 1997.

In conclusion, genomic imprinting of genes results in their mono-allelic expression in a reversible manner. The specific marking is set in a region called the imprint-box which functions for the specific gene only. Expression competition might regulate opposite imprinting for closely linked genes, while an imprint centre has been located on chromosome 15q13 influencing imprinting within a 2 Mb region. The number of imprinted genes in the human genome is estimated to be 100-200 (Barlow, 1995) of which around 15% are cloned at the moment (Table 1.2). Analysis and interpretation of candidate genes has been performed in different ways, like allelic expression (e.g. H19; Bartolomei et al., 1991), allelic methylation (e.g. IGF2R; Kalscheuer et al., 1993), or whether or not expression in hydatiform moles (tumours with a duplicate set of only paternal chromosomes) or dermoid cysts/teratomas (duplicate maternal) is observed (Nishita et al., 1996). A problem in the assignment of imprinting to a specific gene might be the spatial and temporal variation in imprinting.

As an example for the tissue-specificity, the human KvLQT1 gene (an ion-channel gene active in heart and involved with the long QT syndrome) is imprinted in most tissues with the exception of, and thus biallelic expression in, the heart (Lee et al., 1997). Mutations in this gene have been found in a small number of BWS patients (Lee et al., 1997). On the contrary, the UBE3A gene is imprinted only in brain (Rougeulle et al., 1997; Vu and Hoffman, 1997).

1.3.4. Other features linked with genomic imprinting

Some other features associated with genomic imprinting are allele-specific replication timing and sex-specific recombination frequency. These two features support the observation of regional regulation as observed on chromosome 11p15.5 and 15q11-q13, as well as the idea that specific chromatin structures are involved in imprinting.

Imprinted genes appear to lie within chromosomal domains that display asynchronous replication, with the paternal allele replicating earlier at a number of investigated loci (IGF2, H19, SNRPN, IGF2R; Kitsberg et al., 1993; Knoll et al., 1994). However, the relation between allele-specific expression and allele-specific replication timing as measured by FISH is not straightforward, since the paternal allele generally replicates earlier than the maternal allele, regardless of its activity state. Using a bromodeoxyuridine incorporation method, absence of allelic replication asynchrony was observed in both the 15q13 and the 11p15.5 regions, although this might be related to low levels of imprinted gene expression near these loci in the examined lymphocytes, fibroblasts and lymphoblastoid cells (Kawame et al., 1995).

Another chromosome feature that might be part of the imprinting mechanism is reflected by allele-specific recombination rates (Thomas and Rothstein, 1991; Robinson et al., 1995; Påldi et al., 1995). The parental allele that is being imprinted (silenced) has been proposed to undergo local unwinding, so that it is more accessible to the recombination-machinery and will thus show a higher recombination rate in this specific area. Although female recombination rates are in general higher than in male (Dib et al., 1996), reflecting the more condensed state of chromatin in spermatogenesis, specific local differences on e.g. 15q13 could be linked to imprinting (Robinson et al., 1995).

Table 1.2. Imprinted, autosomal, genes in human and mice.

Gene	Chromosome location		Expressed allele		Disease	References
	human	mouse	human	mouse		
H19	11p15	7dis	mat[a,c]	mat[a]		Bartolomei et al., 1991; Zhang and Tycko, 1992; Zhang et al., 1993
IGF2	11p15	7dis	pat[b,c]	pat[a,b]		DeChiara et al., 1991; Ohlsson et al., 1993
Ins2/INS*	11p15	7dis	pat*	pat[a,b]		Giddings et al., 1994; Haig, 1994; Deltour et al., 1995
Ins1		19		pat[b]		Giddings et al., 1994; Deltour et al., 1995
Mash2/HASH2	11p15	7dis	mat[a]	mat		Guillemot et al., 1995; Alders et al., 1997
KVLQT1	11p15	?	mat[b]	?	BWS	Lee et al., 1997
$p57^{KIP2}$	11p15.5	7dis	mat[a]	mat	BWS	Hatada et al., 1995, 1996; Matsuoka et al., 1996
IPL	11p15.5	7dis	mat	mat		Qian et al., 1997
WT1	11p13	2	mat[b,c]	?	WT	Call et al., 1990; Buckler et al., 1991; Jinno et al., 1994
SNRPN	15q11-q13	7	pat	pat[a]	PWS/AS	Cattanach et al., 1992; Leff et al., 1992; Reed and Leff, 1994
ZnF127	15q11-q13	7	pat	pat	PWS/AS?	Jong et al., 1994
PAR1	15q11-q13	?	pat	?		Sutcliffe et al., 1994
PAR5	15q11-q13	?	pat	?		Sutcliffe et al., 1994
IPW	15q11-q13	7	pat	pat		Wevrick et al., 1994; Wevrick and Francke, 1997
UBE3A/E6-AP	15q11-q13	7	mat[b]	mat[b]	AS	Albrecht et al., 1997; Rougeulle et al., 1997; Vu and Hoffman, 1997
NDN	15q11-q13	7	pat	pat		MacDonald and Wevrick, 1997; Jay et al., 1997; Watrin et al., 1997
GABR-B3,-A5,-G3	15q	?	pat	?		Meguro et al., 1997
GNAS1	20q13	2dis	mat	pat	AHO	Williamson et al., 1996
Peg5/Neuronatin	?	2dis	?	pat		Kagitani et al., 1997
IGF2R	6q27	17	mat[c]	mat[b,c]		Barlow et al., 1991; Kalscheuer et al., 1993; Xu et al., 1993; Smrzka et al., 1995
MAS	6q25.3-q26	17	biallelic	pat[a,b]		Villar and Pederson, 1994; Riesewijk et al., 1996
U2af1-rs1/SP2		11prox		pat		Hatada et al., 1993
5HT2R	13q14	14	mat	?	delayed development RB?	Kato et al., 1996
Peg1/MEST	7q32	6prox	pat	pat		Kaneko-Ish et al., 1995; Nishita et al., 1996; Kobayashi et al., 1997
Peg3	19q13.1-3[d]	7prox	?	pat		Kuroiwa et al., 1996
Grf1/Cdc25^{Mm}	?	9	?	pat		Plass et al., 1996
p73	1p36	?	mat	?	neuroblastoma	Kaghad et al., 1997
Impact	?	18	?	pat		Hagiwara et al., 1997
mapped:						
PGL1	11q22-q23	9[d]	?[e]	?	FGT	Heutink et al., 1992; Baysal et al., 1997a; this thesis
PGL2	11q13	19[d]	?[e]	?	FGT	Mariman et al., 1993, 1995
IDDM5	6q22-q23	?	pat (UPD)	?	TNDM	Temple et al., 1995, 1996

* controversial or tentative assignments; polymorphic in [a] different maturation stages, [b] different tissues, [c] individuals; [d] based on the synteny between mouse and human; [e] paternal transmission; ?: not determined. For other diseases showing parental differences in transmission but without gene locus, see Nakao and Sasaki, 1996.

Another possible evolutionary reason why imprinting should exist, not mentioned thus far, is a surveillance mechanism for chromosome loss. Chromosome loss events are known to have deleterious consequences that imprinting could serve to prevent, since most or all chromosome loss events will result in completely loss of expression of at least one gene (Thomas, 1995). One assumption for this mechanism is that imprinted genes should be dispersed fairly evenly throughout the genome on both maternal and paternal chromosomes. UPD-mapping serves as a tool to identify chromosomal regions harbouring imprinted genes (Cassidy, 1995). These imprinted genes should not be tumour suppressor genes, since this would lead to inactivation of one of the two gene copies.

1.3.5 Imprinting and tumorigenesis

Wilms' tumours have been described in association with, or part of, WAGR (Wilms' tumour, aniridia, genitourinary anomalies, and mental retardation), Denys-Drash syndrome or BWS. One of the loci causing Wilms' tumour, WT2, has been located at 11p15.5 and is suspected to be IGF2, since there is a high incidence of loss of parts of the maternal chromosome 11 with the s.r.o. involving the terminal portion of chromosome 11p15.5 (Mannens et al., 1988). In these cases, paternal UPD of 11p15.5 is often found, being one of the possibilities leading to duplication of the dose of paternal expressed IGF2 (reviewed by Deal, 1995).

Loss of imprinting (LOI) of IGF2, resulting in biallelic expression has been first observed in Wilms' tumours (Ogawa et al., 1993; Rainier et al., 1993), and it has been confirmed in other tumours like lung cancer (Suzuki et al., 1994), hepatoblastoma (Rainier et al., 1995), oesophageal cancer (Hibi et al., 1996), or choriocarcinoma (Hashimoto et al., 1995). In BWS patients, LOI of IGF2 could be detected in non-affected tissues (tongue and kidney) which might normally be affected by the disease, indicating that LOI could be causally linked to the pathogenesis of organ overgrowth (Feinberg et al., 1994).

Table 1.3. Imprinting and tumorigenesis in human cancer.

Tumour type	chromosome	alteration	gene	parental allele affected
Neuroblastoma	1p36	LOH	p73	maternal
	2p24	amplification	N-myc	paternal
Acute myelogenous leukaemia	7	monosomy/LOH	?	paternal
Embryonal tumours[a], some carcinomas, germ cell tumours	11p15.5	LOH and/or LOI	IGF2/H19	LOH maternal
Retinoblastoma, osteosarcoma	13q14	LOH	RB	maternal[b]
Chronic myeloid leukaemia	9q24	translocation	ABL	paternal
	22q21	translocation	BCR	maternal
HN-paraganglioma	11q22-q23	LOH	?	maternal

? not determined; [a] Wilms' tumour, rhabdoid tumour, hepatoblastoma, rhabdomyosarcoma; [b] Controversial results. Based on: Squire and Weksberg, 1996; Feinberg, 1993; Tycko, 1994; Devilee et al., 1994; Baysal et al., 1997a; Kaghad et al., 1997.

Furthermore, LOI in tumour tissue was shown to involve a switch of the maternal chromosome to a paternal epigenotype, leading to a double dose of IGF2 expression.
Another locus, WT1 (Table 1.3), has been mapped to 11p13 and this gene suppresses not only IGF2 and IGF1R gene activity, but also its own expression. The effect and the magnitude of it, however, depends on which type of the four alternatively spliced transcripts is used in the transfection assay (Rupprecht et al., 1994). The imprinting status of this gene has been shown to be polymorphic, with biparental expression in Wilms'tumours (Little et al., 1992; Jinno et al., 1994). If an imprinted endogenous gene functions as a tumour suppressor gene, the first hit will be represented by the imprint, presumably silencing one allele of this gene. This would imply that all cells of an individual carry one silenced copy and only one additional somatic hit is required for complete loss of function. Given the rate with which LOH occurs, one would expect that the population incidence of this tumour will be high. The imprinted p73 gene, strongly related to p53, is the putative neuroblastoma tumour suppressor gene showing maternal expression only. Specific maternal LOH of chromosome 1p36 in these tumours indeed silences p73 completely, although these tumours are not common in the general population. p73 May be the first example of a new paradigm for how (imprinted) tumour-suppressor genes are involved in cancer (Kaghad et al., 1997; Dickman, 1997).

A high incidence of tumours due to this 'new' one-hit theory might be circumvented by a small number of target cells or spatial and/or temporal specific imprinting. Loss of imprinting, for example after embryogenesis, in all cells to re-establish bi-allelic expression, seems unlikely, but, again, this is not necessary if tissue- or developmental-specificity is involved. However, the p73 gene is not only imprinted in neuroblastoma cell lines, but also in peripheral blood cells from healthy persons, which might reflect its tissue-specific function (Kaghad et al., 1997). Further investigations need to confirm these initial observations.

1.4 Positional cloning of disease genes

Positional cloning is the isolation and identification of a disease gene on the basis of positional information regarding its location on the chromosome (Collins, 1992). Once this position is sufficiently accurately known, expressed sequences can be isolated by saturation cloning of the region. Identification of the disease gene is generally dependent on the demonstration of functional mutations that co-segregate with the trait. In the case of familial occurrence of a disease, linkage analysis with polymorphic DNA markers is usually the first step to roughly locate the disease causing gene on one of the chromosomes. Knowing the coding sequence of the disease gene, a database query might reveal functional properties of the protein.

Functional cloning of a gene approaches the gene of interest via an alternative route: the knowledge of the malfunctioning protein causing the disease, like a non-functioning enzyme. Using the amino acid composition of the protein, one can deduce the corresponding DNA sequence and clone the gene. For example, the Tay-Sachs disease gene encoding the alpha chain of the lysosomal storage enzyme beta-hexosaminidase was cloned via this route (Proia and Soravia, 1987). Since nothing is known regarding the cellular function of the PGL1 protein, positional cloning was the method of choice to identify the disease gene.

1.4.1 Linkage analysis

Linkage analysis employs the rules of Mendelian inheritance by which each individual receives a paternal and a maternal copy of each autosomal chromosome, plus a pair of sex-chromosomes. The segregation of these chromosomes in the family can be measured by typing polymorphic markers. Positional cloning involves as a first step the typing of a large set of polymorphic markers distributed over the genome in order to search for co-segregation (linkage) of markers with the disease phenotype. DNA polymorphisms broadly fall into two classes: single nucleotide polymorphisms, and variation in the copy number of tandem repeats. The first class was initially revealed as restriction-fragment length polymorphisms in Southern blot analysis. These polymorphisms are recently being developed into dense maps that can be rapidly typed by the polymerase-chain-reaction (PCR) in highly automated setting to guarantee high throughput analysis (Wang et al., 1996b). The second class can be subdivided on the basis of repeat-length. The minisatellites have a repeat core-unit of approximately 25-40 bp (Jeffreys et al., 1988). Many locus-specific minisatellites have been isolated which can be detected by Southern analysis using restriction enzymes that cut outside the repeat block. They can be highly polymorphic but tend to cluster in the telomeric regions of chromosomes (Royle et al., 1988). Microsatellites are mostly di-, tri-, and tetranucleotide repeats and are easily amplified by the PCR using oligonucleotide primers flanking the tandem-repeat. The alleles are separated on the basis of their size on a polyacrylamide gel.

The two homologous chromosomes segregate independently; an allele at one locus on one chromosome, therefore, segregates together with a given allele at another locus on another chromosome with 50% probability. Alleles at loci on the same chromosome should cosegregate with a probability ratio that is related to the distance between them on the chromosome. The probability ratio is the recombination fraction (Θ) of a recombination event occurring between the two loci. Genetic distances are based on the number of recombinations observed per meiosis, and are given in centiMorgans (cM): 1 cM corresponds to a recombination fraction of 1% or a physical distance of approximately 1 Mb. Two loci are said to be genetically linked when $\Theta<0.5$. The object of linkage analysis is to estimate Θ and to test whether an observed deviation from 50% recombination is statistically significant. The lod score is the ^{10}logarithm of the odds ratio between the likelihood of linkage at Θ and the likelihood of loci unlinked ($\Theta = 0.5$). In searching the disease gene, the two loci are a specific marker and the disease gene. Linkage is generally claimed when the lod score exceeds 3.0 (i.e. the likelihood ratio is 1000:1), while exclusion of linkage is shown for lod score values below -2.0 (Terwilliger and Ott, 1994). The lod score can be calculated by the use of computer programs such as the LINKAGE program package or the VITESSE algorithm (Lathrop et al., 1984; O'Connell and Weeks, 1995). To identify the most likely location of the disease gene, lod scores are calculated for a specific marker over different distances between $\Theta = 0$ (loci right next to each other) and $\Theta = 0.5$ (loci far apart or on different chromosomes). The lod score calculation is dependent on e.g. the allele-frequency (a higher allele-frequency will give a lower lod score since the chance of sharing that allele is more likely to be coincidental), the informativity of the marker as given by the PIC-value (polymorphic information content), the penetrance of the disease, and the number of typed individuals -especially the number of generations typed. In the case of analysis of several small -monogenic- families, which are not informative enough to reach a lod score of 3 on their own, lod scores may be summated per marker to obtain enough power for linkage.

Since linkage can be disturbed by genetic recombination, a map of well-defined, evenly spaced markers is a prerequisite in order not to miss it (Dib et al., 1996). Once linkage with a marker is established, analysis of flanking markers can reveal recombination events that can further define the location of the disease gene. This is called haplotyping and will in most instances refine the candidate gene region to a region suitable for positional cloning, although this highly depends on the marker spacing within the region and the number of recombinants observed.

Multipoint linkage analysis

Multipoint linkage analysis involves more than one chromosomal marker in the linkage analysis, and the genetic distances between the markers. The advantage of this calculation is the influence of neighbouring (informative) markers which can lead to increased power of the analysis. In addition, it can provide positional information regarding a more precise localisation of the disease gene, although this can also be derived from haplotype/recombinant analysis. The main disadvantage is the rapid increase of the necessary calculation time.

Table 1.4. Interval showing LD in Finnish disease loci over large (≥3 cM) distances

Disorder Gene	location	Interval (cM)
Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)	21q22.3	3
Cartilage-hair hypoplasia (CHH)	9p21-p13	3
Chorioderemia	Xq21	9
Congenital chloride diarrhoea (CCD)	7q31	13
Congenital nephrosis (CNF)	19q12-q13.1	3
Familial amyloidosis, Finnish type (FAF)	9q33	3,5
Hereditary nonpolyposis colorectal cancer (HNPCC)	3p	10
Infantile onset spinocerebellar ataxia (IOSCA)	10q23.3-q24.1	5
Northern epilepsy (Kainuu epilepsy)	8ptel.	10
Progressive myoclonus epilepsy (PME)	21q22	5
Retinoschisis	Xp22.2-p22.1	10
Salla disease	6q14-q15	10
Usher syndrome, type III (USH3)	3q21-q25	7,5
Variant form of late infantile neuronal ceroid-lipofuscinosis (vLINCL)	13q21.1-q32	11

Based on Peltonen et al., 1995, and Nyström-Lahti et al., 1994.

Linkage disequilibrium analysis

Linkage disequilibrium (LD) is another population genetic phenomenon that can be useful in gene mapping. When the occurrence of pairs of specific alleles at different loci on the same haplotype is not independent, the deviation from independence is termed linkage disequilibrium. Contrary to genetic linkage, where different alleles at a marker can be linked to a disease gene in different families, LD can

be seen as an association between a specific allele at a marker and the disease. With LD calculations, a set of closely linked markers on the same chromosome are analysed for both disease and non-disease chromosomes. If patients with the same disease are due to the same mutation, markers nearby the disease gene will show this association. Especially in founder populations like the Finnish this approach can lead to rapid fine mapping of the disease gene; Hästbacka et al. (1992) used this approach, extended with an adapted Luria-Delbrück analysis, to calculate the genetic distance between a specific marker (CSF1R) and the disease gene DTD responsible for diastrophic dysplasia. Their calculated distance, 0.064 cM, corresponded well with the physical distance (70 kb) once the disease gene was cloned (Hästbacka et al., 1994). LD analysis can be performed within a family or between different families with the same disease. The sharing of certain alleles between a number of families could indicate they originate from the same ancestral founder (reviewed by Peltonen et al., 1995), dependent on the haplotype frequency of these specific alleles. It might also indicate that these markers are closely linked and that this particular haplotype will increase the probability of a mutation occurring in the specified gene; a 'hot-spot' region. Within vicinity of these markers, the disease causing gene will reside (Peltonen et al., 1995). As shown in Table 1.4, several disease loci show LD over large distances; this can hamper easy identification of the disease gene involved.

Locus Heterogeneity

If more than one gene can cause the investigated disease phenotype as observed in different families, this is termed locus/genetic heterogeneity, or nonallelic heterogeneity. The existence of such heterogeneity can strongly influence the power to detect linkage, and if it is detected, it will be at too large genetic distances. More serious is the effect on gene mapping using recombinant analysis by haplotyping, because many of these events are observed in families that are too small to provide sufficient linkage evidence for being due to this disease gene. To weight the significance of this finding, one would like to estimate the posterior probability of linkage under heterogeneity. For this one needs the proportion of families due to a particular disease locus. This can be calculated using the HOMOG programme. Under the assumption of homogeneity (Ott, 1991) this algorithm will first determine if the assumption of homogeneity can be rejected. HOMOG2 can then be used to calculate the statistical evidence for genetic heterogeneity, the proportion of linked families to either locus and the positions of these loci in relation to the markers analysed. HOMOG3 analysis is used to calculate whether all families analysed can be explained by these two loci or that more than two loci are involved.

LOH analysis

LOH-analysis can attribute to the mapping of tumour suppressor gene loci, which is independent of linkage analysis. Most inherited tumors will show loss of the wildtype allele at the disease locus (discussed in section 1.2.3.2). However, the chromosome region affected by LOH can extend greatly beyond the immediate disease gene region, and generally differs between tumors. The smallest region of overlap is therefore expected to incorporate the interval found by linkage analysis, i.e. the disease gene locus. The choice of markers to determine LOH patterns in tumors will therefore generally depend on recombinants observed (linkage) or breakpoints of lost regions (LOH-analysis). The approach of first identifying regions showing LOH in tumour material (using comparative genomic hybridization (CGH)

and subsequently LOH-analysis with markers within the region), followed by linkage analysis with these markers, determined a small interval involved in Peutz-Jeghers syndrome leading to the identification of the tumour suppressor gene LKB1 (Hemminki et al., 1997; Kallioniemi, 1997; Hemminki et al., 1998). This approach turned out to be far less labour intensive than the generally applied linkage searches, which usually requires approximately 300 markers to be typed.

1.4.2 Physical mapping and cloning

The transition from the mapping to the cloning stage depends on the smallest candidate gene region left after all family material has been exhausted to place the disease gene. Preferably one would like to narrow the region to under 1 Mb before starting saturation cloning, but if it remains at 3-5 cM, one is obliged to subclone even this large chromosomal segment. Chromosomal segments can be subcloned by covering the interval with yeast artificial chromosomes (YACs; Burke et al., 1987), P1 artificial chromosomes (PACs; Ioannou et al., 1994), bacterial artificial chromosomes (BACs; Shizuya et al., 1992) or cosmids (Ish-Horowicz and Burke, 1981). The insert sizes ranges from approximately 40 kb for a cosmid up to 1-1.5 Mb for a YAC. The subcloning of the specified region will eventually lead to the ultimate goal: the identification of the disease gene.

Exon-trapping, cDNA library screening or mapping of known expressed sequences -finding genes by computer- within the region are a few examples how to obtain fragments of possible genes (e.g. Weber et al., 1995; Fickett, 1996; Datson, 1997). Having part of such a cDNA, Northern blotting, on zoo-blot and tissue-blot, will for instance reveal whether the isolated fragment is truly an expressed gene (Monaco et al., 1986; Rommens et al., 1989). If so, hybridisation with the specific fragments on a cDNA library, or cDNA selection, could lead to other fragments, thus extending towards the complete cDNA (Sedlacek et al., 1993; Brookes et al., 1994). 5'- and 3'- rapid amplification of cDNA ends (RACE) will give the ultimate complete cDNA sequence (He et al., 1992; Krizman and Berget, 1993). To screen for mutations to identify the disease gene, the genomic structure of the gene might have to be revealed. Southern blotting, generation of a pulse-field-gel-electrophoresis (PFGE)-map, PCR-based strategies to identify intron-positions and sizes are a few examples for this step (e.g. Ophoff et al., 1996). In general, mutation analysis by PCR, single strand conformational polymorphism (SSCP) analysis, Southern- and Northern blotting, the protein truncation test (PTT) and ultimately sequencing are the commonly used techniques (Cotton, 1997) to reveal whether the investigated candidate gene is the disease gene or just another gene in this region. The two latter techniques can be used to screen both at the level of genomic DNA as well as mRNA to detect possible alternative splicing or other aberrations like a truncating mutation. Finally, co-segregation of the mutation with the disease should be confirmed besides testing of a control population to exclude a random polymorphism.

1.4.3 Synteny (in mice)

Disease genes can also be cloned by making use of functional homologues between species, for example mouse or yeast. Candidate genes known in mouse, either by function or map location (syntenic region), can be screened in patient samples for confirmation of involvement. The human chromosome 11q22-q23 region, harbouring the PGL1 gene, has its syntenic region in the mouse on chromosome 9 and this region is conserved as shown in Figure 1.5. A mouse model for HN-paragangliomas is not

known at the moment, neither any new likely candidate genes in this region (MGDB). Since the PGL1 gene is thought to be imprinted, a process in which expression of a gene is dependent on the transmitting parent (see section 1.3), and most imprinted genes are conserved between mouse and human, identification of an imprinted gene within this murine chromosomal interval might lead to the PGL1 gene. However, the murine imprint map of chromosome 9 or the human imprint map of 11q do not show any indication for an imprinted gene to be located on this chromosomal segment (Ledbetter and Engel, 1995; Beechey and Cattanach, 1996; MGDB). On the other hand, murine chromosome 19 neither showed an imprinted gene, while the *Ins1* gene was found to be imprinted and localized on chromosome 19 (Giddings et al., 1994).

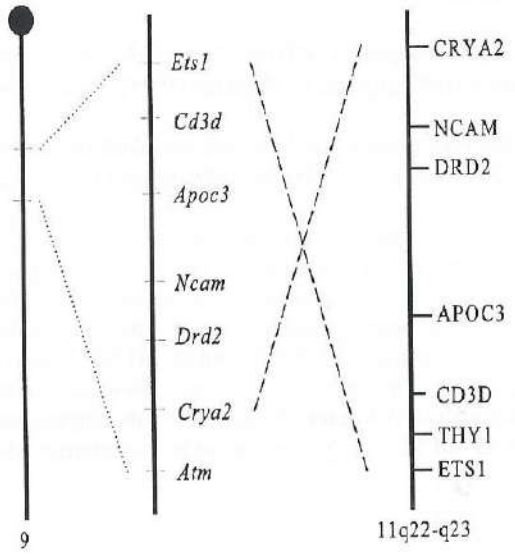

Figure 1.5. Syntenic regions of mouse chromosome 9 and human chromosome 11q22-q23.
Identical genes are shown by dashed lines between the two chromosome regions (left side: murine, right side: human).

1.5 Outline of investigation

Within this thesis, the results of both mapping the disease gene responsible for hereditary paragangliomas of the head and neck region (HN-paragangliomas) and analysis of loss of heterozygosity in tumour DNA are discussed.

Previously, the disease gene PGL1 has been mapped proximal of marker D11S836 on chromosome 11q22-q23 within one Dutch family (Heutink et al., 1992). We performed linkage and haplotype analysis to refine the location of the PGL1 gene (**Chapters 2,3,6**).

At the same time, another Dutch family was shown to be linked to a separate region on 11q13 (Mariman et al., 1993, 1995) and we initiated a heterogeneity analysis for the two possible candidate loci PGL1 on 11q23 and PGL2 on 11q13 (**Chapter 4**).

Haplotype analysis was performed in a set of Dutch families originating in South-West Holland to elucidate possible founder effects (**Chapter 5**).

Analysis of total tumour DNA for loss of heterozygosity (LOH) might indicate whether PGL1 acts as a tumour suppressor gene according to the model suggested by Knudson (1971). Results are discussed in **Chapter 7**.

In **Chapter 8** the LOH analysis has been extended to the sorted aneuploid and diploid fractions of tumours and single-cell microdissected chief cells (one of the two cell types in a HN-paraganglioma).

Physical mapping of the chromosome 11q22-q23 region encompassing the PGL1 locus showed the discordance between the genetic map and the physical map within this region (**Chapter 9**). Also, markers could be ordered physically, resulting in a "zebra-pattern" of segregating and non-segregating markers for family FGT189. Since only two neighbouring markers showed haplotype sharing in family FGT189, we concentrated on this region around markers D11S1327 and D11S1792. Within this region the PLZF gene was mapped by radiation hybrid mapping (James et al., 1994) and we revealed its genomic structure and performed mutation analysis (**Chapter 10**). Two new polymorphic markers were located within intron 4, PLZF-CA and PLZF-CTTT, which turned out to be recombinant within family FGT189.

2

FURTHER LOCALIZATION OF THE GENE FOR HEREDITARY PARAGANGLIOMAS AND EVIDENCE FOR LINKAGE IN UNRELATED FAMILIES

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Paragangliomas of the head and neck are slow growing tumors that rarely show malignant progression. Familial transmission has been described, consistent with an autosomal dominant gene that 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 or female gene carrier until subsequent transmittance of the gene through a male gene 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.

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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, Dutch cancer registration). Paragangliomas are mostly benign; less than 10% develop into proven metastases (Glenner and Grimley, 1974). 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 or a female gene carrier 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 (van Baars et al., 1982; van der Mey et al., 1989; Heutink et al., 1992), a process that 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 can affect gene expression (Reik, 1989).

An explanation for the segregation of hereditary paragangliomas could be the functional inactivation of both alleles of a tumor suppressor gene, whose normal activity is required for the proper development of carotid body tissue (Hulsebos et al., 1990). 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. 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* (Reik, 1989; Hulsebos et al., 1990; Hall, 1990). An alternative explanation has been proposed by Van der Mey et al. (1989); an autosomal dominant (onco)gene 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. For a growing number of human genetic disorders, genomic imprinting appears to be involved in the expression of disease phenotypes (Hall, 1990). 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 (Heutink et al., 1992). 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 6 extended Dutch families with head and neck paragangliomas. In total, 111 meioses were studied. Family FGT1 has been described previously (Heutink et al., 1992). Families FGT3, FGT4, FGT9, FGT10 and FGT18 were recently ascertained (Figures 2.1-2). Clinical procedures were described elsewhere (Heutink et al., 1992) 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 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 (van Gils et al., 1990b). Diagnoses of family members of the other families were based on medical history, physical and otolaryngological examination, and determination of free urinary catecholamine excretion.

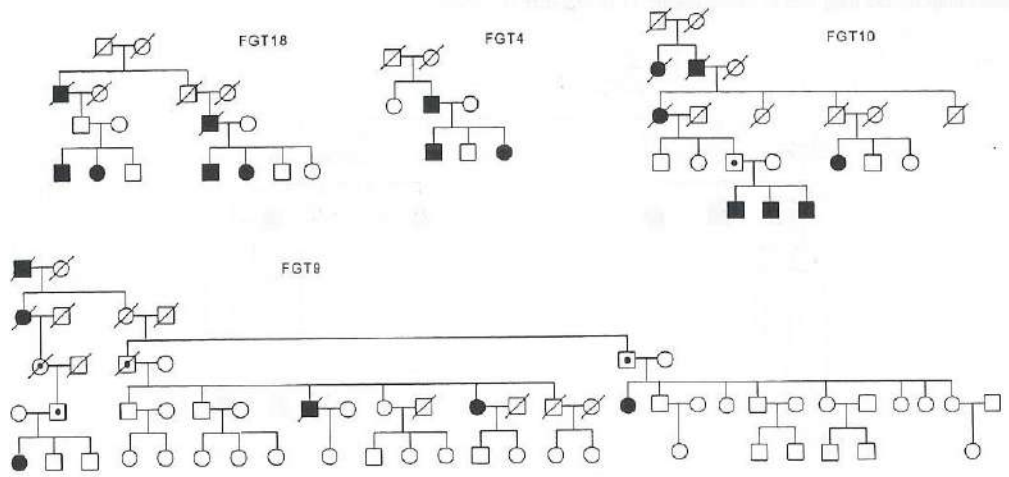

Figure 2.1. Pedigrees of four Dutch families with hereditary paragangliomas. Filled boxes indicate affected individuals. Blank symbols indicate individuals that did not show tumor growth or from whom the disease phenotype could not be established. Dotted symbols indicate individuals subject to genomic imprinting.

DNA studies

Genomic DNA was isolated from peripheral blood as described by Miller et al. (1988). Restriction digestion was carried out according to the manufacturer's recommendations. Gel electrophoresis of 5 µg DNA samples on 0.7% agarose gels, and DNA immobilization by alkaline blotting onto nylon membranes (Hybond +, Amersham), were performed according to standard procedures (Sambrook et al., 1989). Hybridization conditions were as described by Sambrook et al. (1989) 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 (1983). Information and sources of all polymorphic markers used are described in the Human Genome Database (GDB; Pearson et al., 1992) and the NIH/CEPH collaborative linkage map (1992). Microsatellite markers were tested in multiplex reactions essentially as described by Weber and May (1989) using a Perkin-Elmer-Cetus 9600 Thermocycler. Initial denaturation was 10 min at 94°C followed by 25 cycles of 30-second denaturation at 94°C, 30-second annealing at 55°C and 90-second extension at 72°C. After 25 cycles, a final extension time of 5 min at 72°C was used. Gel electrophoresis on polyacrylamide gels was performed as described by Weber and May (1989).

Linkage analyses

Linkage analyses were performed using the LINKAGE program package version 5.1 (Lathrop and Lalouel, 1984; Lathrop et al., 1984). 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 (Heutink et al., 1992). Individuals with an unknown/uncertain genotype were given genotype 0-0 in the linkage analysis. The gene frequency of the disease gene was fixed at 0.001. All lod scores are based on equal allele frequencies in the linkage analysis. Allele frequencies based on independant pedigree members marrying into the family did not affect the end result with more than 10%. Multi-point analysis was performed by subsequent three-point analyses on markers from chromosome 11q13-qter. Sixteen markers spanning the region from INT2 and D11S836 from the NIH/CEPH Collaborative Linkage map (1992) were analyzed. D11S527 was added to this map based on a microsatellite index map for the long arm of chromosome 11 (Kramer et al., 1992).

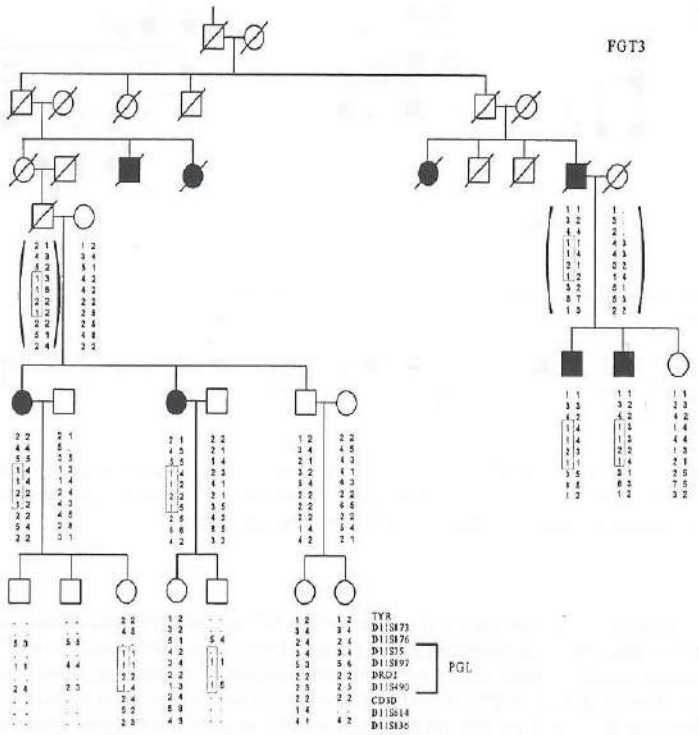

Figure 2.2. Haplotype analysis of family FGT3. Markers of chromosome 11q are ordered according to the NIH/CEPH collaborative linkage map (1992) from centromere to telomere. The shared haplotype that segregates with PGL in both branches of the family is depicted within boxes.

RESULTS

Twenty polymorphic markers localized on chromosome 11q13-qter were typed in family FGT1 in addition to the five markers that were previously reported (Heutink et al., 1992). Two-point analyses were performed between all markers and the disease locus (Table 2.1). Several markers generate significant evidence for linkage with PGL. With marker APOC3, a maximum lod score of Z=6.527 at

Θ=0.0 was obtained. These findings strengthen our earlier reported evidence for linkage that placed PGL on chromosome 11q23-qter (Heutink et al., 1992). Based on multi-point analysis, the most likely position of PGL is between markers STMY and D11S836 (Figure 2.3).

Table 2.1. 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
CD3D[2]	4.511	4.393	3.920	3.366	2.334	1.427	0.591
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
D11S836	-∞	1.017	1.944	1.993	1.548	0.947	0.361

[1] Order of the loci is presented from centromer to telomer; [2] described in Heutink et al., 1992.

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 or female gene carrier (Figures 2.1-2). Two-point analysis revealed positive lod scores with markers for 11q13-qter in each of these families. Although none of these families by themselves are 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 2.2). This marker maps to the candidate region of PGL defined in family FGT1. Multi-point analyses raised the lod score in these five families to Z=5.4 at marker DRD2. Combined two-point (Table 2.3) and multi-point analysis (Figure 2.3) of all six pedigrees determines the most likely position of PGL between STMY and D11S836. Haplotype analysis of marker data revealed a recombination event in family FGT1 (Figure 2.4, individual II-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 (individuals II-1, III-3) define STMY as the proximal boundary of the candidate region (Figure 2.4).

In family FGT3, the haplotype linked with the disease locus in one part of the family only segregates in part to the other segment of the family (Figure 2.2). 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 (NIH/CEPH, 1992).

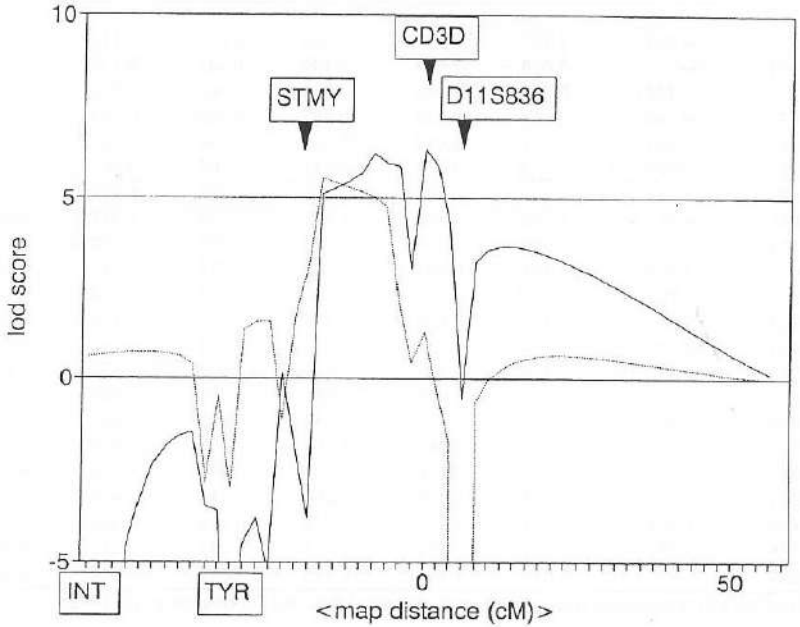

Figure 2.3. Multi-point analysis of 16 markers from chromosome 11q on family FGT1 (solid line). Multi-point analysis of 8 markers from chromosome 11q on families FGT3, FGT4, FGT9, FGT10 and FGT18 (dotted line). Inter marker distances and marker order were based on NIH/CEPH mapping data (1992) and Kramer et al., 1992.

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 evidence for linkage in five unrelated families with hereditary paragangliomas and a more detailed localization 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 in this report are not informative enough to detect linkage by themselves, but the cumulative lod scores obtained from the multi-point analysis with all families are well above the accepted level of significance for linkage

(Z=5.4 at marker DRD2). In all families segregation of hereditary paragangliomas is consistent with genomic imprinting although definite proof for genomic imprinting could not be stablished in families FGT3, FGT4 and FGT18 (Figures 2.1-2). In a number of obligate carrier males, the disease phenotype could not be established, due to either non-penetrance or un-retrievable anamnesis. Using the imprinting model in the linkage analysis for these families yields identical results as a model assuming an autosomal dominant gene with reduced penetrance.

Table 2.2. 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
APOC3	0.963	0.946	0.866	0.750	0.498	0.268	0.097
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.

Table 2.3. Cumulative pairwise lod scores between chromosome 11q13-qter markers in six families with hereditary paragangliomas.

	Recombination fraction (Θ)						
Locus[1]	0.000	0.010	0.050	0.100	0.200	0.300	0.400
D11S527	-∞	-6.573	-1.868	-0.333	0.691	0.820	0.459
TYR	-4.959	-1.966	-0.702	-0.257	0.008	0.038	0.021
D11S873	-11.786	-5.227	-1.692	-0.403	0.342	0.342	0.152
D11S35	-1.639	0.212	1.386	1.715	1.515	0.968	0.415
D11S897	6.615	8.080	8.054	7.323	5.343	3.180	1.306
DRD2	4.092	3.991	3.586	3.080	2.078	1.176	0.501
APOC3	7.490	7.354	6.791	6.053	4.500	2.882	1.254
D11S490	2.160	3.756	4.199	4.038	3.201	2.080	0.958
D11S614	1.373	3.065	3.488	3.325	2.519	1.539	0.622
D11S528	-1.113	2.618	3.479	3.382	2.646	1.979	0.602

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

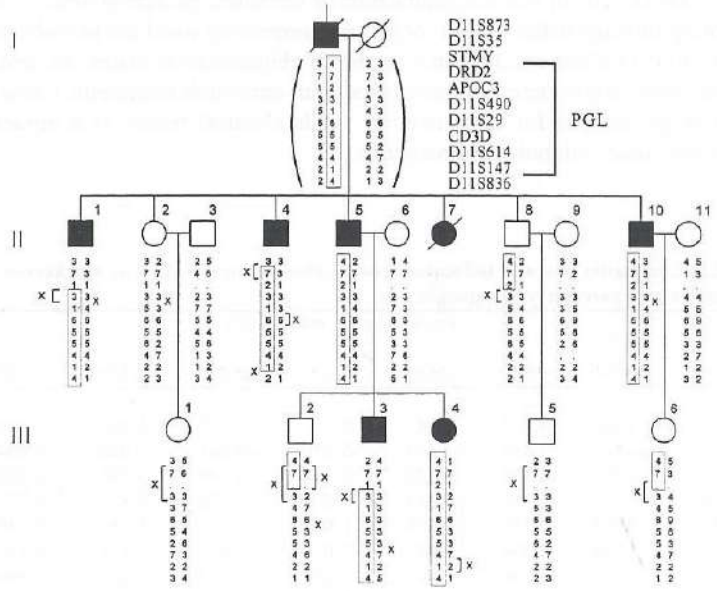

Figure 2.4. Haplotype analysis of family FGT1 C branch. Markers of chromosome 11q are ordered according to the NIH/CEPH collaborative linkage map (1992) from centromere to telomere. X= recombination events observed. Haplotypes between brackets were conmstructed using information from all available family members including from branches A and B (data not shown).

However, the use of a model without imprinting on families FGT1, FGT9 and FGT10 would result in reduced lod scores for linked markers. 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 in the haplotype analysis.
Furthermore, clinical manifestation of hereditary paragangliomas is age-dependent and this implies that unaffected individuals will not be informative either, 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 (data not shown), 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 they are at risk of developing the disease phenotype during the years to come. In the future, these individuals will become fully informative for the haplotype analysis. Two other individuals who have inherited the complete disease-associated haplotype are not expected to develop

the disorder because it was inherited from their affected mother (data not shown).

Recently, a possible second locus for hereditary paragangliomas with markers INT2 and TYR at chromosome 11q13-q14 was reported (Mariman et al., 1993). We have tested several 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 to determine 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 are 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 (Devilee et al., 1994). Additional information for the location of PGL may be obtained through datailed mapping of the regions on 11q undergoing these genetic changes.

Genomic imprinting appears to be responsible for irregular patterns of inheritance and variable expressions in human disorders (Hall, 1990). 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, giving indirect molecular evidence that a tumor suppressor gene might be involved (Pal et al., 1990; Scrable et al., 1989; Toguchida et al., 1989). For hereditary paragangliomas, both a dominant (onco)gene and a tumor suppressor gene have been proposed (van der Mey et al., 1989; Hulsebos et al., 1990). The low incidence of hereditary paragangliomas argues against the involvement of a tumor suppressor gene. The maternal allele of PGL is imprinted. Each individual in the population carries only a single active allele at PGL in all its somatic cells. Subsequently, a single mutation in the active allele would be sufficient to give rise to tumor growth. A much higher incidence of paragangliomas in the population is then expected unless a second event takes place. A loss of the imprint on PGL during late childhood could be such an event. An example of such a mechanism was reported in recent studies on Wilms tumors (WT). These studies have shown that in 70% of WT not undergoing loss of heterozygosity the mormal imprint on IGF2 was lost (Rainier et al., 1993; Ogawa et al., 1993). In the normal situation the genomic imprint represses the expression of one copy of a growth promoting gene. If the imprint is disturbed the gene will be expressed and this will lead to tumor growth.
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 (Cedar, 1988). 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 (Keshet et al., 1986; Doerfler, 1983). Mutant mice embryos lacking DNA methyltransferase activity do not control differential expression of genes known to be genomically imprinted (Li et al., 1993; Surani, 1993). However, at this point it is still not clear whether DNA methylation plays a functional role in establishing the genomic imprint. Possibly there are additional transcriptional elements for pairs of alleles that have a role in determining the parent of origin-specific expression.

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, rather than genes involved in later stages of malignant tumor progression. In this study, the cell adhesion molecule NCAM is localized within the candidate region for PGL but it lacks a polymorphism that is informative enough to determine its possible role in paraganglioma development.
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.

3

CONFINEMENT OF PGL, AN IMPRINTED GENE CAUSING HEREDITARY PARAGANGLIOMAS, TO A 2-CM INTERVAL ON 11q22-q23 AND EXCLUSION OF DRD2 AND NCAM AS CANDIDATE GENES

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Paragangliomas of the head and neck region, also known as glomus tumours, are mostly benign tumours of neuro-ectodermal origin. We mapped the familial form by linkage analysis in 6 families to chromosome region 11q22-q23, between the markers STMY and CD3D which currently span a 16 cM interval. Here, we performed detailed haplotype analysis of this region in a single Dutch multibranch 7-generation family. A region of 2 cM between the markers D11S938/D11S4122 and D11S1885 was shared between all patients of whom disease haplotypes could be reconstructed. In support of this localization, a recombination observed in a small French family with 2 affected nieces places the PGL gene proximal to marker D11S908, genetically coincident with D11S1885.

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INTRODUCTION

Paragangliomas of the head and neck region, also known as glomus tumours or chemodectomas, are slow growing, mostly benign tumours of neuro-ectodermal origin. Their incidence has been estimated to be approximately 1:100,000 and they manifest roughly between the age of 18 and 60 (Zak and Lawson, 1982).

The familial form of the disease displays an autosomal dominant mode of inheritance (Zak and Lawson, 1982; van der Mey et al., 1989). However, the tumours only develop in individuals who have inherited the gene paternally, whereas maternal transmission results in non-affected carriers only (van der Mey et al., 1989). This has been interpreted as evidence that the underlying gene defect is subject to 'genomic imprinting' (van der Mey et al., 1989), an epigenetic mechanism whereby, in a reversible process, a gamete-specific modification in the parental generation leads to functional differences between maternal and paternal genomes in the offspring (Barlow, 1994).

In an earlier study we have mapped the disease gene, termed PGL (OMIM 168000), by linkage analysis in one large Dutch family (FGT01) to chromosome 11q22-q23 (Heutink et al., 1992). The reported meiotic recombinants in the families positioned PGL between the markers STMY and CD3D, separated at a 26 cM genetic distance (Heutink et al., 1994). More recent estimates fix this distance at 16 cM (Dib et al., 1996). Although no statistical evidence was obtained for the involvement of genetic heterogeneity (Heutink et al., 1994), a single family has been shown to link to markers for 11q13, and not to be due to the PGL locus on 11q22-q23 (Mariman et al., 1993, 1995). This suggests that a second locus may exist.

Because paragangliomas are rare, few families become available for recombinant analyses, and the current size of the gene region of 16 cM is too large to seriously attempt the positional cloning of PGL. We present here the detailed haplotype analysis of this region in a single Dutch multibranch 7-generation family. This allowed the definition of a shared haplotype between all affected descendants, narrowing the candidate gene region to an interval of 2 cM between the markers D11S938/D11S4122 and D11S1885.

MATERIALS AND METHODS

Family ascertainment

Diagnosis of paraganglioma was based on medical history, physical and otolaryngological examination, and/or determination of free urinary catecholamine excretion (van der Mey et al., 1989; Heutink et al., 1992; Oosterwijk et al., 1996). In 94 individuals, MRI of the head and neck region was performed as well (van Gils et al., 1991). Part of the large family (branches E-H in this study, Figure 3.1) has been presented earlier as FGT01, in which the initial linkage was obtained (Heutink et al., 1992). Branches A, C and D were previously presented as family FGT09 (Heutink et al., 1994).

DNA isolation and PCR analysis

Blood samples were collected from 190 individuals and genomic DNA was isolated from peripheral blood lymphocytes as described by Miller (Miller et al., 1988). PCR conditions to visualize microsatellite polymorphisms were as described (Heutink et al., 1994; Weber and May, 1989). All primer sequences for these markers are retrievable online from GDB (http:/gdbwww.gdb.org/) or Généthon (http:/www.genethon.fr/) databases, and all oligonucleotides were manufactured by Isogen Inc.

Linkage analysis

Linkage analysis was performed using the LINKAGE program package version 5.1 (Lathrop and Lalouel, 1984). Briefly, 8 liability classes were defined to account for age of onset and the absence of penetrance in children of female gene carriers (Heutink et al., 1992, 1994). The population incidence of glomus tumours has been estimated to be 1:100,000, but we believe this is probably an underestimation. In addition, because the disease shows incomplete penetrance, the number of gene carriers is probably higher than the number of patients. Hence we have used a conservative estimate for the disease gene frequency of 0.001 in order not to inflate lod scores. Allele lengths of the markers were determined using an M13 sequence as reference, and were as expected from GDB. Lod scores were computed using allele frequencies that were determined in 41 unrelated Dutch individuals from the same area in the Netherlands from which the 7 generation family originated. Twenty of these were spouses marrying into this family, the others were spouses marrying into 3 families with familial atypical multiple mole melanoma syndrome (Gruis et al., 1995).

For haplotype analysis we used a marker order which was derived from the NIH-CEPH Collaborative Mapping Group (NIH-CEPH 1992; Litt et al., 1995), complemented with data from the Généthon group (Dib et al., 1996; Gyapay et al., 1994) and a radiation hybrid map (James et al., 1994). For all markers, the odds for their mutual order was 1,000:1 unless stated otherwise.

The investigation of the inferred second locus for hereditary paragangliomas on 11q13 was performed with the markers D11S554, D11S905, D11S956, D11S480, PYGM, and FGF3.

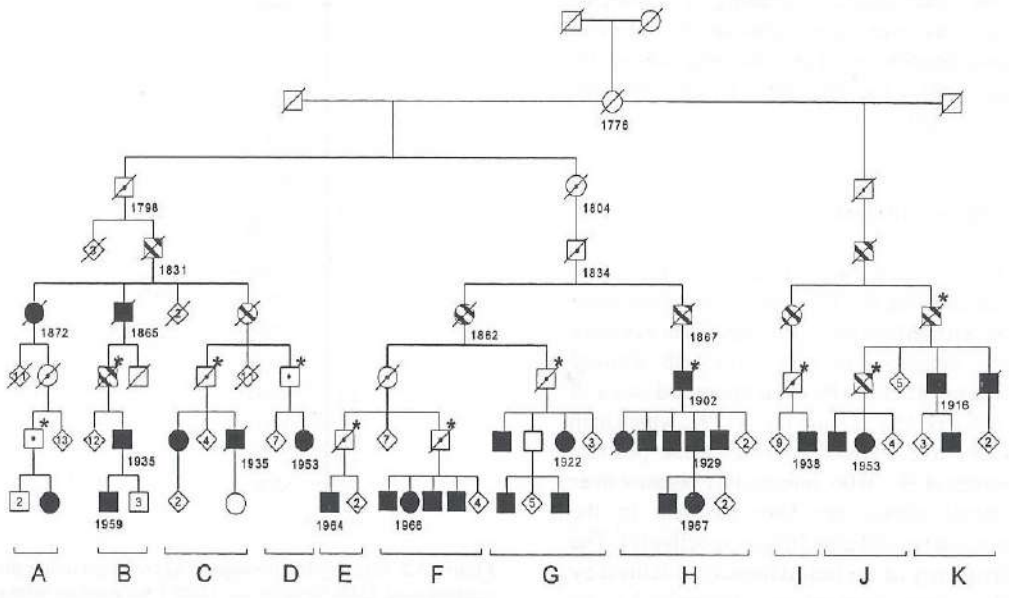

Figure 3.1. Simplified pedigree of family FGT189. Circles are females, squares males. Solid symbols represent patients (according to medical record), open symbols non-affected persons; dashed symbols are persons of whom we were not able to retrieve the medical record, but who should be affected according to the mode of inheritance. Symbols with a dot are obligate (imprinted) carriers. An asterisk indicates the person in the branch (A through K) as far back in genealogy as possible of whom we can deduce the haplotype with certainty. For orientation purposes, the year of birth is given below for some individuals. The man in branch G (gray symbol) did not want to cooperate in this study.

RESULTS

In an attempt to further map PGL more accurately, a search for recombinants in the disease-associated haplotype was initiated by typing a total of 19 families with the markers D11S897, NCAM, D11S490 and CD3D, which map to both extremes of the 16 cM candidate region reported previously (Heutink et al., 1992, 1994). Both linkage and haplotype analysis failed to provide evidence for such recombinants, possibly because many of the kindreds analyzed were of relatively small size, and few patients in at least two generations were available for marker typing. By genealogical analysis, we were able to link two of these families into the kindred in which the initial linkage to 11q22-q23 was reported (FGT01, Heutink et al., 1992), resulting in a large 7-generation multibranch family derived from a small geographic area in the Netherlands (Figure 3.1). The comparison of the disease haplotypes of all affected individuals would in principle allow the detection of ancestral recombination events. To this end, a total of 190 individuals, including 25 affected subjects, were genotyped at 21 different polymorphic markers mapping at regular intervals across the entire 16 cM candidate region (Figure 3.2).

Linkage analysis

Table 3.1 shows the 2 point lod score calculations for 12 of the 21 markers used. Seven markers provided significant evidence for linkage, but only D11S908 showed linkage at $\Theta = 0.00$ (maximum lod score of 3.99). D11S1327 and D11S1792, which map close to D11S908, showed weak positive scores at $\Theta = 0.00$, presumably because their linked alleles are very frequent in the population (64 and 76%, respectively). The frequency of the linked haplotype defined by these three markers was determined to be 18% in our reference population. By recoding the three marker haplotype to a single 'marker', a lod score of 5.77 was obtained at $\Theta = 0.00$ (data not shown).

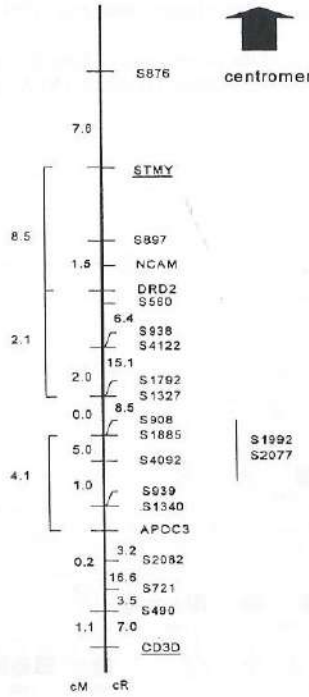

Figure 3.2. Map of chromosome 11q22-23 containing the markers used. Odds for order are 1,000:1 for markers with a horizontal line; a vertical line represents markers of which the location is not exactly known. Distances are given in cM (on the left) or in cR (on the right) (NIH-CEPH 1992; Gyapay et al., 1994; James et al., 1994; Litt et al., 1995; Dib et al., 1996). The markers underlined represent the previous borders of the PGL containing region (Heutink et al., 1994).

Haplotype analysis

A total of 29 affected persons were ascertained in the lower 3 generations, and the disease-linked haplotypes were reconstructed with data from 21 markers in 25 of these (Table 3.2). No recombinants were detected in the disease haplotype in those cases where marker data were available for affected or carrier parents as well as their affected child(ren). Therefore, for the sake of comparison, all sibships with an affected case were considered a separate branch of the family, designated A through K (Figure 3.1), and each could be represented by a single disease-associated haplotype (Table 3.2).

Table 3.1. Two-point lod scores for family FGT189 (8 liability classes) using population based allele frequencies.

	Recombination fraction							
Marker[1]	0.000	0.010	0.050	0.100	0.200	0.300	MAX lod	Θ
NCAM	-∞	5.90	6.81	6.66	5.38	3.53	6.83	0.06
DRD2	-∞	0.35	0.96	1.06	0.84	0.47	1.06	0.09
D11S560	-∞	7.93	8.74	8.45	6.90	4.71	8.74	0.05
D11S938	-∞	0.61	1.82	2.03	1.65	0.91	2.03	0.10
D11S4122	-∞	4.29	5.27	5.22	4.19	2.66	5.32	0.06
D11S1792	0.88	0.86	0.79	0.67	0.39	0.14	0.88	0.00
D11S1327	1.42	1.38	1.22	1.03	0.66	0.34	1.42	0.00
D11S908	3.99	3.92	3.60	3.18	2.24	1.28	3.99	0.00
D11S1885	-∞	7.22	7.23	6.61	4.96	3.09	7.36	0.02
D11S2082	-∞	2.86	4.80	5.16	4.51	3.14	5.16	0.10
D11S490	-∞	2.74	2.93	2.61	1.72	0.87	2.96	0.03
CD3D	-∞	2.61	3.58	3.57	2.82	1.74	3.63	0.07

[1]Markers are displayed from centromere to 11q telomere.

Since all these branches descend from the same ancestral female, all patients are presumed to carry the same gene defect and alleles of markers closely bordering this gene are expected to be identical by descent. This phenomenon was observed with the same 3 markers that showed tight linkage, i.e. D11S1327, D11S1792, and D11S908 (Table 3.2). This suggests that ancestral recombinants have occurred between PGL and D11S938/D11S4122 (these markers are genetically not separated) and between PGL and D11S1885. These markers span a sex-average genetic distance of 2 cM (Dib et al., 1996). Notably, the haplotypes C through K appear identical over a much larger region of chromosome 11q, namely between the markers D11S876 and D11S4092, a distance of approximately 10 cM. Haplotypes A and B are most divergent from this, and share only the region D11S938-D11S1885 between them.

While this work was in progress, a small family of French origin (FGT21) was referred to our lab. After haplotyping, the 2 affected nieces (ID number 8 and 10 in Figure 3.3) appear only to share the region proximal to marker D11S908. This implies a recombination between markers D11S1327 and D11S908 in either one of the parents. Unfortunately, we do not have DNA samples from the grandmother to determine where this recombination occurred. These results suggest that D11S908 can be excluded from the region containing PGL.

Table 3.2. Haplotype analysis in the family FGT189.

Marker[1]	Disease haplotype in branch												
	A	B	C	D	E	F	G	H	I	J	K	Freq.[2]	Alleles[3]
D11S876	6	6	6	6	4	4	4	1	7	7	7	6	11
D11S897	2	2	1	1	1	1	1	1	1	1	1	7	8
NCAM	7	5	3	3	3	3	3	3	3	3	3	7	12
DRD2	2	3	2	2	2	2	2	2	2	2	2	52	5
D11S560	8	4	3	3	3	3	3	3	3	3	3	4	8
D11S938	1	2	3	3	3	3	3	3	3	3	3	58	6
D11S4122	7	7	5	5	5	5	5	5	5	5	5	10	7
D11S1792	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**76**	**4**
D11S1327	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**64**	**4**
D11S908	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**38**	**6**
D11S1885	4	2	2	2	2	2	2	2	2	2	2	11	10
D11S1992	2	3	3	3	3	3	3	3	3	3	3	ND	6
D11S2077	2	2	1	1	1	1	1	1	1	1	1	ND	2
D11S4092	4	4	5	5	3	3	3	3	5	3	3	46	6
D11S939	4	4	4	4	2	2	2	2	2	2	2	45	4
D11S1340	2	2	5	5	2	2	2	2	1	1	1	22	6
APOC3	1	4	5	5	1	1	1	1	9	9	9	2	15
D11S2082	8	4	1	1	10	10	10	10	10	10	10	6	13
D11S721	7	4	4	4	11	11	11	11	11	11	11	ND	12
D11S490	4	6	6	6	6	6	6	6	6	6	6	32	11
CD3D	2	4	1	1	4	4	4	4	4	4	4	27	6
Patients[4]	1	2	2	1	1	3	4	8	1	2	3		

[1]Markers are shown from centromere to 11q telomere. [2]Freq. = Percentage of the most frequent disease-allele among 82 chromosomes marrying into the family. ND = Not determined. [3]Total number of different alleles within this family. [4]Number of patients in which the haplotype is found. The marker STMY (previous border) is located between the markers D11S876 and D11S897.

Locus heterogeneity

A second locus for PGL has been mapped to 11q13 in a single family between the markers D11S956 and PYGM, a sex average genetic region of 5 cM (Mariman et al., 1993, 1995). These and several other markers, including D11S480, the only marker showing complete allele-sharing in that family, were investigated here. No common haplotype could be defined among the branches E-H and among the branches A, C and D (data not shown). Also in family FGT21 no shared haplotype could be detected between the 2 affected individuals, of whom marker D11S480 is shown in Figure 3.3.

DISCUSSION

We have presented evidence that PGL maps to a 2 cM interval on 11q22-q23 by haplotype sharing between affected persons in a large Dutch family and a small French family. Both kindreds are consistent with the sex-dependent modification of gene expression ('imprinting'). Recently, genomic

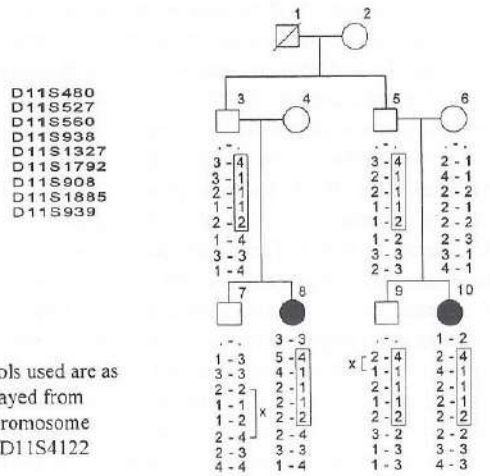

Figure 3.3. Pedigree of family FGT21. The symbols used are as in Figure 3.1. Chromosome 11q markers are displayed from centromere to telomere. The shared segment of chromosome 11q23 between the two patients is boxed. Marker D11S4122 is not informative in this family.

imprinting in families with paragangliomas of the head and neck region was also confirmed independently for 9 US families (McCaffrey et al., 1994). Our results represent a significant reduction of the candidate gene region, which stood at 16 cM after recombinant analysis of 6 families (Heutink et al., 1994; Dib et al., 1996), and now excludes the dopamine receptor 2 gene (DRD2) and neural cell adhesion molecule gene (NCAM) as candidates for the disease. The PGL region maps between the markers D11S938/D11S4122 and D11S908/D11S1885. The physical localization of these marker pairs in relation to each other is not yet known.

The order of the markers used for the reconstruction of haplotypes was compiled from different types of maps: genetic maps (Dib et al., 1996; NIH-CEPH 1992; Litt et al., 1994) and a radiation hybrid map (James et al., 1994), which overlap partially in terms of markers used. One inconsistency of particular concern for this study was the position of D11S1327. RH mapping placed it at the same position as D11S1792 (James et al., 1994), which is supported by characterization of YACs in this region [M. James, unpubl.]. Meiotic recombinant analysis in CEPH families, however, located this marker 1 cM proximal to D11S938 (Dib et al., 1996), i.e. 3 cM proximal to D11S1792/D11S908. On the basis of the depth of the YAC contig at this position, the RH map, our own cosmid map (unpublished), and the potential errors in CEPH family analysis, we have here assumed that D11S1327 and D11S1792 are tightly linked.

A potential pitfall in the analysis of the haplotype sharing might be that all family members are from a small isolated community living in a small geographic area in the Netherlands. Thus an identical haplotype might have been brought into the family by an unsuspected consanguineous relationship. Consequently, certain recombination events might have been missed. Indeed, the two large blocks of shared haplotypes interrupted by a discordant block in branches E-K (Table 3.2) might be reflecting such an event. However, since all affected members of the family carry the same mutation by descent, this can only lead to overestimation of the candidate region, but not to a false candidate region. On the other hand, underestimation of the candidate region could occur by mutations in the markers bordering

this region. Since frequencies for such events are low (10^{-3}-10^{-4}, Weber and Wong, 1993) and any 2 patients in the large family are at most separated by 14 meiotic events, this seems less likely here. The power of linkage disequilibrium mapping and/or haplotype sharing for gene isolation in founder populations has been demonstrated before (Hästbacka et al., 1994; Ramsey et al., 1993). Due to the high frequencies of the linked alleles at the three shared loci, the statistical support for this region is just significant. The isolation of additional markers within the current gene region with a low frequency of the disease-linked allele should increase this significance. The localization reported here is supported independently by our analysis of loss of heterozygosity in several glomus tumours, both sporadic and familial (Devilee et al., 1994). These tumours all seem to affect the region distal to marker D11S560, which includes the currently defined 2-cM candidate gene region.

The absence of meiotic recombinants in 17 families, although they were of various sizes, is unexpected. At least 50 informative (i.e. of paternal origin) meioses could be scored, and hence about 8 recombination events were expected, although an excess of female over male recombination has been noted in this region (Litt et al., 1995). On the other hand, regions as large as 10 cM have been found to be transmitted randomly without recombination through multiple generations in several extended families (Gruis et al., 1995; Nyström-Lahti et al., 1994; Houwen et al., 1994). Our inability to identify recombinants in the initial screen of 17 families might thus be purely coincidental, particularly as we found evidence for at least a few ancestral recombination events. It is nonetheless tempting to consider that if recombination suppression existed in this region, it may be related to the disease-causing mutation. A link has been proposed between genomic imprinting and sex-specific recombination (Påldi et al., 1995), while a class of mutations has been proposed that might interfere with the process of genomic imprinting (Reik et al., 1995; Buiting et al., 1995). We cannot exclude that the mutation in PGL would render an otherwise non-imprinted gene susceptible for genomic imprinting, concomitantly affecting meiotic recombination.

We were unable to obtain evidence for the involvement of an inferred second locus for PGL at 11q13 (Mariman et al., 1993, 1995), not in the two families reported here, nor in other families (Heutink et al., 1994; our unpublished data). This suggests that, if a second exists, it will play a minor role in inherited paraganglioma, but formal heterogeneity analysis will be required to confirm this.

Although our results represent significant progress towards identifying PGL, a genetic distance of 2 cM would imply a physical size of approximately 2 Mb, and therefore a further reduction of this region is still required. A detailed physical map of the region, in conjunction with linkage disequilibrium measurements should enable this.

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4

GENETIC HETEROGENEITY ANALYSIS OF HEREDITARY PARAGANGLIOMAS SHOWS THAT THE LARGE MAJORITY ARE DUE TO PGL1 AT 11q22-q23

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Two genes have been implicated as causing an inherited form of hereditary paragangliomas of the head and neck region (HN-paragangliomas). One has been assigned to 11q22-q23 in a set of 6 families (PGL1) and another to 11q13 (PGL2) in one family. Both are thought to undergo genomic imprinting. To investigate whether there is statistical evidence for genetic heterogeneity or that a single overlapping region may exist between the two loci, we performed HOMOG analyses using multipoint lod scores obtained with 4 markers at each locus in 17 families, including the ones in which PGL1 and PGL2 were detected previously. A maximum lod score of 4.42 for the PGL2 locus is located between the markers D11S956 and PYGM, and of 11.10 for the PGL1 locus between D11S1327 and D11S490. HOMOG analysis supports the hypothesis of 2 separate disease genes within the genome, both located on chromosome 11q. The proportions of families linked to PGL1 and PGL2 were estimated to be 0.93 and 0.07, respectively. A founder effect described for 9 of the 17 families was shown to affect these proportions only marginally. The statistical evidence for genetic heterogeneity was found to be entirely dependent on the addition of a single PGL2-linked family.

Submitted.

Introduction

Hereditary paragangliomas of the head and neck region (HN-paragangliomas) are rare, slow growing tumors of the parasympathic paraganglion system, which are usually of benign nature. Familial transmission has been described consistent with an autosomal dominant gene subject to genomic imprinting. Only after paternal transmission do tumors arise (van der Mey et al., 1989; Heutink et al., 1992; McCaffrey et al., 1994), although tumor development shows age-dependent penetrance (van Baars et al., 1982; Heutink et al., 1992) and usually becomes manifest between the ages of 18-60 years. The proportion of familial cases is estimated to be 50% (van der Mey et al., 1989), although this might be an underestimation due to generation skipping upon maternal transmission.

Linkage analyses of a small number of families have detected two loci, both on the long arm of chromosome 11. The PGL1 gene has been assigned to a 16 cM interval in the 11q22-q23 region in 5 Dutch families by recombinant mapping (Heutink et al., 1994), which was recently further refined to 2 cM by haplotype-sharing in a single extended multibranch family (van Schothorst et al., 1996). The PGL2 gene was located in a 5-cM interval in the 11q13 region in another single Dutch family (Mariman et al., 1993, 1995) in which linkage to the PGL1 locus was excluded. Additional independent linkage studies of 6 North-American families (Baysal et al., 1997a) and 3 other families of unknown geographical origin (Milunsky et al., 1997) showed that in 6 families the disease was due to PGL1, whereas the involvement of the PGL2 region could be excluded in 8 families.

Since PGL1 and PGL2 map to the same chromosome arm, and HN-paragangliomas are a rare occurrence, we sought to provide further statistical support for the existence of both genes as separate disease causing loci. We now present the detailed analysis of 17 families, which supports the existence of genetic heterogeneity. However, this result was entirely dependent on the admixture of the one large family linked to 11q13, since exclusion of this family from the data set indicated that HN-paragangliomas in all other families are due to PGL1.

Material and methods

Family ascertainment

Diagnosis of HN-paraganglioma was based on medical history, physical and otolaryngological examination, and radiological imaging (van der Mey et al., 1989; van Gils et al., 1994; Mariman et al., 1995; van Schothorst et al., 1996). Families FGT1, FGT8, and FGT9 were independently ascertained, but later shown to constitute a single kindred (renamed FGT189) by genealogy analysis (van Schothorst et al., 1996); families FGT3, FGT4, FGT10 and FGT18 have been described by Heutink et al. (1994) and families FGT5, FGT11, FGT20, and FGT25 are described elsewhere (van Schothorst et al., submitted). FGT2 has been described by Mariman (therein mentioned as family 1; Mariman et al., 1995).

Criteria for other, recently ascertained families included in this analysis were: at least two affected individuals, preferably from more than one generation. These families are FGT14, FGT15, FGT22, FGT24, and FGT26. Family FGT26 is the only family not ascertained in the Netherlands, but in Newfoundland, Canada.

After obtaining informed consent, blood was removed from familymembers and used for genomic DNA isolation.

DNA isolation and PCR analysis

DNA isolation and PCR analysis was performed as described (van Schothorst et al., 1996) with the following modifications: the markers within the 11q13 region were amplified with an end-labeled primer, while the markers for the 11q22-q23 region were labeled by incorporation of ^{32}P-dCTP.

Markers used for the 11q13 region were (from centromere to telomere): D11S905, D11S554, D11S956 and PYGM

with the location of the PGL2 gene near D11S480 between D11S956 and PYGM (Mariman et al., 1995). The marker D11S480 has not been included in multipoint analyses, since at the time linkage with this marker was found, these multipoint analyses were already running.

The markers D11S927, D11S1327, D11S490 and CD3D span the 11q22-q23 region, with the location of the PGL1 gene close to the marker D11S1327 (Heutink et al., 1994; van Schothorst et al., 1996).

Linkage analysis

Two-point and multipoint linkage analyses were performed with the Vitesse linkage algorithm (O'Connell and Weeks 1995) run on a PC under Unix. Eight liability classes were used to account for age-of-onset and the phenomenon of imprinting, and a disease gene frequency of 0.001 was used as described previously (van Schothorst et al., 1996). Briefly, liability class (LC) 1 comprise affected or married in individuals; class 2 children (imprinted) of an affected mother, class 3 to 8 unaffecteds, at risk, at an age of: < 15 years (3); 15-20 years (4); 21-30 years (5); 31-40 years (6); 41-50 years (7) and >51 years (8).

For analyses involving two or more marker loci, genotypes were recoded to obtain maximum 5 alleles at each marker locus with approximately equal allele frequencies. Recoding of alleles did not substantially alter the two-point lod scores (results not shown). Genetic map sex-average distances used were : D11S905- (3 cM) -D11S554- (5 cM) -D11S956- (5 cM) -PYGM for the PGL2 region (Mariman et al., 1995) and D11S927- (5 cM) -D11S1327- (5 cM) -D11S490- (2 cM) -CD3D for the PGL1 region (Guyapay et al., 1994; Litt et al., 1995). Multipoint lod scores were calculated for 27 locations of the disease locus in the two regions, at 1 cM intervals within each region. The genetic distance between these two regions is at least 35 cM.

Multipoint lod scores were calculated in each of the two regions independently. Whenever one of the four markers in a region was uninformative or not tested, that marker was omitted from the analysis and recombination fractions were adjusted wherever necessary, according to Haldane's mapping function. In the remaining families overlapping four-point analyses were carried out, since five-point analyses proved to be too time-consuming. In a few families, the two overlapping four-point analyses yielded different lod scores for some map locations. In those families it was decided on the basis of two-point lod scores and visual inspection of haplotypes which marker combination delineated most clearly the recombinants present in that family, and the corresponding lod scores were included in the combined multipoint and heterogeneity analysis.

Heterogeneity analysis

Analysis of heterogeneity was carried out with the HOMOG programmes (Ott, 1991). The hypothesis of locus homogeneity was compared with that of locus heterogeneity (the existence of one disease locus, located in the map of markers, and one additional disease locus unlinked to the map) via HOMOG. Via HOMOG2 we evaluated the hypothesis that two disease loci are located within the marker map, while the presence of yet a third, unlinked disease locus was modeled via HOMOG3.

Results

HOMOG analysis

Multipoint linkage analyses were carried out in 17 families with HN-paragangliomas in two distant candidate regions: PGL1 on 11q22-q23, and PGL2 on 11q13. The pedigrees of 5 families, not reported previously, are shown in Figure 4.1. Three families show only paternal transmission, in agreement with the phenomenon of genomic imprinting as observed earlier (van der Mey et al., 1989; McCaffrey et al., 1994), while the other two (FGT14 and FGT26) are not conclusive in this regard.

Under the assumption of locus homogeneity, locations of the disease gene between the four PGL1 markers yielded modestly positive lod scores (peak lod score of 2.81, 2 cM distal of D11S1327), while strongly negative lod scores were obtained for the PGL2 region (peak lod score of -28.93 at 1 cM

distal of D11S905)(not shown). A test for locus homogeneity via the HOMOG programme yielded strong evidence in favor of locus heterogeneity (odds $2x10^8$:1) with a peak lod score for linkage and heterogeneity of 11.10 at a location 2 cM distal of D11S1327 in the PGL1 region. Only family FGT2 yielded a strongly negative lod score of -9.48 (Table 4.1, Figure 4.2), while another four families yielded mildly negative (-0.7<Z<0) lod scores for this location (Table 4.1). The proportion of families linked (α) to PGL1 was estimated to be 0.85.

A second, albeit much lower peak in the curve of multipoint lod scores under heterogeneity was observed in the PGL2 region, at a location 2 cM distal of D11S956 (peak lod score 4.42, Figure 4.2). Family FGT2 contributed mostly (Z= 5.79) to this peak, with some additional moderate contributions from some other families (Table 4.1).

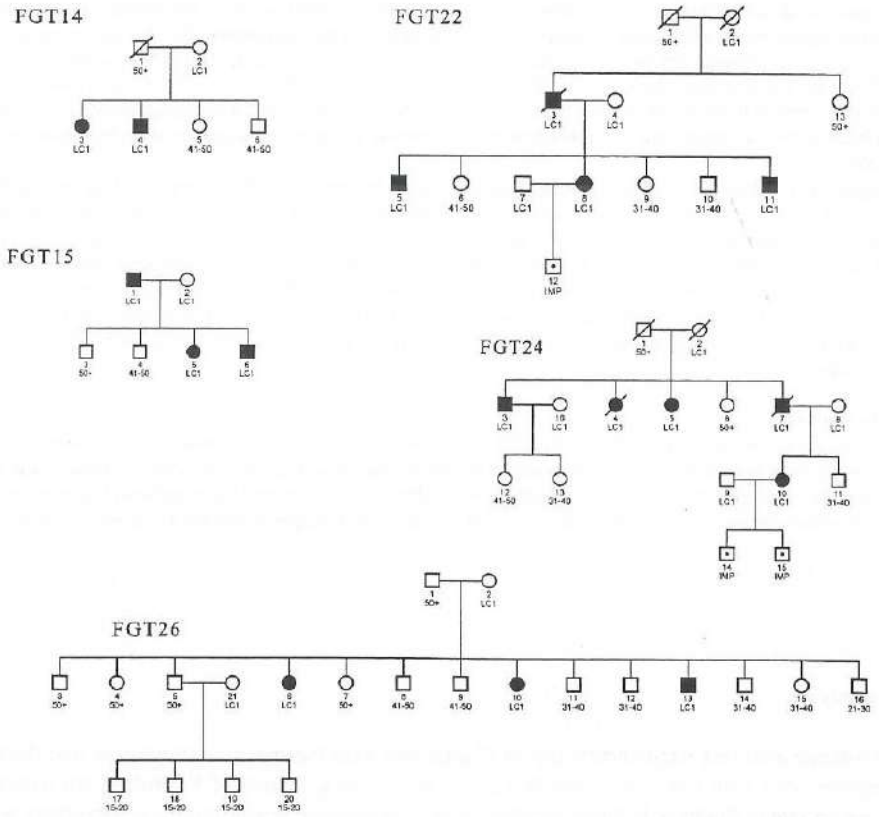

Figure 4.1. Pedigrees of 5 recently ascertained HN-paraganglioma families.
Solid symbols represent patients, symbols with a dot children of an affected mother (no risk). The liability class is depicted beneath the symbol (see Material and methods). DNA was available from all subjects, except for FGT14.1, .2; FGT22.1-4, .13; and FGT24.1, .2, .4, .7, .8, .16.

Table 4.1. Maximum lod scores for PGL1 and PGL2.

	PGL1 (11q22-q23)			PGL2 (11q13)		
Family	Max[a]	Marker[b]	theta[c]	Max[a]	Marker[b]	theta[c]
FGT1	5.51	CD3D	0.00	-7.15	S905	0.01
FGT2	-9.13	S1327	-0.02	5.79	S956	0.02
FGT3	1.14	S1327	0.00	0.92	PYGM	0.00
FGT4	0.86	CD3D	0.00	0.48	PYGM	0.00
FGT5	-0.50	S1327	0.02	-1.70	PYGM	-0.02
FGT8	0.42	S927	0.00	0.56	S905	0.00
FGT9	0.80	S1327	0.00	-4.82	PYGM	-0.02
FGT10	0.54	S490	0.00	-0.89	S554	0.01
FGT11	-0.11	S490	-0.01	0.34	S905/S554/S956	0.00
FGT14	-0.14	S927	0.00	-0.17	S905/S554/PYGM	0.00
FGT15	0.84	S927/CD3D	0.00	0.03	S905	0.01
FGT18	1.94	S927	0.01	-1.62	S905	0.00
FGT20	0.30	S927/CD3D	0.00	0.30	S905	0.00
FGT22	1.20	CD3D	0.00	-0.01	S554	0.02
FGT24	0.87	S927	0.00	-0.49	S905	0.00
FGT25	0.12	S490	-0.01	-4.07	PYGM	-0.01
FGT26	-0.03	CD3D	0.00	-5.62	PYGM	-0.02

[a] Maximum lod score under heterogeneity analysis calculated at 1 cM intervals. The nearest marker ([b]) is given without the prefix D11 with the distance ([c]); - denotes proximal to the marker. If more markers are listed, it was not possible to distinguish between these markers. Families which are underlined show a possible founder effect.

While these multipoint lod score computations were underway, tight linkage was found in FGT2 between PGL2 and marker D11S480, which is located 1 cM distal of marker D11S956 (Mariman et al., 1995). Subsequent typing of D11S480 in the other 16 families revealed evidence against linkage in 7 families, while the marker was not informative in 5 families. Sharing of an allele between all patients was noted in 4 families (data not shown). However, FGT8 and FGT11 are two of these 4 families. FGT8 has recently been shown to share a common ancestor with families FGT1 and FGT9, and all patients in these families share a 2-cM region on 11q22-q23, containing PGL1 (van Schothorst et al., 1996). The disease-linked haplotype as defined by markers D11S905 (11q13) and CD3D (11q23), and over 15 markers in between, did not recombine in either family FGT8 or FGT11 (data not shown), thus explaining linkage/haplotype sharing at both the PGL1 and PGL2 locus.

HOMOG2 and HOMOG3 analysis

The HOMOG2 programme yielded a combined lod score of 16.53, thereby supporting the presence of locus heterogeneity and of two PGL loci in the regions of chromosome 11 that we investigated. The difference between the results of the HOMOG and the HOMOG2 analyses, which amounted to 5.43 lod score units can be regarded as evidence in favor of the second PGL locus being located within either

of the regions investigated (HOMOG2), as opposed to elsewhere in the genome (HOMOG). The two locations implicated in the HOMOG2 analysis are exactly the same locations that yielded peaks in the PGL1 and PGL2 regions in the HOMOG analysis. The proportion of families linked to the PGL2 region (α_{PGL2}) was estimated to be 0.07, with a corresponding estimate for α_{PGL1} of 0.93. Analysis with the HOMOG3 programme yielded no evidence for further locus heterogeneity.

Exclusion of family FGT2, linked to PGL2, and subsequent HOMOG analysis, indicated that all other 16 families are linked to PGL1 (α_{PGL1}=1.00) with a maximum lod score of 12.26 (Figure 4.2). The haplotype analysis of family FGT2 with the 4 markers defining the PGL1-region is indeed fully consistent with its exclusion (Figure 4.3). The 4 markers defining the PGL2-region also do not share an allele among all affected individuals, but tight linkage was observed for the marker D11S480 mapping between D11S956 and PYGM (Mariman et al., 1995). Presumably, this is due to at least two ancestral recombinants between D11S956 and PYGM in the predecessors of the two distant branches (Figure 4.3). Since a typing error is difficult to reconcile with our haplotype analysis and phenocopies are an unlikely occurrence with such a rare disease as HN-paragangliomas, we conclude that the disease in family FGT2 is most likely caused by PGL2.

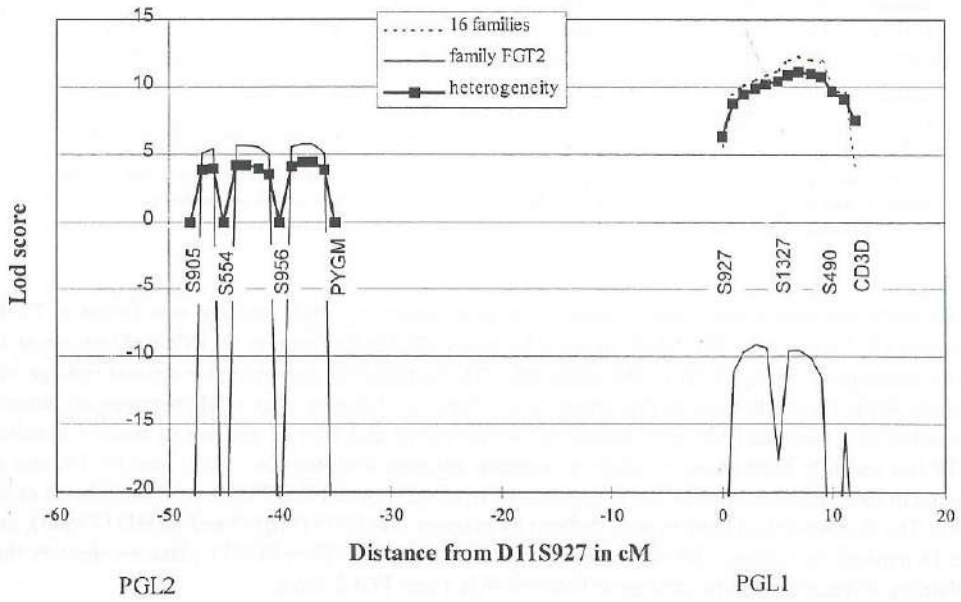

Figure 4.2. Multipoint lod scores for the PGL1 and PGL2 loci on chromosome 11q.
The solid line represents the analysis under heterogeneity for all 17 families, while the thin line represents the multipoint analysis of family FGT2, and the dotted line the analysis of the remaining 16 families. For the PGL2 region, the analysis of the 16 families resulted in values at or near 0 and is therefore excluded from this figure. Sex-average genetic distances (cM) are given in relation to marker D11S927 (arbitrarily chosen).

Influence of a founder effect on the heterogeneity estimates

Analysis of the haplotypes reconstructed with the PGL1-markers also revealed that they are identical among a number of the families analyzed here, suggesting they share a common ancestor (van Schothorst et al., submitted). In general, it is expected that a founder effect would lead to an underestimation of locus heterogeneity, since the model assumes all families to be genetically completely independent, although even within a large family in an isolated population locus heterogeneity has been reported (van Soest et al., 1994). Genealogy analysis had already identified a common founder for the three families FGT1, FGT8 and FGT9 (van Schothorst et al., 1996), and the disease-linked haplotype segregating in this family has now also been observed in FGT3, FGT5, FGT11, FGT18, FGT20, and FGT25 (van Schothorst et al., submitted). We therefore repeated the HOMOG2 analysis using the sum of the lod scores of these families as a single observation. In this way these families are all considered as descendants from a single ancestor; a multipoint lod score analysis of the entire kindred was not feasible as the distant relationships would have led to unacceptable computation times. Results were not appreciably different compared to the results assuming independence of the families. The estimate for α_{PGL2} increased from 0.07 to 0.12, and accordingly the α_{PGL1} decreased from 0.93 to 0.88, while maximum lod scores for either locus increased at most 0.16. The locations of the disease genes did not alter, nor did the significant evidence for locus heterogeneity.

Discussion

Occurrence of hereditary HN-paragangliomas has been linked to two genes on chromosome 11q: PGL1 on 11q22-q23 (Heutink et al., 1992, 1994) and PGL2 on 11q13 (Mariman et al., 1995). We have shown here in a set of 17 families, that this disease is genetically heterogeneous, although this finding was dependent on a single family in which the disease showed strong evidence for being due to PGL2. Accordingly, the proportion of families due to PGL2 was estimated to be very low (7-12%). The obtained peak lod score for the PGL1 region mapped 2 cM distal of D11S1327. This is slightly outside the region defined by haplotype-sharing in family FGT189 (van Schothorst et al., 1996), but the peak is very flat and the 1-LOD unit support interval does include it. Similarly, the maximum lod score of 4.42, under heterogeneity, for the PGL2 region maps at 2 cM distal of D11S956. This is close to D11S480, which shows tight linkage in FGT2 (Mariman et al., 1995).

It should be noted that many of the 17 analyzed families are relatively small and are thus unable to provide conclusive linkage information on their own (Table 4.1). In addition, the linkage model we used (Heutink et al., 1992) is sensitive to non-penetrant paternally derived gene carriers, particularly if these are currently at higher ages. Consequently, in some branches of the more extended kindreds, maximum informativity was not obtained. For example, weak negative lod scores were obtained for the PGL1 region in family FGT26 (Figure 4.1). All 3 patients shared a paternally derived haplotype, but this also segregated to 4 clinically unaffected individuals. Possibly, the penetrance of PGL1 is much lower than previously estimated (van Baars et al., 1981), or heterogeneous across families. Our results might thus reflect the coincidental admixture of a few families that can be unambiguously assigned to either locus by linkage analysis, and as such might in fact be typical for the Dutch population. Despite this concern, a recent linkage study of 11 North- American HN-paraganglioma families indicated that

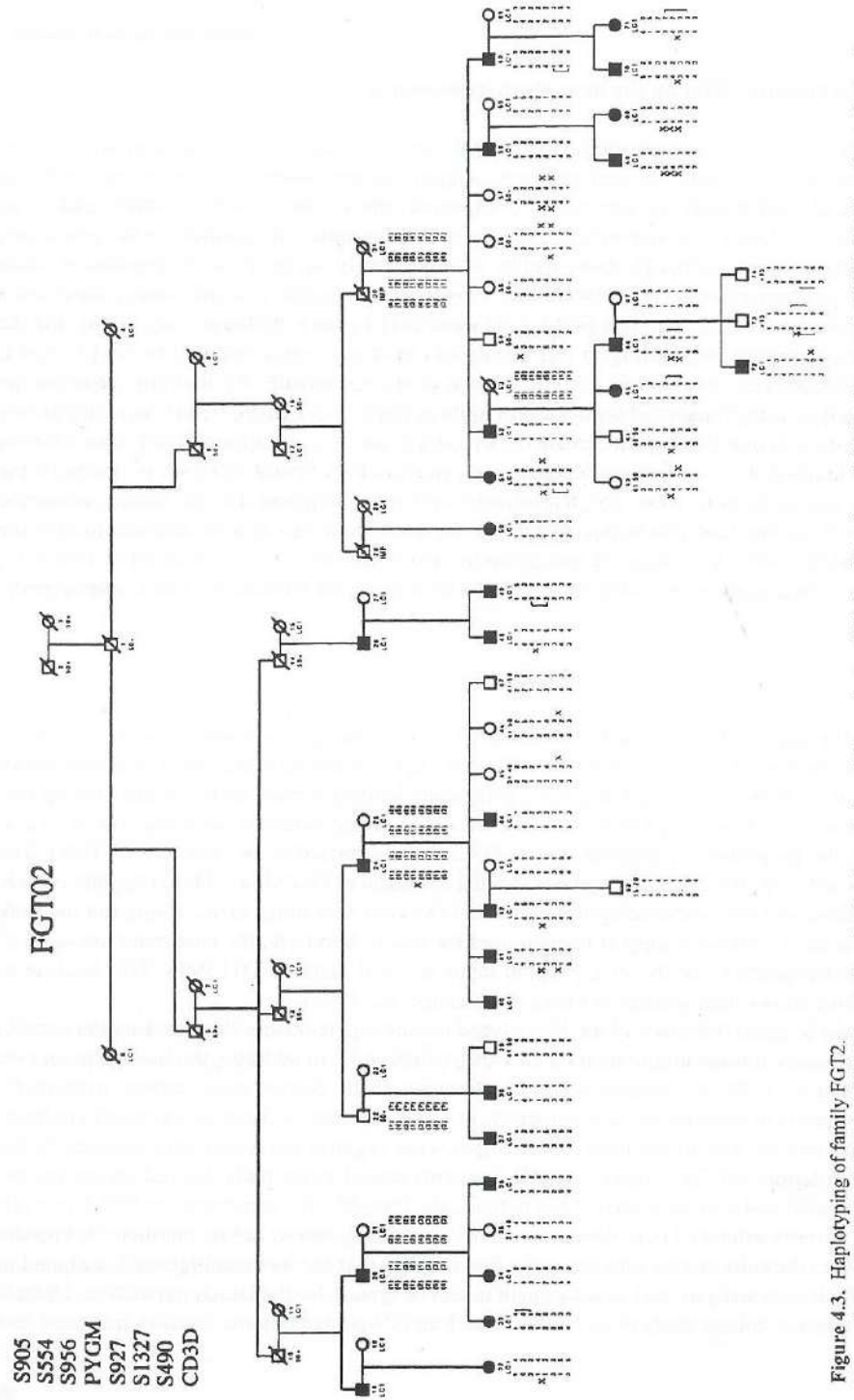

Figure 4.3. Haplotyping of family FGT2.
Descendants of individual ID 16 and ID 17 were described as branch A and descendants of ID 5 and 7 as branch B (Mariman et al., 1995). Markers (top to bottom) are as indicated. The first 4 flank the PGL2 and the latter 4 the PGL1 locus. Inferred haplotypes are given in brackets. A cross between markers indicates a recombination event. The disease-linked haplotype defined by the 4 PGL2-markers in the descendants of ID 5 (1-4-3-1) is partially observed in ID 50 (1-4-3-3). Therefore, assuming that this latter haplotype derives from the paternal grandmother ID 16, a recombinant needs to be inferred between the markers D11S956 and PYGM to obtain the linked haplotype 5-1-4-3 in the descendants of ID 30.

of 6 informative families, 3 were strongly linked to PGL1, while PGL2 could be excluded in 5 (Baysal et al., 1997a). Hence family FGT2 remains the only family reported to date that can be unambiguously assigned to PGL2.

Another factor that might have influenced the analysis is that both PGL1 and PGL2 are on the same chromosome arm. Even though the genetic distance between these loci is large (30-35 cM), they may be inherited without recombination through a small number of meioses, which becomes particularly evident in the smaller kindreds (e.g., families FGT8 and FGT11). These families will hence provide linkage evidence for both loci. While this situation is remarkable for such a rare disease, other examples do exist. Two genes for retinitis pigmentosa (RP2 and RP3) are located on the X-chromosome, separated by an estimated distance of 16 cM (Teague et al., 1994). Another potential pitfall of such a situation is that untyped regions in between the two identified loci might in fact contain a single locus that co-segregates with the disease in all families. In particular when, as in our case, a relatively widely separated map of a few markers is used. However, the difference in lod scores for the distal marker of the PGL2 region (Z=-92) and the proximal marker of the PGL1 region (Z=-24) is too large to assume a region in between which is shared by all families, but further microsatellite-marker analysis should formally exclude whether such a region exists. These considerations would in any case not be expected to affect the outcome of the heterogeneity analysis.

The recent discovery of a possible founder effect in Dutch families (van Schothorst et al.,, submitted), involving a subset of the families analyzed here, could have an effect on the heterogeneity analysis. We therefore repeated the HOMOG analyses, but the results indicated that, although distantly related families might influence the α-values marginally, it did not change the general conclusion of locus heterogeneity in this case. To our knowledge, this is the first practical example illustrating the limited impact of a founder effect on the linkage-based estimates of genetic heterogeneity.

In summary, we have shown that PGL1 on 11q22-q23 is the major locus underlying hereditary HN-paragangliomas. A very small proportion of cases appears to be due to PGL2. Mutation analysis of the culprit genes should now resolve whether or not other families exist in which the disease is caused by PGL2, in addition to the single family known to date. An intriguing possibility is that PGL1 acts in *cis* to regulate PGL2 (or *vice versa*), whose disregulated expression would then be the primary cause of tumor growth. The function of PGL1 could be compromised either through interference with the normal imprinting process, presumed to occur at PGL1 (van der Mey et al., 1989; McCaffrey et al., 1994), or by changes in its primary coding DNA sequence. Examples of such gene-interaction in conjunction with genomic imprinting abnormalities already exist for developmental disorders such as Prader-Willi and Angelman syndromes (Ferguson-Smith, 1996). Identification of PGL1 and PGL2 will represent an important contribution in the further elucidation of the role of imprinting in the process of tumorigenesis.

Acknowledgments

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5

FOUNDER EFFECT AT PGL1 IN DUTCH HEREDITARY HEAD AND NECK PARAGANGLIOMA FAMILIES

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PGL1, an imprinted gene responsible for hereditary paragangliomas of the head and neck, was recently confined to a 2-cM interval on chromosome 11q22-q23 by linkage and haplotype sharing analysis in a large multibranch Dutch family. We determined the disease-linked haplotype, as defined by 13 markers encompassing an approximately 50-cM interval on 11q13-q24, in 10 additional families ascertained from the same geographical locale. Alleles were identical for 6 contiguous markers, spanning a genetic distance of 6 cM and containing PGL1. Despite this strong indication of a common ancestor, no kinships between the families could be demonstrated through genealogical surveys, going as far back as 1800 AD. We conclude that a single ancestral mutation is responsible for most, if not all, hereditary paragangliomas in this region of the Netherlands, and that strong founder effects may exist at the PGL1 locus.

A modified version of this chapter is in press in The American Journal of Human Genetics.

INTRODUCTION

Paragangliomas are rare, usually benign tumors of the extra adrenal paraganglion tissue associated with the autonomous nervous system (Parry et al., 1982). Most paragangliomas occur in the head and neck region, where they may lead to cranial nerve deficit. Characteristically the tumor progresses slowly and although the age of onset is variable, most patients develop symptoms after puberty. Familial nonchromaffin paragangliomas of the head and neck (HN-paragangliomas, MIM 168000) inherit as an autosomal dominant disease with reduced penetrance (van Baars et al., 1982; McCaffrey et al., 1994). Affected offspring is observed only after paternal transmission, which has been taken as evidence that the underlying gene-defect is subject to genomic imprinting (van der Mey et al., 1989).

Linkage analysis of a single large Dutch pedigree mapped the gene, termed PGL1, to 11q22-q23 (Heutink et al., 1992). This result was replicated in additional families (Heutink et al., 1994), and confirmed in North American families (Baysal et al., 1997a; Milunsky et al., 1997), but the detected recombination events did not assign the gene any more accurate than to an approximately 10-cM interval. We recently identified a common ancestor, born in 1776, of three families originating from the same geographical region, including the one in which the original linkage was found. A 2-cM haplotype, presumably containing PGL1, was shared among all patients in the two lowest generations of these families (van Schothorst et al., 1996). Although a second locus has been implicated to reside on 11q13 in one Dutch paraganglioma family (Mariman et al., 1995), all other informative families analyzed to date have revealed only linkage evidence for the distal PGL1 locus on 11q22-q23 (Heutink et al., 1994; Baysal et al., 1997a; Milunsky et al., 1997).

Recently another 10 families with HN-paragangliomas were ascertained from the same geographical region as from which the large PGL1-linked family originated. Assuming that such conspicuous geographic clustering of a rare disorder might reflect a founder effect, which could be exploited for further gene localization, we performed a genealogical survey and determined the disease-linked haplotypes for all 10 families. Although no family relationships could be demonstrated by geneaology, haplotype analysis provided strong evidence for a common founder in this population.

MATERIALS AND METHODS

Family ascertainment
Since 1950, 183 patients with head and neck paragangliomas were referred to our ENT Department. Queries were sent to those patients with a recorded family history of HN-paragangliomas, enabling us to ascertain 27 pedigrees with at least 142 patients. Three families (FGT1, FGT8, FGT9) originating from the central western part of the Netherlands could be traced to a common ancestor (van Schothorst et al., 1996), and was renamed FGT189. Fourteen other families originated from the same province as did family FGT189, and we obtained DNA samples to reconstruct the disease haplotype from 10 of these. FGT3, FGT11 and FGT18 were partially described previously (van der Mey et al., 1989; Hart and Maartense, 1992; Heutink et al., 1994), others are reported here for the first time.

Disease ascertainment
Diagnosis of HN-paragangliomas was based on clinical signs and in most patients confirmed by histological or radiological investigation. Twelve probable affected progenitors were identified by evaluating their medical history. Ten of these putative patients, of whom two died during surgical intervention, are known to have had lateral neck masses. The other two are known to have had ear complaints leading to bleeding or loss of facial nerve function.

Genealogy analysis
Familial ancestries were traced back starting from the oldest known common ancestor of affected family members, and included both the paternal and maternal line. Most data were obtained from the civil registration founded in approximately 1800. Generations older than this were not studied. Three ancestral lines were not completed: in family FGT27 data on one generation in the maternal line was not available; in family FGT32 an in-marrying spouse born approximately 1850 could not be traced; in FGT20 the genealogical search was thwarted by an adopted ancestor. The Dutch fore-bearers were usually farmers or handicraftsmen; marriages outside the native village were common but little migration to other regions was observed until after World War II.

DNA isolation and PCR analysis
Blood samples were collected and genomic DNA was isolated from peripheral blood lymphocytes (Miller et al., 1988). PCR and gel-electrophoresis conditions to visualize microsatellite polymorphisms were as described previously (van Schothorst et al., 1996). All primer sequences for these markers are retrievable online from the Genome Data Base, and all oligonucleotides were manufactured by Isogen Inc. (Maarssen, The Netherlands).

Haplotype analysis
For haplotype analysis we used a marker order as described by van Schothorst et al. (1996). The genetic map (NIH-CEPH Collaborative Mapping Group, 1992; Litt et al., 1995; Dib et al., 1996) was complemented with data obtained by physical mapping of the region between markers D11S897 and D11S4111 (Baysal et al., 1997b). The markers selected covered a genetic distance of approximately 50 cM. Allele lengths were determined using an M13-sequence as reference. Allele frequencies in the control population were determined in 20 in-marrying spouses of family FGT189, and 21 unrelated members of families with the familial atypical multiple mole melanoma syndrome, and originating from the same area as FGT189 (Gruis et al., 1995). Allele-lengths and frequencies thus determined were not appreciably different from those reported in the Genome Data Base.

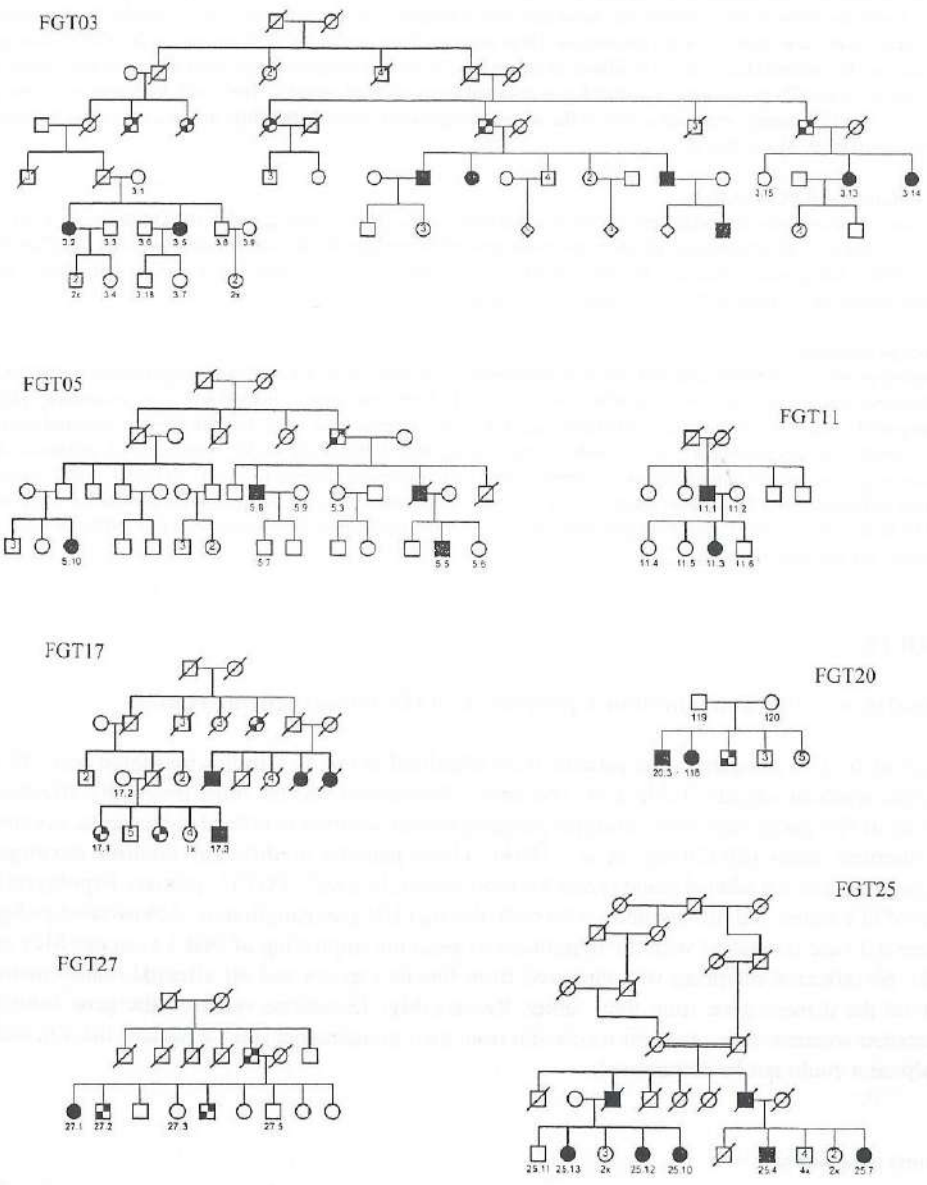
RESULTS

Clinical description and inheritance patterns in 10 HN-paraganglioma families

A total of 63 HN-paraganglioma patients were identified in the 10 families presented here, 42 with complete medical records (Table 5.1). The carotid bifurcation was the most frequently affected site (57% of all HN-paragangliomas). Multiple paragangliomas occurred in 66% of the patients, as expected for inherited cases (McCaffrey et al., 1994). Three patients in different families developed a paraganglioma of the adrenal gland (pheochromocytoma). In family FGT11, primary hypothyroidism occurred in a father and his daughter, who both also had HN-paragangliomas. Abbreviated pedigrees (Figure 5.1) are consistent with the hypothesis of genomic imprinting of PGL1 (van der Mey et al., 1989). No affected offspring was observed from female carriers and all affected family members received the disease gene from their father. Remarkably, 42 patients received the gene from their grandfather whereas only 2 patients received it from their grandmother (for 19 patients the transmitting grandparent could not be determined).

Haplotype analysis

Blood samples were obtained from 136 family members, including 35 patients (Table 5.1, Figure 5.1). DNA was genotyped at 13 markers encompassing an approximately 50-cM interval on 11q13-q24.



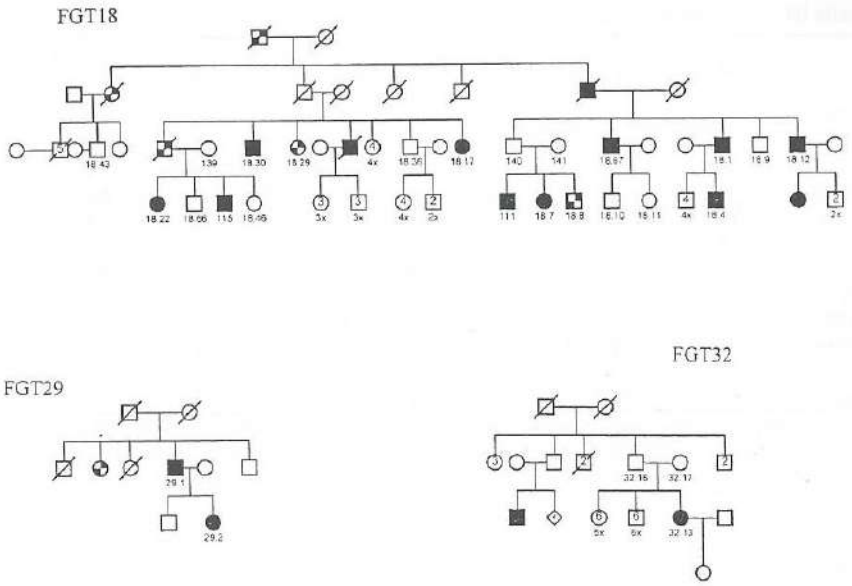


Figure 5.1. HN-paraganglioma kindreds.
Filled symbols represent affected individuals. Chequered symbols represent individuals for whom the affected status was ascertained only anamnestically. Multiple unaffected siblings are represented by collapsed symbols indicating the number of individuals. Numbers underneath symbols indicate sample number, except for collapsed symbols, where they indicate the total number of blood samples from that sibship.

Previously, we identified a 3-marker haplotype of about 2 cM, defined by markers D11S1792, D11S1327 and D11S908, which was conserved among all the patients from a large multibranch family FGT189 (summarized in Figure 5.2). However, a much larger haplotype of approximately 10 cM, defined by markers between D11S876 and D11S4092, was shared among the patients from branches C-K, and this sharing extended beyond CD3D in branches E-H. We compared this 'E/H-haplotype' with the disease-linked haplotypes of the 10 families included in this study (Table 5.2). No recombinants were detected between the disease and any marker mapping between D11S1647 and D11S908, which were previously shown to be recombinant in two independent families (van Schothorst et al., 1996; Baysal et al., 1997a). All patients shared a haplotype defined by 6 markers and bracketed by D11S927 and D11S908. This haplotype was not observed among 41 unrelated individuals (82 chromosomes) from the same geographical region, supporting the hypothesis that all patients in these 11 families are genetically identical by descent. Notably, several families, i.e., FGT11, FGT18 and FGT20, appear to share a very large region with family FGT189, including all but the most distant markers tested.

Table 5.1. Clinical data from 10 HN-paraganglioma kindreds

Family ID	Number of Patients			Tumors[c]				Religion[d]
	Total[a]	Verified[b]	Haplotyped	CBT	VBT	JTT	PH	
FGT3	13	8	4	6	4	7	0	RC
FGT5	5	4	3	5	2	0	0	P
FGT11	2	2	2	1	0	2	0	P
FGT17	7	4	2	3	0	2	1	RC
FGT18	18	13	12	15	2	0	0	P
FGT20	3	2	2	4	3	1	1	P
FGT25	7	5	5	4	1	1	0	RC
FGT27	4	1	2	1	0	0	1	RC
FGT29	2	1	2	1	0	1	0	P
FGT32	2	2	1	2	3	3	0	RC
Totals	63	42	35	42	15	17	3	

[a] Total number of patients identified anamnestically; [b] As documented by medical records; [c] CBT = carotid body tumor, VBT = vagale body tumor, JTT = jugulo-tympanicum tumor, PH = phaeochromocytoma; [d] RC = roman catholic, P = protestant

Genealogy

Records on 72 ancestors of the 10 families were retrievable. They were born between 1770 AD and 1830 AD, and originated from several rural areas of the central western part of the Netherlands, all within a radius of approximately 40 kilometres (Figure 5.3). Half of the families belonged to a Protestant church whereas the others were of Roman Catholic faith (Table 5.1). No marriages between members of different convictions were observed. Despite the strong geographical clustering and this sharing of religious faith, none of the studied families could be proven to be interrelated.

DISCUSSION

Paragangliomas of the head and neck usually follow a slow benign natural course, and generally occur above the age of 18 (Parry et al., 1982; van Baars et al., 1982; van Gils et al., 1992). As a result, they are expected not to impede reproductive fitness. From pooled data from Dutch pathological laboratories, we estimated an annual rate of 0.11 per 100,000 (Oosterwijk et al., 1996). Lack et al., (1977) retrieved 69 paragangliomas among 600,000 surgical cases seen at the Sloan-Kettering Memorial Cancer Centre from 1937 to 1975. Assuming a relevant population size of 1 to 2 million, this would suggest a comparable incidence rate. However, because of the late onset and benign course of the disease, an unknown proportion of the patients will not be hospitalized, leading to an underestimation of its incidence (van Gils et al., 1992). For similar reasons, the proportion of familial cases will be underestimated. This proportion has been reported to be 5-10% (Grufferman et al., 1980;

Parry et al., 1982), but in the Netherlands we are seeing a much more conspicuous occurrence of familial cases (van der Mey et al., 1989; van Baars et al., 1982). A founder effect is therefore not unexpected, in particular because this has also been observed for a number of families with atypical multiple mole melanoma syndrome originating from the same geographical locale (Gruis et al., 1995).

Recently, we described a 2-cM haplotype shared by all patients in the lowest two generations of the large multibranch family FGT189 (van Schothorst et al., 1996). However, conservation of this haplotype extended over much larger regions in a subset of the patients (the 'E/H-haplotype', Figure 5.2). We have shown here that parts of this particular haplotype are conserved in another 10 families originating from within a radius of approximately 40 kilometres from the town from which FGT189 originated. The minimal region shared with the 'E/H-haplotype' covers about 6 cM, and encompasses the previously identified 2-cM haplotype presumably containing the PGL1 locus. This haplotype was not detected in 82 chromosomes from unrelated individuals from the same region. Under Hardy-Weinberg equilibrium the population frequency of this haplotype would be 0.04%, making it highly unlikely that it occurs linked to PGL1 in 11 families by chance. Since we included 10 of the 14 HN-paraganglioma families ascertained from this region, these data therefore strongly indicate that a single ancestral mutation in PGL1 is responsible for most paragangliomas occurring in the central western part of the Netherlands.

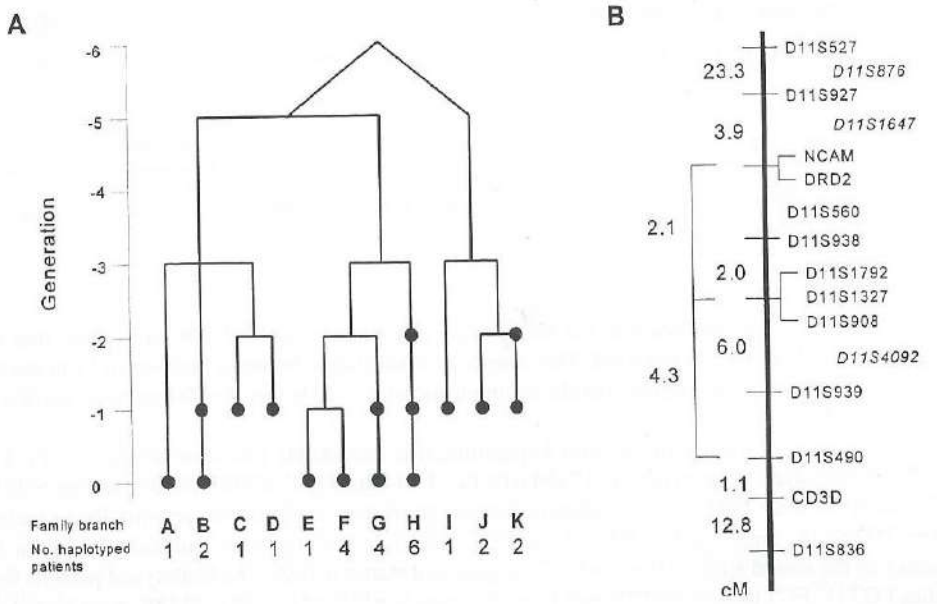


Figure 5.2. A. Abbreviated version of the pedigree-structure of the extended family FGT189, described in detail by van Schothorst et al. (1996). The family was arbitrarily subdivided in branches A-K. Filled circles represent sibships in which at least one patient has been verified by medical records. The number of haplotyped patients is indicated below each branch. **B.** Genetic map of the markers used in this study; cM = distance in centiMorgans. The underlined markers showed allele-sharing in all patients of family FGT189. Markers in italics are indicated as a reference.

Table 5.2. Disease haplotypes of 10 HN-paraganglioma families and branches E-H of family FGT189

	Linked allele-size (bp) in family FGT											Frequency (%)[a]
Marker	189	11	18	20	32	25	3	17	5	29	27	
D11S527	159	159	147	157	151	159	157+151[b]	155/159[c]	165	147/157[c]	161/155[c]	16
D11S927	135	135	135	135	135	135	135	135	135	135	141/137[c]	18
NCAM	126	126	126	126	126	126	126	126	126	126	126	7
DRD2	82	82	82	82	82	82	82	82	82	82	82	52
D11S560	87	87	87	87	87	87	87	nd[d]	75/87	nd[d]	77/87	4
D11S938	212	212	212	212	212	212	212	212	212	212	212	58
D11S1792	269	269	269	269	269	269	269	269	269	269	269	76
D11S1327	250	250	250	250	250	250	250	250	250	250	250	64
D11S908	147	147	147	147	147	147	147	147	149	149	151	38
D11S939	247	247	247	247	247	241	241	241	241	249	247	45
D11S490	159	159	159	159	161	149	167	159	159	149	159	32
CD3D	89	89	89	89	89	85	93+91[b]	89/93[c]	89	nd[d]	89	27
D11S836	70	72	72	74	74	72	74+66[b]	74	74	74/70[c]	74	8

Families are ordered according to the extent of haplotype sharing with family FGT189. [a] Frequency of alleles which define the 'E/H-haplotype' in FGT189 (shaded) in control population; [b] A recombination event was observed in this family; [c] Phase unknown; [d] Not determined.

Genealogy of these 10 families was not able to link any of them to FGT189, suggesting that this mutation must at least be 200 years old. The absence of assimilation between families of Protestant or Roman Catholic faith even suggests that the common ancestor lived before the Reformation in the 16th century.

In the light of the age of this PGL1-mutation, it is remarkable that several families (FGT11, FGT18, FGT20) share a region of over 15 cM with the 'E/H-haplotype' of FGT189. In families FGT11, FGT25, and possibly FGT17, the haplotype conservation may even extend proximally to include marker D11S527, mapping to subband 11q13.5, although this might be coincidental given the frequency of the shared allele at this marker. The genetic distance between the haplotyped patients from families FGT11, FGT18, and FGT20, and from the branches E-H of family FGT189, must be at least 16 meioses. We have suggested a deficit of recombination-events involving the disease-linked haplotype in paraganglioma families (van Schothorst et al., 1996), but this lacks statistical support so far. In fact, such events must have occurred more recently in branches A and B of family FGT189, as these confine the haplotype sharing to 2 cM. Moreover, a more than two-fold excess of female versus male recombination has been reported for this region of chromosome 11 (Litt et al., 1995). Thus the

size of the conserved haplotype might partly be explained by the over-representation of male transmission in our families. Finally, strong haplotype conservation has been reported in other founder populations as well, and might not be uncommon (Peltonen and Uusitalo, 1997). A 10-cM haplotype has been reported to be conserved for 450 years in Finnish families with hereditary non-polyposis colorectal cancer (Nyström-Lahti et al., 1994). Nevertheless, the finding that genomic imprinting may

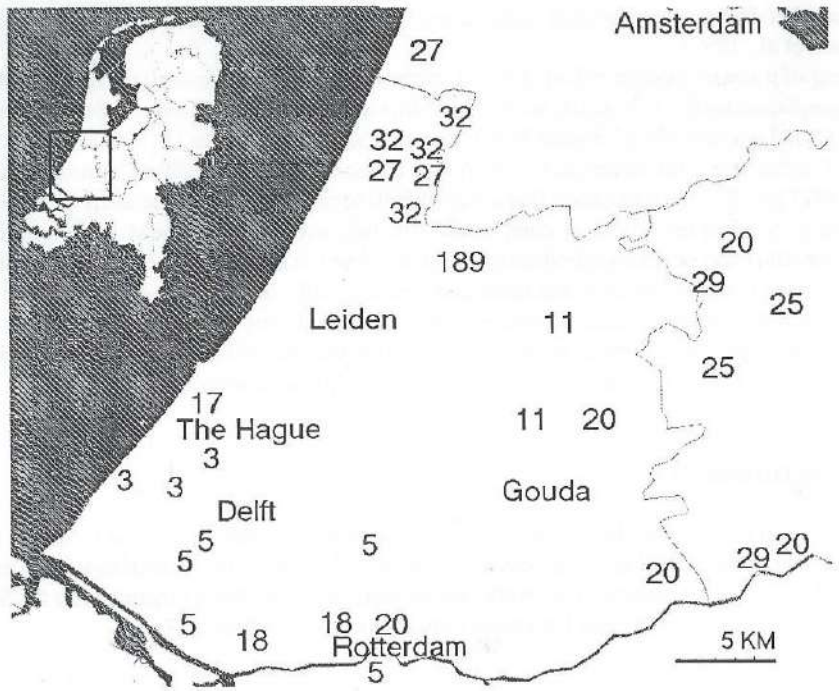


Figure 5.3. Map of the studied region. Numbers indicate the birthplaces of the oldest ancestors identified in corresponding HN-paragangliomas families. The ancestors from the maternal line are omitted. The inset shows a full map of the Netherlands with a square indicating the study region.

interfere with sex-specific recombination rates (Pàldi et al., 1995; Robinson and Lalande, 1995) makes it tempting to speculate that the mutation in PGL1 responsible for HN-paragangliomas also affects recombination rates in this region of chromosome 11.

The over-representation of paternal and grand-paternal transmission we have noted here might be explained by an ascertainment bias owing to the fact that HN-paragangliomas develop only after paternal transmission (van der Mey et al., 1989). Even though PGL1-carrying females would on average have the same chance of having affected grandchildren as PGL1-carrying males, their affected

grandchildren are less likely recognized as hereditary cases because they obtain the gene via their non-penetrant father. This would imply that an unknown proportion of allegedly sporadic paraganglioma patients are in fact hereditary.

Another interesting feature of this founder mutation in PGL1 is its apparent ability to predispose to pheochromocytomas (the paraganglioma of the adrenal gland). We detected 5 cases of this rare tumor in the 11 families studied here (Table 5.1 and (van Gils et al., 1992)), confirming earlier suggestions of an association with HN-paragangliomas (Sato et al., 1974; Bogdasarian et al., 1979). PGL1 might thus be another factor in the already heterogeneous genetic basis of familial pheochromocytomas (Woodward et al., 1997).

Our finding of a strong founder effect at PGL1 contrasts with haplotype analysis of North American HN-paraganglioma families, in which no obvious, large regions of allele sharing were apparent (Baysal et al., 1997a). Two families of distant Polish ancestry shared alleles for D11S938, D11S1792, and D11S1327 in that study, the latter two of which map to the 2-cM region defined by haplotype sharing in family FGT189. Yet the population frequency of 0.30 of this haplotype precluded an unambiguous conclusion of a common origin. A comparable situation exists for the 2-cM haplotype shared in FGT189, for which the population frequency was determined to be 0.18 (van Schothorst et al., 1996), but for which the common ancestor was identified genealogically. The founder effect reported here can now be exploited further for gene-mapping purposes, by analysing linkage disequilibrium across a closely spaced, highly polymorphic marker-map covering the shared 11q23 region and its immediate flanking regions, in more extended series of HN-paraganglioma patients of Dutch origin.

ACKNOWLEDGEMENTS

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6

STRONG LINKAGE DISEQUILIBRIUM BETWEEN 11q22-q23 MARKERS AND HEREDITARY HN-PARAGANGLIOMAS IN DUTCH PATIENTS REFINES THE LOCATION OF PGL1

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Paragangliomas of the head and neck are rare, slow growing, mostly benign tumours. The majority are believed to be due to inherited defects in PGL1, which has been assigned to a 6-cM interval on chromosome 11q22-q23 by recombinant analysis, and to a 2-cM interval by analysis of haplotype sharing among all patients of a large Dutch family. However, the population frequency of the linked haplotype was still relatively high so that chance sharing could not be fully excluded. More recently indeed, two new markers within this interval were found not to co-segregate with the disease in this family. Haplotype analysis of 25 families, of which 19 are of Dutch origin, with new markers from the 6-cM region resulted in an 8-marker haplotype co-segregating with the disease in the large family and in 10 additional families. This haplotype defines a 2-cM interval, between the markers D11S1986 and D11S897, and has a population frequency of <0.009%. Linkage-disequilibrium analysis underscores this region as the most likely candidate gene region for PGL1. Using a stepwise mutation model, a plausible common ancestry could be shown for most Dutch families, although distinct haplotypes and thus discordance from a general founder effect could be observed in some other families mainly originating outside the Netherlands.

Introduction

Paragangliomas of the head and neck (HN-paragangliomas) are also known as glomus tumours or chemodectomas. These tumours are slow growing, mostly benign tumours of neuroectodermal origin. This rare disease has an age of onset roughly between 18 and 60 with a penetrance at age 60 of around 95% (Heutink et al. 1992). A substantial proportion of all tumours is thought to be familial, showing an autosomal dominant inheritance pattern with phenotypic expression only after paternal transmission. This has been explained by genomic imprinting (van der Mey et al. 1989; McCaffrey et al. 1994).

The responsible gene, PGL1, has been located previously by recombinant analysis in a set of 6 families between the markers STMY and CD3D, separated by approximately 16 cM (Heutink et al. 1994). Further analysis of six North-American families and one French family positioned the PGL1 locus more accurately in a 6-cM region between the markers D11S1647 and D11S908 (Baysal et al. 1997a; van Schothorst et al. 1996). Genealogy studies enabled us to reconstruct a 7- generation pedigree, designated FGT189. In the absence of further recombinants, allele-sharing among all patients in this family at the markers D11S1792, D11S1327 and D11S908 defined a region of about 2 cM (van Schothorst et al. 1996) presumably containing PGL1. However, high population frequencies for the disease-linked alleles were observed, leaving the possibility that this sharing occurred by chance.

In the course of analyzing the PLZF-gene as a candidate gene for PGL1, we developed 2 new polymorphic markers (Baysal et al., 1997b; chapter 9, 10). We report here that these markers are recombinant in family FGT189, indicating that the previously observed haplotype sharing might in fact be a chance occurrence. We therefore initiated a search for new markers within the region as defined by recombinant analysis (D11S1647-D11S908). This resulted in the identification of an 8-marker haplotype, covering again a 2-cM interval located about 3 Mb proximal of PLZF. Linkage disequilibrium analysis of 25 families with a set of 14 microsatellite markers around the PGL1 region on 11q22-q23 suggested that most families are due to a founder effect, caused by a limited number of different ancestral mutations.

Materials and methods

Family ascertainment

Most families have been described earlier (van Schothorst et al.; chapters 3,4,5). Other families included in this analysis are an American family (FGT28), FGT30 (Belgium), FGT34 (India) and three Dutch families (FGT13, FGT41, and FGT42). These 6 families clearly present familial inheritance with multiple patients only after paternal transmission in several generations, in accordance with the genomic imprinting model (van der Mey et al. 1989, McCaffrey et al. 1994). DNA samples were only available from one affected individual in families FGT13 and FGT34, complicating the assignment of the disease-haplotype. Besides the three new families originating not from the Netherlands, families FGT21 (France), FGT26 (Canada), and FGT15 (Germany, Luxembourg) also originated abroad. DNA was available from at least two affected individuals per family (23/25=92%) and from at least two generations for unequivocal haplotyping (21/25=84%).

DNA isolation and PCR analysis

Genomic DNA was isolated from peripheral blood lymphocytes as described by Miller et al. (1988). Microsatellite markers were analyzed as described by Weber and May (1989). Markers were ordered as described elsewhere (van Schothorst et al. 1996, Baysal et al. 1997b, Genethon, CHCL), and allele-frequencies used are as described (van Schothorst et al. 1996). For the markers D11S1347, D11S3178 (Genethon), and D11S1987 (CHLC), amplification conditions are as described in GDB (http://gdbwww.gdb.org). For the new markers pDJ-CA, -TA, -TAT, -GT1, and -GT2, the annealing conditions are

given in Table 6.2; PCR conditions were not remarkable different from that used by Weber and May (1989); marker pDJ-TAT is amplified with the addition of DMSO in the PCR-mix (10%).

Linkage disequilibrium analysis

As a measure for linkage disequilibrium, P_{excess} values (also denoted as δ or λ; Devlin and Risch, 1995) per marker were calculated according to Hästbacka et al. (1992). Briefly, $P_{excess} = (P_D - P_N)/(1-P_N)$, in which P_D and P_N denote the frequency of the disease-associated allele on disease-bearing and normal chromosomes, respectively.

MST-program

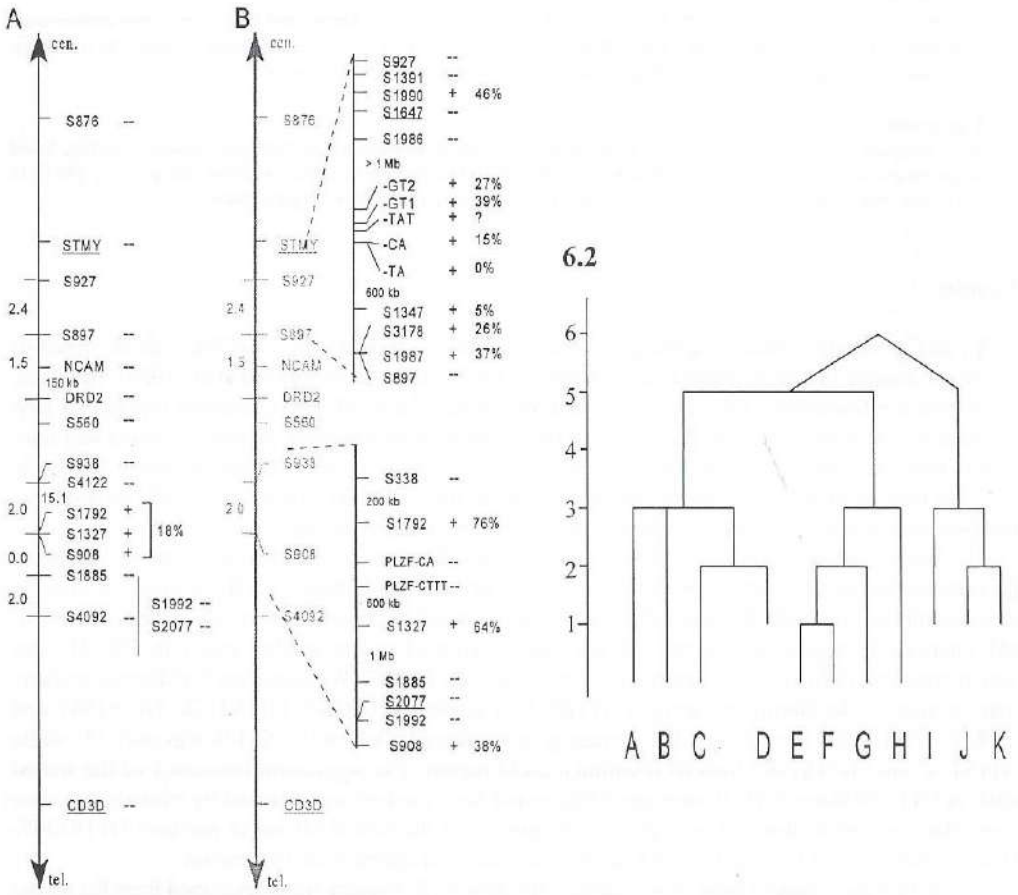
The MST-program (Minimal Spanning Tree) is used to determine possible relationships between different families, based on a stepwise mutation model using an Euclidean distance matrix (Excoffier et al. 1992; Excoffier and Smouse, 1994). In this way, each observed repeat-unit difference at a marker is thought to arose by a single mutation.

Results

PGL1 has previously been assigned to a 2-cM interval on chromosome 11q22-q23 on the basis of haplotype sharing in a large Dutch family (Figures 6.1A and 6.2; van Schothorst et al., 1996). However, the population frequency of this disease-linked haplotype is high (18%). Subsequent typing of 2 new markers for the candidate gene PLZF within this interval created a zebra-pattern of shared and non-shared markers, suggesting that the observed allele-sharing occurred by chance (Figure 6.1B, Table 6.1). We then resorted to (re-)investigate all markers in the 6-cM (D11S1647-D11S908) interval that had become available or more accurately positioned since our last report.

We haplotyped 25 families with up to 30 markers. Disease-linked haplotypes were reconstructed for each family parsimoniously by minimizing the number of recombinations. In all these families no new recombinant(s) in the disease-linked chromosome could be detected within the approximately 6-cM interval. This analysis located the previously reported recombination event in FGT21 (van Schothorst et al. 1996) more accurately, i.e., proximal to D11S2077. We identified 5 additional markers that showed allele sharing in family FGT189: D11S1990, D11S1347, D11S3178, D11S1987 and 5HT3R (Table 6.1). Intriguingly, the frequency of the shared allele at D11S3178 was only 5% while D11S1347 and D11S1987 flanked it within a 2-cM region. The population frequency of the shared allele at D11S1990 and 5HT3R were much higher and these markers were flanked by markers that were discordant in this analysis. The haplotype frequency of the linked alleles at markers D11S1347-D11S3178-D11S1987 was 0.58% and we therefore focused attention on this region.

Within proximity of these three markers, five new STR-markers were developed from the partial sequence of PAC pDJ15901. These five markers (pDJ-TA, pDJ-CA, pDJ-GT2, pDJ-GT1 and pDJ-TAT) are located within a 100 kb region (Baysal et al., submitted). Primer sequences and marker characteristics are given in Table 6.2. These five markers showed also complete allele-sharing within family FGT189 (Figure 6.1B, Table 6.1), thus extending the shared region to a total of 8 markers and decreasing the haplotype-frequency to at most 0.009%. In fact, the shared 341 bp allele at pDJ-TA has so far only been observed in patients, and not among 41 controls. Analysis of the other HN-paraganglioma families confirmed that the complete 6-cM region was shared in 11 founder-families (FGT 189, 3, 5, 11, 17, 18, 20, 25, 27, 29, 32; Table 6.3), but that also other families (FGT 14, 15, 22), previously not recognized as due to the same founder-effect, segregate the same haplotype. The single discordant marker D11S1347 in the haplotype of family FGT29 could reflect a mutation at the marker



Figures 6.1 (left) and 6.2 (right).

Figure 6.1. Marker-order for the PGL1 region on chromosome 11q22-q23; cen.=centromere, tel.=telomere. Distances are given in cM. Markers underlined are the borders as defined by recombinant analysis. **A**: Order and co-segregation of markers (+) in family FGT189 as previously published (van Schothorst et al., 1996). **B**: Markers are re-ordered on the basis of physical mapping (Baysal et al., 1997b; Arai et al., 1996; Baysal et al., in prep.). The five new markers located on PAC pDJ15901 have the preface - and are shown with flanking markers defining the new location of PGL1. Population frequency of co-segregating alleles are given next to markers.

Figure 6.2. A schematic representation of family FGT189 to define the different branches as published before (van Schothorst et al., 1996).

Table 6.1 Haplotyping of family FGT189.

Marker[1]	Disease haplotype in branch											Freq.[2]	Alleles[3]
	A	B	C	D	E	F	G	H	I	J	K	%	
*D11S876	6	6	6	6	4	4	4	4+1	7	7	7	6	11
D11S927	3/8	8	3	3	5	5	8	8	8	8	8	ND	11
D11S1391	8	8	2	2	6	6	8	8	8	8	8	7	8
D11S1990	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**46**	**5**
#D11S1647	3	3	1	.	.	3	.	3	.	.	.	ND	≥3
D11S1986	1	1	3	.	.	7	.	1	.	.	.	ND	≥7
pDJ15901-GT2	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**1/3**	**1/3**	**3**	**27**	**3**
pDJ15901-GT1	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**2/3**	**3**	**3**	**39**	**5**
pDJ15901-TAT	**6**	**6**	**6**	**.**	**.**	**6**	**.**	**6**	**.**	**.**	**.**	**ND**	**≥6**
pDJ15901-CA	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**15**	**7**
pDJ15901-TA	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**13**	**0**	**14**
D11S1347	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**5**	**8**
D11S3178	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**.**	**.**	**3**	**26**	**4**
D11S1987	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**37**	**5**
*D11S897	2	2	1	1	1	1	1	1	1	1	1	7	8
*NCAM	7	5	3	3	3	3	3	3	3	3	3	7	12
*DRD2	2	3	2	2	2	2	2	2	2	2	2	52	5
*D11S560	8	4	3	3	3	3	3	3	3	3	3	4	8
*D11S4122	7	7	5	5	5	5	5	5	5	5	5	10	7
5HT3R	**2**	**1/2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1/2**	**2**	**57**	**2**
*D11S938	**1**	2	3	3	3	3	3	3	3	3	3	58	6
***D11S1792**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**76**	**4**
PLZF-CA	9	9	6	6	6	6	6	6	6	6	6	ND	10
PLZF-CTTT	5	5	6	6	6	6	6	6	6	6	6	ND	9
***D11S1327**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**64**	**4**
*D11S1885	4	2	2	2	2	2	2	2	2	2	2	11	10
*D11S2077	2	2	1	1	1	1	1	1	1	1	1	ND	2
*D11S1992	2	3	3	3	3	3	3	3	3	3	3	ND	6
#*D11S908	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**38**	**6**
*D11S4092	4	4	5	5	3	3	3	3	5	3	3	46	6
*CD3D	2	4	1	1	4	4	4	4	4	4	4	27	6
Patients[4]	1	2	2	1	1	3	4	8	1	2	3		

[1]Markers are shown from centromere to 11q telomere. Markers in bold segregate in all branches of FGT189 (Figure 6.2), and markers preceded by an asterisk were shown previously (van Schothorst et al., 1996). # denotes the previous markers flanking the PGL1 locus (Baysal et al., 1997a; van Schothorst et al., 1996). A + indicates a recombinant, while a / indicates that the phase is unknown. [2]Freq. = Percentage of the most prominent linked disease-allele among 82 chromosomes marrying into the family. ND = Not determined. [3]Total number of different alleles within this family. [4]Number of patients in which the haplotype is found.

locus. Alternatively, it might define a new border of the candidate gene locus. At this moment, it is not possible to distinguish between these two possibilities, and further marker typings are required to resolve this. Hints for a more precise localization of PGL1 are provided by the fact that at least 20 of 25 families share alleles at D11S3178 and D11S1987. More localized sharing might also be apparent

around marker pDJ-TAT, but the complete set of families has not yet been typed for this particular marker. In families where only a few individuals, sometimes just one affected (e.g., in FGT13 and FGT34), could be typed, allele sharing was also confined to parts of the 6-cM founder-haplotype (Table 6.3); these families (including FGT30, FGT41 and FGT42) were not included in the linkage disequilibrium analysis and MST program.

Table 6.2. Primer sequences and fragments.

Markers:		size (bp)	# alleles	% het[1].	T_a (° C)
pDJ15901-TA	-f GGTAAATTTTATGTTATGTGTGTG				
	-r TGAGAGCAGTCTGGCCAATG	291-341	14	0.87	55
pDJ15901-TAT	-f AAACTCCTAGACTGAAGTGA				
	-r GATCACCAGGAAACACCTGAC	366-375	4	.	58
pDJ15901-GT2	-f GGTTGACTCTAGTAGCCATC				
	-r GATTTCTCAGGAAACTGGGC	357-369	4	0.77	58
pDJ15901-CA	-f GATCTTACATGTGGAAAACCCT				
	-r TTGTGTCCTGCCACTTTGCTG	104-114	7	0.86	55
pDJ15901-GT1	-f CCGAGTAATGAGGATTACAGG				
	-r ATCCTTTGGGAAGCTAAGGTAG	162-174	5	0.79	58

[1] Percentage heterozygotes as observed within at most 24 individuals marrying into the founder families.

Linkage disequilibrium analysis.

Linkage disequilibrium (LD) analysis for markers of this new candidate gene region, as well as markers from the previous candidate region, support the new region as shown in Figure 6.3. P_{excess} values were initially calculated using only haplotyped patients from the 20 families (FGT189-FGT15; maximum N=86), since within these families the disease-linked haplotype could be determined reliably. When we included also all imprinted carriers and non-penetrant disease-chromosome carriers (since they do not have any recombination in this large segment) from these families, the set contained 146 disease-linked chromosomes.

A maximum value for P_{excess} of 0.96 is observed at marker D11S1987, although this should be taken cautiously because not all individuals were typed for this marker. A high plateau-value can be observed within this region, including the maximum of 1.00 for 6 of the 7 markers when only the founder-families were analyzed. Comparison of the two possible PGL1 regions, around D11S1327/D11S1792 and pDJ-markers, showed that indeed the latter region provided more evidence for gene location since higher P_{excess} values are observed, supported by more markers and a much lower haplotype-frequency.

Analysis with the program MST to possibly group families on the basis of their shared disease haplotype of these 7 markers, as shown in Figure 6.4, indicate that most Dutch families seem to share

a common ancestor. The three Dutch families not sharing this haplotype (FGT4, FGT10 and FGT24) are very likely related to each other, and seem not to be related to the other founder families. All families originating outside the Netherlands (indicated by an asterisk) carry distinct disease-haplotypes not related to this particular Dutch haplotype. Thus, with the possible exception of FGT15, these families might be due to other mutations than those underlying the FGT189- and FGT4- related clusters.

Discussion

We report here the refinement of PGL1, the gene underlying hereditary HN-paragangliomas. The previously reported 2-cM interval on chromosome 11q22-q23 was based entirely on the haplotype-sharing in a large Dutch family (FGT189; van Schothorst et al., 1996). This haplotype had a high population frequency of 18%. We have now provided evidence to indicate that this sharing was

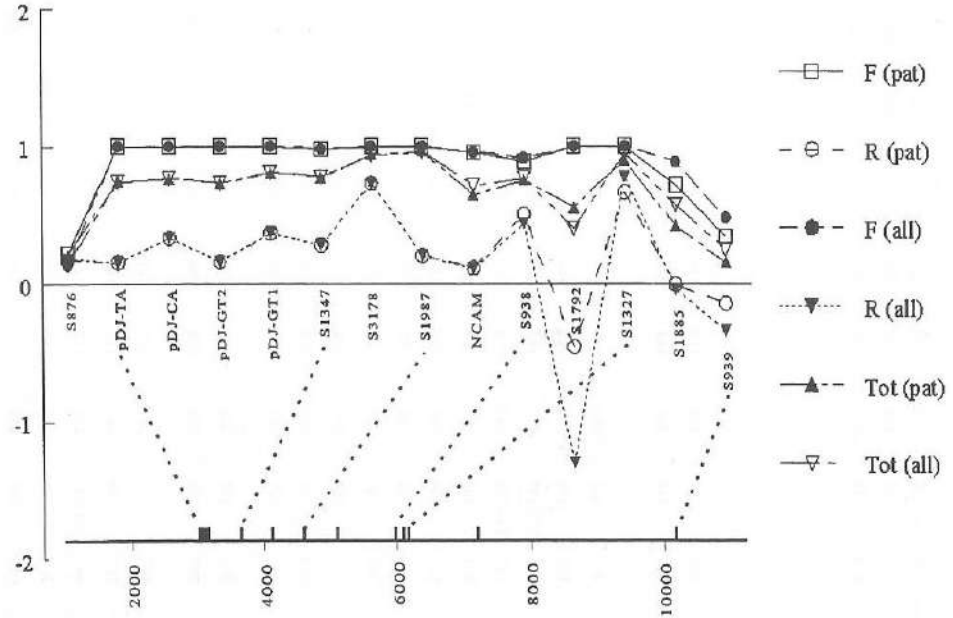


Figure 6.3. Linkage disequilibrium analysis. P_{excess} values are shown on the Y-axis, with the 14 different analysed markers on the X-axis. Below this figure are the approximate distances given between these markers (kb). F: all 11 founder families (see text); R other 10 families; tot: all 21 families; pat: only patients; all: patients and (imprinted) carriers.

Table 6.3 Haplotype analysis in FGT families (Page 1 of 2)

Marker	Family 189[1]	18	20	5	11	32	25	3	27	17	29	21*	4	10	24	22	14
D11S527	159	147	157	165	159	151	159	151+157	155/161	155/159	147/157	.	.	151/165	151	165	143/155
D11S927	135	135	135	135	135	135	135	135	137/141	135	135	.	149	145	143	135	.
D11S1793	134	134	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D11S1990	158	158	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D11S1647	250	250	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D11S1986	228	228	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
pDJ-CA	110	110	110	110	110	110	110	110	110/114	110	110	114	106/110	106	106	110	110
pDJ-TA	341	341	341	341	341	341	341	341	321/341	341	341	295	291	291	291	341	.
pDJ-TAT	375	375	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
pDJ-GT2	361	361	361	361	357/361	361	361	361	357/361	.	.	357	.	357	357	361	.
pDJ-GT1	162	162	162	162	162	162	162	162	162/164	162/164	162	164	162/168	168	168	162	162
D11S1347	197	197	197	178/197	197	197	197	178/197	197	197	199/201	178	195	195	195	197	197
D11S3178	247	247	247	247/253	247	247/249	247	247	247	247	247	247	247	247	247/249	247/249	247
D11S1987	201	201	201	201	201	201	201	201	197/201	201	201	201	201	201	197/201	201	197/201
NCAM	126	126	126	126	126	126	126	126	126	126	126	124	118	118	120	126	126
DRD2	82	82	82	82	82	82	82	82	82	82	82	82	82	84	78	84	82
D11S560	87	87	87	75/87	87	87	87	87	77/87	.	.	75	77	77	77	77	87
D11S4122	255	251/255	255	251/255	255	255	255	255	.	.	.	263	251	251	253	257	.
5HT3R D11S938	212	212	212	212	212	212	212	212	212	212	212	216	218	212	212	212	212
D11S1792 EST241777 PLZF CTTT	269	269	269	269	269	269	269	269	269	269	269	269	255	269	267/269	267/269	267/269
D11S1327 NNMT D11S2276E D11S1885	302	302	302	302	302	302	314	310	.	314	314/318	302	306	310+306'	306	306	306/314
	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250
	267	.	267	.	267	267	267	255	255	255	255	255	267	259	269	255/265	255
D11S908	147	147	147	149	147	147	147	147	151	147	149	147+143	147	143	145	147	149/147
D11S939	247	247	247	241/247	247	247	241	241	247	241	249	249+245	243	241	241	241/247	249
APOC3	349	349	349	315/336	352	349	339	.	.	.	.	.	301	307	346	336	325
D11S490	159	159	159	159	159	161	149	167	159	159	149	.	159	149	165	159	149/165
CD3D	89	89	89	89	89	89	85	91+93	89	89/93	.	89	95	87/93	89	89	93/95

Haplotype analysis in FGT families... continued

Marker	Family (189)	26'	28'	15'	13	41	42	30'	34'
D11S527	(159)	159	.	155	.	.	.	.	151/155
D11S927	(135)	139/149	.	147	.	153	.	.	137/143
D11S1793	(134)	120	.	122/136	.	.	.	.	.
D11S1990	(158)	152	.	158	.	.	.	.	.
D11S1647	(250)	250	.	250/266	.	.	.	.	.
D11S1986	(228)	208	.	224/228	.	.	.	.	.
pDJ-CA	(110)	110	106	110	106	106	110	.	106/114
pDJ-TA	(341)	331	291	341	291	295	341	.	313/325
pDJ-TAT	(375)	375	372	375	.	.	.	.	.
pDJ-GT2	(361)	361	357	361	.	.	.	.	.
pDJ-GT1	(162)	162	168	162	168	168	162/164	174	166/168
D11S1347	(197)	178	195	193	195	197	199	201	193/195
D11S3178	(247)	247	243	243/247	.	247	.	.	247
D11S1987	(201)	197	201	201	197/201	201	201	201	197/201
NCAM	(126)	120	124	124	122/126	.	120/126	118/124	.
DRD2	(82)	80	80	78	82/84	80	80/82	82/84	82/84
D11S560	(87)	77	.	79	75/77	77	75/77	75	.
D11S4122	(255)	253	.	257	.	.	.	.	.
5HT3R D11S938	(212)	212	212	212	212/216	212	212	212/216	218
D11S1792 EST241777 PLZF CTTT	(269)	273	269	267	.	271	269	269/275	269
	(302)	306	306/310	306	.	.	302	.	.
D11S1327 NNMT D11S2276E D11S1885	(250)	250	250	254	250	250	250	254	.
	(267)	255	255	255	265/269	.	255/269	255	.
D11S908	(147)	151	147	147	.	.	.	145/147	.
D11S939	(247)	241	247	243	243	.	241/247	241/247	241
APOC3	(349)	341	.	343	.	.	.	.	333
D11S490	(159)	165	.	159	.	.	.	.	104/112
CD3D	(89)	89	.	95	.	.	.	.	93

Legend Table 6.3.
Markers are displayed from centromere to telomere of 11q; markernames displayed in bold face are the shared markers in FGT189[1]. Underlined are some of the mapped genes/ESTs. Size of disease-allele in bp.
[1] This family is described by van Schothorst et al. (1996);/ phase unknown;+ a recombination occurred resulting in two different alleles segregating with the disease within this family ;' not from Dutch origin;' representing most likely a mutation at the markerlocus.

probably a chance observation, since additional markers in this interval were recombinant. In fact, an 8-marker haplotype of approximately 2 cM, located slightly proximal of the original allele-sharing region, was shown to be present in all patients of 12 families. The extremely low population frequency of this haplotype strongly suggests that this is the region containing PGL1.

We determined the disease-linked haplotype in 25 families with 30 different markers spread over a distance of at least 20 cM on chromosome 11q13-q23. Among 246 meiotic events, we detected 14 recombinants (5.7%) in the 6-cM culprit interval containing PGL1 in non-penetrant carriers or imprinted individuals. Although this corresponds well with the CEPH genetic map, it did not provide unambiguous further mapping of PGL1, as we could not detect a recombinant in any of the 75 proven affecteds. Thus, the suggestion that the under-representation of recombinants in HN-paraganglioma patients might reflect the effect of the PGL1-mutation (van Schothorst et al., 1996) is still open for debate.

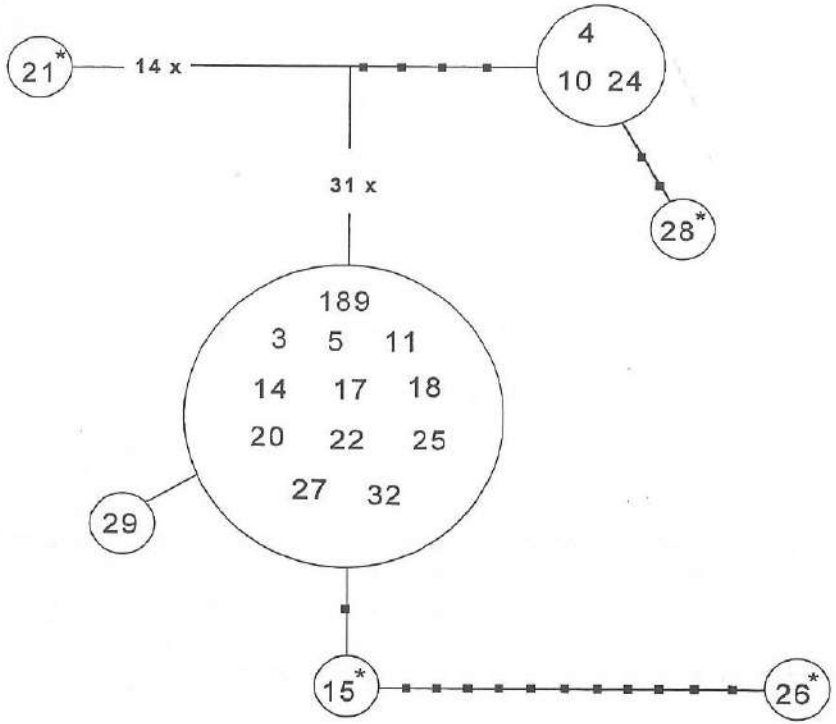


Figure 6.4. Minimal spanning tree on the basis of the markers pDJ-TA, -CA, -GT2, -GT1, D11S1347, D11S3178 and D11S1987 defining the PGL1 interval. Each mutation step is represented with a dot. For convenience, only the most likely haplotype for a family is chosen when the phase is unknown for a specific marker.

A second locus, PGL2, has been implicated by analysis of another Dutch family (FGT2), with its location on chromosome 11q13 (Mariman et al. 1995). Genetic heterogeneity analysis of 16 families, excluding FGT2, indicated that all other 16 families are linked to PGL1 (van Schothorst et al.; chapter 4). We therefore analyzed here only the PGL1-region on chromosome 11q22-q23, and all families - except one- display disease-linked haplotype sharing for this region. Family FGT2 did not show one unique disease-linked haplotype, supporting the exclusion of this region (data not shown).

Linkage disequilibrium analyses have been used to identify the position of different disease causing genes (Jorde, 1995; Ramsay et al., 1993; Goddard et al., 1996; Schleutker et al., 1995; Hästbacka et al., 1994) and can be very helpful in fine-scaling the region containing the gene of interest. Complete disequilibrium in the founder between the disease mutation and alleles at neighbouring linked markers is expected to dissipate through recombination over succeeding generations so that after sufficient time the disease mutation is associated with only the markers that are closest to it. Relatedness between different families (as measured as the number of generations ago they share a common ancestor) is thus inversely proportional to the size of the region in linkage disequilibrium. In the case of HN-paragangliomas, our data shows that the most likely location for the disease gene PGL1 is indeed between the markers D11S1986 and D11S897. Luria-Delbrück analysis of this data set, extended with a set of North-American families, might possibly further refine the position of PGL1 within this region.

Genealogy studies of a set HN-paraganglioma families sharing a haplotype, were unable to identify a common ancestor as far back as the beginning of 1800 (van Schothorst et al.; chapter 5). This suggests that this PGL1 mutation is at least 200 years old. Families originating from the central part of the Netherlands (FGT14), Germany/Luxembourg (FGT15) and South-West Holland (FGT22) can be added to this set of families on the basis of their concordant haplotype (Table 6.3). The sharing of some of these markers in other families might indicate that the mutation can in fact be much older, and might locate the PGL1 gene more precisely: FGT4, FGT10 and FGT24 share a haplotype for the pDJ-markers different from the other families, but an identical haplotype for the markers D11S1987 and D11S3178 with a haplotype frequency of approximately 10%. The assumption that all families are due to one ancestral founder mutation will have to be validated once the PGL1 gene and its mutation(s) have been identified. The P_{excess} values and the results from the MST-program could, however, indicate that at least some families (like FGT4, 10 and 24) might have another origin (haplotype) and/or mutation or that recombinations between the specific mutation and the flanking markers occurred.

So far, only a small number of diseases were shown to display a founder effect within the Dutch population: familial melanoma (FAMMM, CDKN2 gene; Gruis et al. 1995), Batten disease (CLN3 gene; Taschner et al. 1995), familial breast and ovarian cancer (BRCA1 gene; Peelen et al. 1997), fragile X (FRM1 gene; Buyle et al. 1993), variegate porphyria (VP, PPO gene; Meissner et al. 1996) and familial hypercholesterolemia (FH, LDLR gene; Defeche et al. 1993, 1996). The latter two were evident in an isolated subpopulation descending from a common Dutch founder settling in South-Africa (VP, FH) and in western Canada (FH). The Dutch population might thus show founder effects in many more familial diseases, comparable with that observed in Finland (Peltonen et al. 1995), but further haplotype analysis of disease-linked chromosomes and genealogy studies should support this. In the cases where the disease gene has not been cloned yet, linkage disequilibrium analysis in these populations might help to elucidate the precise location of the disease gene involved.

The confirmation of a founder effect will also facilitate genetic counselling on the basis of the

disease-linked haplotype. Families too small to show linkage on their own, e.g. FGT3, can now be referred to a clinical geneticist for counselling (Oosterwijk et al. 1996), and be offered presymptomatic testing on the basis of haplotype identity.

Acknowledgements

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7

ALLELOTYPE OF HEAD AND NECK PARAGANGLIOMAS: ALLELIC IMBALANCE IS CONFINED TO THE LONG ARM OF CHROMOSOME 11, THE SITE OF THE PREDISPOSING LOCUS PGL

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Paragangliomas of the head and neck region are usually slow growing, benign tumors. A considerable fraction has a positive family history, and the predisposing locus, PGL, has recently been assigned to 11q22-q23. The inheritance pattern of the disease suggests that PGL undergoes maternal genomic imprinting. We have investigated 26 tumor samples from 22 patients with head and neck paragangliomas for the occurrence of loss of heterozygosity (LOH) on all non-acrocentric autosome arms. LOH was found only on chromosome 11, with a marked clustering on the distal half of the q-arm. However, in many cases the resulting allelic imbalance relative to normal DNA was weak, suggesting that only part of the tumor showed this abnormality. In all eight cases where we were able to determine the parental origin, the allele undergoing loss was maternally derived. Clonality analysis with a polymorphic marker for the X-chromosome indicated that 2 out of 3 informative female cases were polyclonal, although a number of tumors carry aneuploid stemlines in DNA flow cytometry. We conclude that either tumor heterogeneity or polyclonality may explain the partial allele loss events seen in certain cases.

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INTRODUCTION

Paragangliomas of the head and neck region, also called glomus tumors, are slow growing and heavily vascularized tumors, which rarely metastasize. They originate from the tiny glomus bodies, and consist of two types of cells, called the sustentacular cells and chief cells (Heath, 1991). The disease usually becomes manifest before the age of 50, but rarely under the age of 18 (van Baars et al., 1982). The histological appearance of a glomus tumor resembles that of a normal glomus body structure, although it is believed that it does not represent hyperplasia, but true neoplasia, because of the presence in some tumors of confluent necrosis, vascular and lymphatic invasion, and, invariably, mitotic figures (Lack et al., 1979). The finding of aneuploid DNA stemlines in 37% of histologically and clinically benign paragangliomas (van der Mey et al., 1991) also supports the notion that these tumors represent true clonal proliferations. Nevertheless, the neoplastic nature of paragangliomas has been debated (Stiller et al., 1975).

The incidence of paragangliomas is estimated to be 1 in 20,000-40,000 (Lack et al., 1979; Zak & Lawson, 1982; van Baars et al., 1982), and up to 50% may be inherited (van der Mey et al., 1989; MIM number 168000). Familial cases are much more likely to be multicentric than sporadic cases. The inheritance pattern is autosomal dominant, but shows a remarkable non-Mendelian aspect. All affected persons inherit the gene from their father, while the disease is not expressed when transmitted by the female (van der Mey et al., 1989). This strongly suggests the as yet ill-understood phenomenon of genomic imprinting, in which gene expression is being influenced during germ-cell differentiation (Hall, 1990). We have recently mapped the predisposing locus, PGL, by linkage analysis in a large Dutch pedigree to 11q23-qter (Heutink et al., 1992).

In order to address the question of whether or not paragangliomas are true neoplasms, and to find out if PGL is subject to a two-hit inactivation mechanism (Cavenee et al., 1983; Knudson, 1989), we have investigated all chromosomes for the presence of loss of heterozygosity (LOH) in 26 tumor samples derived from 22 patients.

MATERIALS AND METHODS

Patient-subjects
All patients were referred to either of three Academic Hospitals (Leiden, Rotterdam, Utrecht) in the period 1988-1992. Surgically resected tumor tissue was snap-frozen in cold isopentane and stored at -70°C. A positive family history was recorded in 15 patients, resulting in 10 glomus tumor pedigrees. These patients have at least one first- or second degree relative with paragangliomas as ascertained by MRI, histology, or family interviewing (van der Mey et al., 1989; Heutink et al., 1992). Two of these patients had a phaeochromocytoma as a second malignancy, but these tissues were not included in this study. Three other patients had bilateral paragangliomas from different sites (i.e. caroticus and jugulare, caroticus and vagale, and one with two tumors of unknown origin). To determine the percentage of tumor cells in each specimen, a haematoxylin-eosin stained section was examined histologically. All chief and sustentacular cells were considered tumor cells.

DNA extraction
Frozen tumor tissue was sliced into 40µ sections in the cryostat, and genomic DNA was extracted essentially as described earlier (Devilee et al., 1991). DNA extracted from blood lymphocytes served as normal control.

Southern blot analysis
Methods for restriction enzyme digestion, gel-electrophoresis, capillary transfer onto Nylon filter-membranes, and radioactive probe-hybridization, have been described (Devilee et al., 1991). Many of the DNA-probes used detect multi-allelic variable number of tandem repeats (VNTR) loci and all have been published (Solomon and Rawlings, 1991). The probes used were: pYNZ2 (D1S57), pMUC10 (MUC1), pYNH24 (D2S44), p5-1-32 (D2S3), pBH302 (THRB), LIB40-37 (D3S617), pEFD64.1 (D3S42), pH30 (D4S139), pJO71H (D5S20), pJCZ30 (D6S37), pTHH5 (D6S39), pRMU7.4 (D7S370), pMETH (MET), pXV-2C (D7S23), p82B (D8S2), pHNFL (NEFL), pMCT128.2 (D8S39), pEFD126.3 (D9S7), p7A9 (D10S24), pEFD75 (D10S25), pEJ6.6 (HRAS), SS6 (INT2), L7 (D11S29), pTHH14 (D12S16), pDL32B (D12S7), p68RS2.0 (RB1), pCMM101 (D14S13), pCMW1 (D15S24), 3'HVR (D16S85), p79-2-23 (D16S7), p144D6 (D17S34), pCMM86 (D17S74), B74 (D18S3), OLVII-E10 (D18S8), OLVII-A8 (D18S7), pERT25 (D18S11), pLDLR-2HH1 (LDLR), pHW60 (D19S13), NJ3.6 (APOC2), pCMM6 (D20S19), 22Cl-18 (D22S10).

PCR analysis
We have used the polymerase chain reaction (PCR) to detect polymorphisms at microsatellite loci. These loci consist of a repeated stretch of 2 to 4 nucleotides, which may carry up to 20 different alleles (Hearne et al., 1992). The primers used to amplify these markers are all available on line from GDB or Généthon (Cohen et al., 1993). The standard conditions for the PCR was 27 cycles consisting of 1 minute at 94°C, 2 minutes at 55°C, and 1 minute at 72°C, in the presence of ^{32}P-dCTP. The markers used were Mfd36 (D2S72), Mfd17 (D3S196), cBJ56 (D4S127), Mfd154 (D5S211), Mfd61 (D6S105), Mfd141 (D9S54), Mfd164 (D10S111), cCI11-411 (D11S560), c1,16 (D11S490), JG4 (D11S527), c13,1 (D11S528), Mfd231 (D11S897), AFM022te1 (D11S898), AFM267yh5 (D11S939), TP53, Mfd120 (D19S177), Mfd25 (D20S27), Mfd95 (D21S171), Mfd51 (D22S257). The order of chromosome 11 markers was derived from the NIH-CEPH Collaborative Mapping Group (1992), supplemented with data from the Généthon group (Weissenbach et al., 1992).

Assessment of allelic imbalance
Autoradiograms obtained by Southern analysis were scanned by soft-laser densitometry and band-intensities semi-quantitatively determined (Devilee et al., 1991). Gels containing PCR-products were exposed overnight to a PhosphorImaging screen (Molecular Dynamics), and the number of counts per band were quantified using the linear PhosphorImaging system. The ratio of allele intensities were determined in the control and tumor lane (from blood and tumor DNA, resp.). We have previously defined the allelic imbalance factor (AIF) in the tumor as the ratio between these two ratios (Devilee et al., 1991). This factor thus expresses the extent with which the allele intensities in the tumor lane differ from those in the control lane. Notably, we found the AIF to be relatively insensitive to the presence of the so-called stutter or shadow-bands of the alleles, a well known by-product of the amplification reaction (Hearne et al., 1992), as long as care was taken to in- or exclude these bands in an identical way for both alleles. For Southern analysis, an empirically determined cut-off AIF of 1.30 was taken as an indication for the presence of allelic imbalance (Devilee et al., 1991). For polymorphisms detected by PCR, this cut-off was determined to be 1.5 (see text for details). All AIFs of chromosome 11-markers, and those >1.5 of non 11-markers, were determined at least twice in independent experiments, and the final AIF was the average of all determinations.

DNA flow cytometry (FCM)
Procedures for measurement of cellular DNA content of the tumor samples have been described earlier (van der Mey et al., 1991).

RESULTS

The tumor characteristics of the patients are listed in Table 7.1. Fourteen of the 22 patients have a positive family history, and 12/26 tumor samples showed evidence for the presence of a DNA-aneuploid peak in DNA FCM in addition to a DNA-diploid peak. Both observations are in agreement with earlier observations (van der Mey et al., 1989, 1991). It must be noted that the aneuploid population in a number of tumors represented only a small subpopulation, despite the fact that they

contained >70% chief and sustentacular cells as judged by histology (e.g., tumors 117, 170, 173). In an initial whole-genome search for allele loss, we used Southern analysis and VNTR-markers to screen all but 6 autosomal chromosome arms in 26 tumor specimens. This analysis revealed no significant changes in allele intensities between normal and tumor DNA for all markers not mapping

TABLE 7.1: PARAGANGLIOMAS INVESTIGATED IN THIS STUDY

Patient Number	*Family History*[1]	*% tumor cells*[2]	*DNA-indices of observed $G_{0,1}$ populations*
002	P	80-90	1.00
040	P	25	1.00
042	P	70	1.00
110		90-95	1.00
111-right	P	80-95	1.00[4]
111-left	P	40-80	1.00
114	P	90-95	1.00[4]
115	P	60-90	1.00
117	P	80-95	1.00 + 1.30[3]
118	P	60-90	1.00
121-caroticus	P	90	1.00 + 1.66
121-jugulare	P	80-90	1.00
122	P	90	0.90 + 1.79
125-caroticus	P	80	1.00 + 1.74
125-vagale	P	90	1.00 + 1.85
133		90	1.00
143		90-100	1.00 + 1.50 + 1.80
144	P	90	1.00
147	P	70	1.00 + 1.14
148		70	1.00
170		90	1.00 + 1.46[3] + 1.95[3]
172(1)		80	1.00 + 1.78
172(4)		60	1.00 + 1.86[3] + 1.94[3]
173	P	80	1.00 + 1.86[3]
174		80	1.00
2.11	P	N.D.	1.00

[1]P = positive; blank = negative or unknown; [2]Chief cells + sustentacular cells; N.D. = not determined; [3]Found in small subpopulation (<20% of the cells); [4]High G_2+M-fraction comprising approximately 20% of the cells.

to chromosome 11 (data not shown, see Materials & Methods for markers used). At least 30% of the cases were constitutionally heterozygous (i.e. informative for this analysis) at the majority of investigated loci (average: 55%; 19/33 arms were informative in ≥50% of the cases). In three tumors, a weak decrease in the intensity of one of the alleles was noted with all informative markers for chromosome 11 (data not shown).

To further investigate this specificity of chromosome involvement, and to further study those sites that

had not been part of this screening by Southern analysis (i.e., 4p, 5q, 6p, 9p, 20p, and 21q), or had not been sufficiently informative with the probes used (i.e., 2q, 3q, 10p, 19p, and 22q), we employed a number of highly informative microsatellite markers mapping to these chromosomes as well as to chromosome 11. Upon visual examination of the resulting autoradiograms again a weak imbalance in allele ratio in the tumor *versus* normal control was frequently observed with markers mapping to the long arm of chromosome 11, whereas this was rarely noted with markers mapping outside chromosome 11. An example of such an analysis is shown in Figure 7.1.

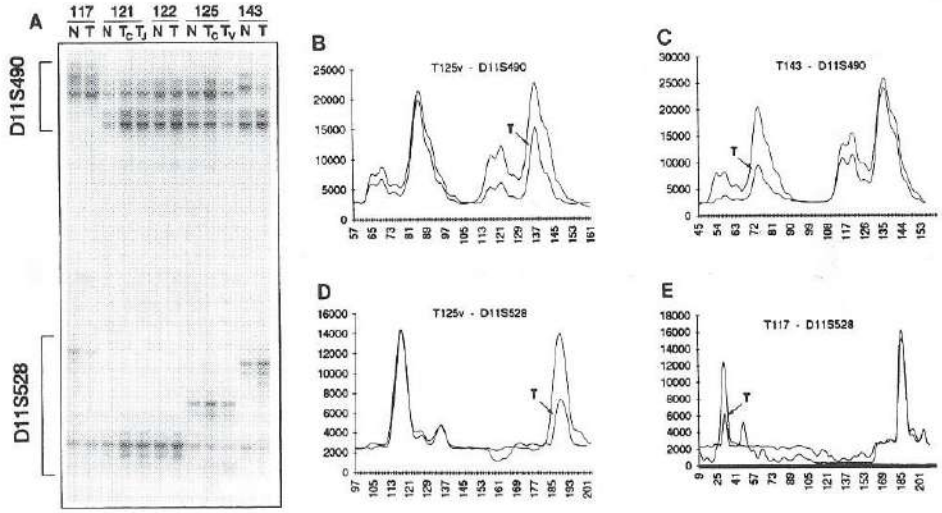

Figure 7.1. Allelic imbalance measured at microsatellite loci on 11q. (A) Multiplex PCR detecting D11S490 and D11S528 simultaneously. N = normal white blood cells DNA, T = tumor DNA, T_v = vagal tumor, T_c = caroticus tumor, T_j = jugulare tumor. Case numbers are above each pair of lanes. (B-E) PhosphorImaging analysis of same gel-result. T = tumor lane results. X-axis: scan position top-bottom, Y-axis: absolute number of ^{32}P-counts.

Inspection by naked eye reveals that the upper allele at D11S490 and the lower allele at D11S528 in tumor 143 are dramatically decreased in intensity in the tumor relative to the control lane, which is verified by LPI-analysis (panel B). For tumors 117 and 125, however, these differences are less explicit visually, but they nevertheless clearly and reproducibly bear out by LPI (panels A, C, D). Note that both tumors were assessed to contain >80% tumor cells histologically (Table 7.1). Because of difficulties in controlling exactly the amount of input-DNA in the PCR, it is impossible to distinguish between the two possible explanations for the observed intensity-differences, i.e. the loss of the upper allele or the duplication of the lower allele or *vice versa*. We therefore lump any of these events under the term allelic imbalance (AI) (Devilee et al., 1991). Because most imbalances were often weak, and to exclude

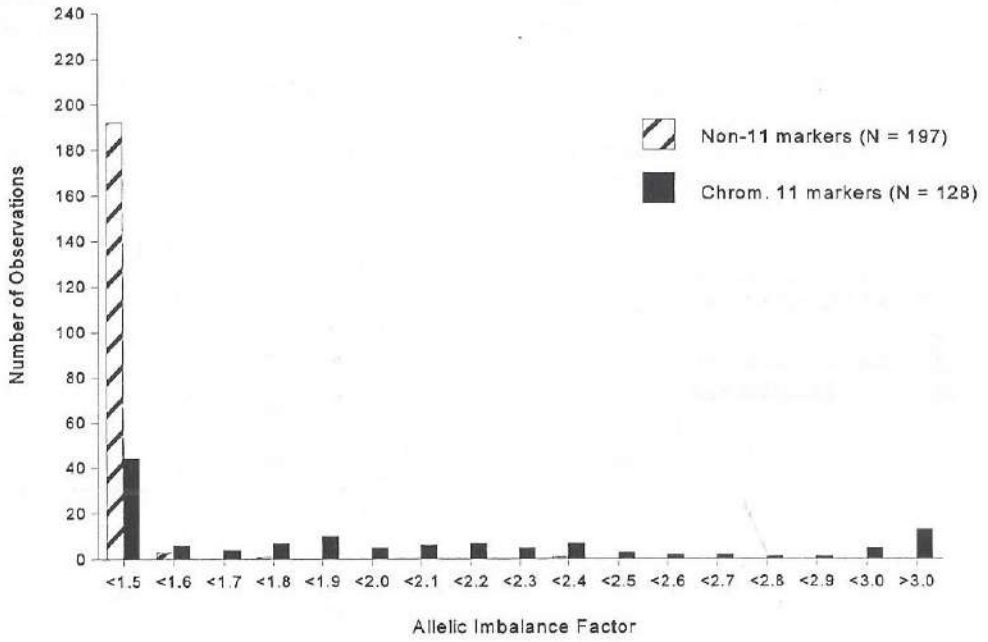

Figure 7.2. Histogram of allelic imbalance factors obtained with chromosome 11 markers and non-11 markers. One observation for chromosome 11 markers is the average of at least two independent experiments. For non-11 markers, only AIFs >1.5 were reproduced, averaged, and counted as a single observation. All others were measured only once.

the possibility that they were in fact PCR-artefacts, all AIFs >1.5 were confirmed at least once independently.

In addition, the extent of the imbalances were quantified using linear phosphor-imaging (LPI). When all AIFs thus obtained are plotted against the number of observations, distinguishing chromosome 11q-markers from non-11q-markers (Figure 7.2), it is obvious that AIFs >1.5 are almost exclusively found with markers for 11q. Since in total 13 chromosome arms were investigated with markers typed by PCR, partially overlapping the set of chromosomes studied by Southern analysis, which also revealed a complete absence of AI at non-chromosome 11-loci, this strongly suggests that AI in head and neck paragangliomas occurs specifically on chromosome 11. It is of interest to note that 11q contains the gene predisposing for this tumor (Heutink et al., 1992).On the basis of the results presented in Figure 7.2, we decided to use a cut-off AIF of 1.5, above which we assumed the presence of AI in the tumor. Employing 7 markers for 11q, we found that 21/26 tumor samples (81%) had AIFs >1.5 with at least one of these 7 markers. All tumor samples were tested with all 7 markers and all were informative with at least two. There was no correlation with DNA ploidy as both tumors with a DNA-index of 1.00 (diploid), and those with a DNA-index other than 1.00 (aneuploid) showed AI. Notably, in all 8 familial cases where we were able to determine the parental origin of the alleles, the imbalance event could be

explained either by the loss of the maternal copy or duplication of the paternal copy (Figure 7.3).To define a smallest common region affected by AI on 11q in glomus tumors, we compared AI-patterns in the different tumors (Figure 7.4). Of the 11 tumors informative for this analysis, 8 showed "zebra"-patterns, i.e. alternating regions of retention and imbalance of allele intensities (e.g., tumor 144). Other tumors carried an interstitial region of imbalance between two markers, e.g., between D11S897 and D11S939 (tumor 118). It should be noted, however, that many AIFs were in between 1.5 and 2.0. Given that markers outside chromosome 11 also sometimes show an AIF>1.5 (Figure 7.2), the relevance of these values is not always clear, particularly when it concerns a single observation in a tumor where all other flanking markers show AIFs ≤1.50 (tumors 110, 111_{right}, 118).

In any case, the results did not pinpoint a single common region of AI among the tumors studied here, although all imbalance events do seem to affect the PGL gene region distal to D11S560 (case 110 is not informative in this region).

One possible explanation for the presence of weak imbalances in parental copies of 11q

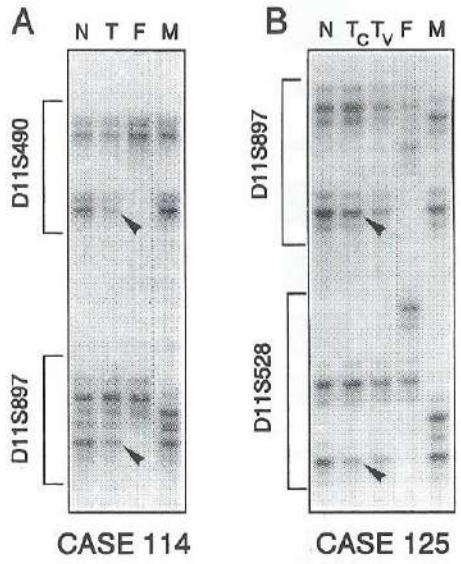

Figure 7.3. Determination of parental origin of alleles involved in AI. **(A)** Case 114. **(B)** Case 125. N = normal white blood cells DNA, T = tumor DNA, T_v = vagal tumor, T_c = caroticus tumor, F = normal DNA from father, M = normal DNA from mother. The arrows indicate the allele undergoing loss in the tumor.

could be that the tumors are not clonally derived. We therefore used the X-inactivation assay (Hendriks et al., 1992) to determine the clonality of 5 female-derived tumors in our series. Three patients were informative for this analysis with probe M27β (DXS255), and in tumors 110 and 133 the band-intensities in the *Hpa*II-restriction patterns from blood and tumor DNA were virtually identical (Figure 7.5A), suggesting these tumors had an oligoclonal origin.

In case 143, however, allele intensities in the *Hpa*II-digest of the tumor clearly differed from that of blood lymphocytes (Figure 7.5B), suggesting that in this tumor a significant clonal population existed. Interestingly, tumors 110 and 133 are diploid, while tumor 143 shows the presence of aneuploid peaks in DNA flow cytometry (Table 7.1). In addition, tumor 133 shows no AI on 11q (not shown), while tumor 143 does (Figure 7.1A).

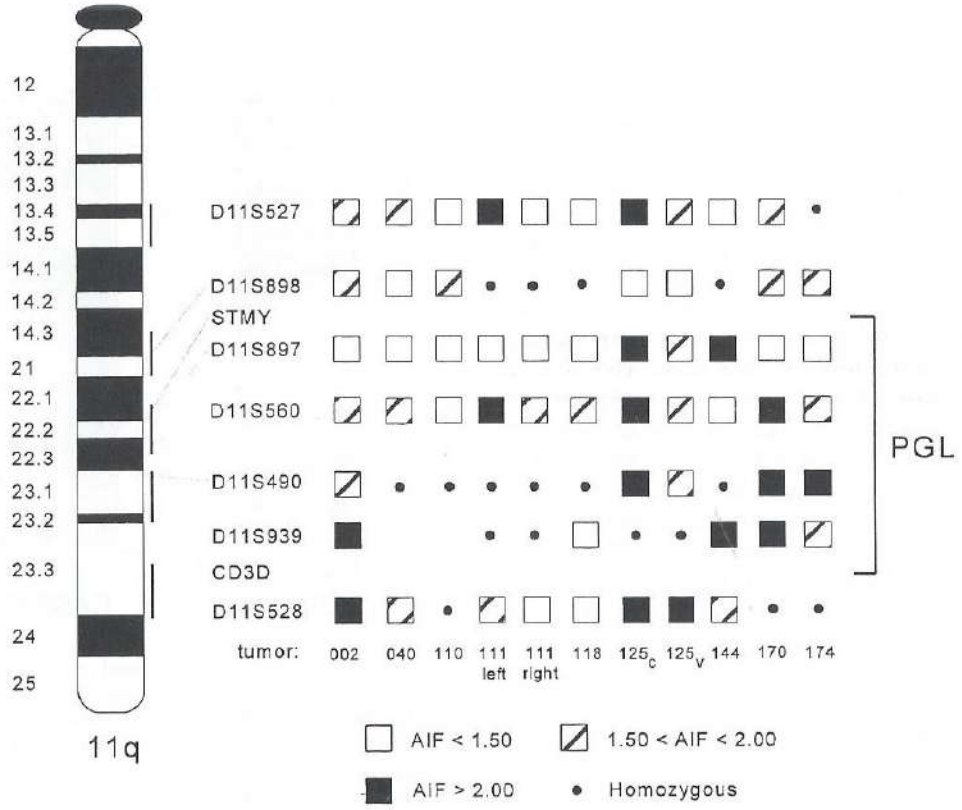

Figure 7.4. Map of allelic imbalance factors in 11 paragangliomas showing alternating regions of retention and allelic imbalance. Case numbers are below each column of results. PGL marks the region defined by meiotic recombinants in pedigree-analysis of linked families (Heutink et al., 1994).

DISCUSSION

We have presented a complete allelotype study of 26 head and neck paragangliomas, and show a frequent and highly specific parental imbalance in copies of chromosome 11. This study is the first to identify somatic genetic changes at the chromosome level in this type of tumor. No cytogenetic data by classical chromosome banding procedures yet exists, probably because of difficulties in culturing the tumor cells and obtaining metaphase spreads, and the extremely low incidence of this tumor in the population.

The rationale for this study was to investigate the genetic mechanism operating on the predisposing

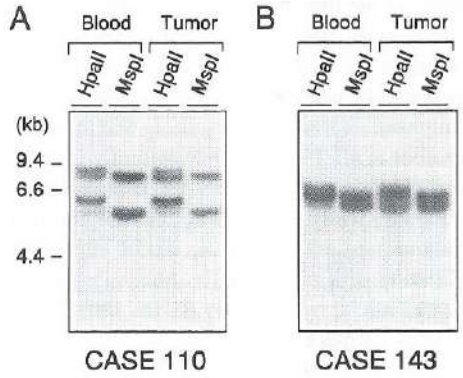

Figure 7.5 X-inactivation assay using M27β as probe in Southern analysis. Restriction enzymes are as indicated. Note the decrease in intensity of the middle band of the triplet in the *Hpa*II-lane of tumor 143 relative to that of blood DNA.

PGL gene on 11q, given the strong indication that PGL is subject to genomic imprinting. We have mapped PGL by linkage analysis in a large Dutch pedigree to 11q23-qter (Heutink et al., 1992). More recently, this assignment was further refined in additional families, placing PGL between STMY and CD3D (Heutink et al., 1994; Figure 7.4). A possible second locus has been detected in a single other family, and assigned just proximally of this region to 11q12-q13 (Mariman et al., 1993).

While the finding that 81% of paragangliomas show allelic imbalance specifically on 11q is suggestive, several other observations complicate the conclusion that Knudson's two-hit theory (Knudson, 1989) operates on PGL. This theory explains the coexistence of sporadic and inherited forms of a tumor by assuming that two inactivating mutations are needed to fully silence the activity of both alleles of a tumor suppressor gene. While the first event may be inherited, the second event is usually a somatic chromosomal mechanism leading to loss of heterozygosity (Cavenee et al., 1983). Complicating the application of this model to PGL are the inheritance pattern of paragangliomas, which strongly suggests that PGL undergoes genomic imprinting (van der Mey et al., 1989), and the finding that allele losses are weaker than expected on the basis of the percentage of tumor cells in the specimen.

Genomic imprinting is still mainly an operational definition thought to apply to all genes who undergo parent-of-origin specific modification of their expression (Hall, 1990; Sapienza, 1991). Much evidence for this phenomenon has been collected from experiments with transgenic mice (e.g., DeChiara et al., 1991), but a number of human genes are also known to display allele-specific expression (Feinberg, 1993). There are strong indications that DNA methylation may be involved in genomic imprinting either as the imprint itself or as a consequence of it (Surani, 1993). Clinically, genomic imprinting may become apparent when it affects disease genes to cause parent-of-origin specific effects such as juvenile onset or decreased penetrance of the phenotype (Hall, 1990). The latter was found for familial paragangliomas, where all affected persons inherit the gene from their father, while the disease is not expressed when transmitted by the mother (van der Mey et al., 1989). In further support of the notion that PGL is imprinted is our finding that familial tumors always show either loss of the maternal allele or duplication of the paternal allele.

of the maternal allele or duplication of the paternal allele.
Assuming that PGL is expressed mono-allelically, two simple models explaining its inheritance pattern emerge. In the first, a dominantly acting mutation in an oncogene-like gene causes tumor growth (van der Mey et al., 1989). In that case, when the gene carrying the mutation is passed through the male germline it becomes activated and the offspring expresses the mutant phenotype. When the mutation is maternally inherited, it is imprinted and not expressed. Alternatively, the inheritance pattern is equally well explained by assuming that the mutation causes the inactivation of a tumor suppressor gene (Hulsebos et al., 1990). When paternally inherited, the inactive allele is then paired with an imprinted maternal allele, resulting in a null mutant at this locus (DeChiara et al., 1991).

Paradoxically, the finding that the chromosomal site where PGL is located often shows allelic imbalance argues against the tumor suppressor gene model (Feinberg, 1993). It is not clear how duplication of a mutationally inactivated paternal allele, or the loss of the imprinted, i.e. inactive, maternal allele, would provide the critical impetus towards tumor growth. Furthermore, if every individual carries at birth only a single active allele at PGL in all somatic cells, only a single mutation would suffice to give rise to a tumor. This seems difficult to reconcile with the low incidence of sporadic disease in the population, even though the number of at risk cells, residing in the glomus bodies of the head and neck region, may be very low. One possibility is that the imprint on PGL is lost during the development of the paraganglia in late childhood. A loss of imprint has recently been demonstrated for IGF2 in Wilms' tumors (Ogawa et al., 1993; Rainier et al., 1993). It should be noted that deletions or loss of heterozygosity in the 11q22-q23 region have been observed in a number of malignancies, including those of haematological origin (Kobayashi et al., 1993), melanoma (Tomlinson et al., 1993), neuroblastoma (Srivatsan et al., 1993), and colorectal cancer (Keldysh et al., 1993). It is of course not known whether these events are targeting PGL or another tumor suppressor gene located close to PGL.

If, on the other hand, mutant PGL encodes an oncogene-like or growth promoting product, it could be that a dosage or threshold-effect demands an increase in PGL-copy number in order for it to be effective in eliciting tumor growth (Feinberg, 1993). For sporadic cases only mutations in the active paternal copy will be effective, and two genetic events will be required for tumor development to occur, explaining their low population incidence. One genetic mechanism leading to paternal allele duplication is somatic recombination (Cavenee et al., 1983). This mechanism causes the loss of the maternal allele, which in this case would be of no functional significance. An example of a gene in which a dominant mutation gives rise to inherited cancer is the RET oncogene, which has been found to cause the multiple endocrine neoplasia type 2A syndrome and familial medullary thyroid carcinoma (Mulligan et al., 1994). It is of interest to note that many genes shown to undergo genomic imprinting are associated with growth abnormality syndromes (Moore and Haig, 1991), and a number encode growth factors or growth factor receptors. Since paraganglioma could be viewed as an overgrowth disorder, this parallel might be extrapolated to PGL as well, to support the view that (mutant) PGL encodes a growth promoting factor.

Another level of complexity in the understanding of the development of paragangliomas is added by our finding that many allelic imbalances are weaker than expected, and that a substantial number of paragangliomas might be oligoclonal by X-inactivation analysis. On the other hand, the aneuploid peaks in flow cytometry in about one third of the tumors suggest the presence of populations of cells with a monoclonal origin. Finally, it is difficult to see how a hypotetraploid DNA-index would be

caused entirely by a (weak) imbalance exclusively in chromosome 11 copy number, which would be suggested by our finding of chromosome specificity.

Paragangliomas of the head and neck are biphasic tumors (Lack et al., 1979; Heath, 1991), and the paradox between DNA flow cytometry outcome and clonality assay might thus be explained by assuming that only one type of cell, i.e. the chief or the sustentacular cell, is actually responsible for the monoclonal increase in tumor mass, while the other multiplies polyclonally. This would explain the detection of minor aneuploid populations (monoclonal) in addition to a major diploid population (polyclonal) in a number of cases (Table 7.1), as well as the observed weak allelic imbalances. Specific subpopulations in the tumor mass could thus be carrying the detected genetic changes. Our results with tumors 110, 133 (both diploid, no AI at PGL), and 143 (showing aneuploid peaks and AI at PGL) suggest, but do not prove, that AI at PGL is an important mechanistic step towards monoclonal growth within the proliferating tumor mass. Further analysis of allelic imbalance and clonality in flow-sorted aneuploid nuclei might resolve this question.

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8

PARAGANGLIOMAS OF THE HEAD AND NECK REGION SHOW COMPLETE LOSS OF HETEROZYGOSITY AT 11q22-q23 IN CHIEF CELLS AND THE FLOW-SORTED DNA ANEUPLOID FRACTION

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Non-chromaffin paragangliomas of the head and neck region, also known as glomus tumours, are usually benign neoplasms consisting of clusters of chief cells surrounded by sustentacular cells arranged in so called '*Zellballen*'. These tumours are in at least 50% of the cases familial in origin. In a previous study, examining all chromosome arms, we found LOH predominantly at the chromosome 11q22-q23 region, where the disease causing gene PGL1 has been located by linkage analysis. However, all tumours showed only partial loss of allele signal-intensities and it was not clear whether this represented allelic imbalance or cellular heterogeneity. In the present study, we have performed LOH analysis for the 11q22-q23 region on DNA-aneuploid tumour cells, enriched by flow sorting, and on purified chief cell fractions obtained by single-cell microdissection. Complete LOH was found for two markers (D11S560 and CD3D) in the flow sorted aneuploid fractions, whereas no LOH was found in the diploid fractions of three tumours. The microdissected chief cells from two of these tumours also showed complete LOH for both markers, indicating that the chief cells are clonal proliferated tumour cells. These results indicate that the PGL1 gene is likely to be a tumour suppressor gene, that is inactivated according to the two-hit model of Knudson. Furthermore, it shows that chief cells are a major, if not the only, neoplastic component of paragangliomas.

Human Pathology (in press)
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INTRODUCTION

Paragangliomas of the head and neck region (hereafter called HN-paragangliomas) are rare, usually benign tumours (Lack et al., 1979). They consist of large, oval-shaped cells or chief cells (type I cells) of neuroectodermal origin. These cells are arranged in clusters surrounded by flat, elongated satellite or sustentacular cells (type II cells) in so called *'Zellballen'*. In about 1% of the paragangliomas, the chief cells contain neurosecretory granules that can release catecholamines. Sustentacular cells lack these granules and neither secrete nor contain catecholamines (Glenner and Grimley, 1974).

HN-paragangliomas often present as a familial disease (MIM 168000). An estimation for the proportion of familial cases is at least 50% (van der Mey et al., 1989), but this could be an underestimation due to the skipping of generations after maternal transmission. The observed inheritance pattern is autosomal dominant with genomic imprinting: only after paternal transmission do tumours arise (van der Mey et al., 1989; Heutink et al., 1992; McCaffrey et al., 1994; van Schothorst et al., 1996; Baysal et al., 1997a). Multicentricity, either uni- or bilateral, is an important feature of hereditary paragangliomas (van Gils et al., 1990b). The PGL1 gene causing the disease was assigned by linkage analysis in a few large pedigrees to a small area on chromosome 11q22-q23 (Heutink et al., 1994; van Schothorst et al., 1996; Baysal et al., 1997a). In a previous comprehensive allelotype study (Devilee et al., 1994), this region also showed almost exclusive loss of heterozygosity (LOH). The gene region distal to marker D11S560 seemed to be affected in all informative tumours. Non-random LOH is generally interpreted as representing the second hit inactivating a tumour suppressor gene (Knudson, 1989). However, in the case of HN-paragangliomas, such a model is complicated by the genomic imprinting hypothesis based on the typical hereditary transmission pattern (Devilee et al., 1994). In addition, since most tumours showed only partial loss for all tested 11q-markers, it was not clear whether this represented mere allelic imbalance, rather than loss, in both sustentacular and chief cell populations, or whether one cell type showed complete loss of an allele while the other did not. In the first case, PGL1 would not likely be a tumour suppressor gene, and both types of cells would be neoplastic constituents of these paragangliomas. The last case would be consistent with PGL1 being a tumour suppressor gene, and either one of the cell types being the major neoplastic component.

To further investigate these possibilities, we have purified tumour cell fractions from three tumours by flow sorting of DNA-aneuploid cells. In addition, from two of these, chief cells were isolated by single-cell microdissection. Concordant results were obtained for the flow-sorted, as well as microdissected cell fractions from the same tumours in which we were able to demonstrate complete loss of one allele of two different microsatellite markers in the 11q22-q23 region in all cases.

MATERIAL AND METHODS

Tumour tissues
Fresh-frozen tumour material was obtained from the Department of Surgery at the Academic Hospital Utrecht, The Netherlands. Part of this material was fixed in formalin and embedded in paraffin. For this study, we selected two patients with carotid body tumours (T143 and T172) which were previously shown to contain DNA-aneuploid populations (DNA-index of 1.80 and 1.78, respectively) by DNA flow cytometry and which contained a high, microscopically estimated percentage (>80%) of tumour cells (Devilee et al., 1994). The family history of these two patients was not available. Case T143 was operated at age 34, and case T172 at age 20. A third patient (VB) was operated at age 36 for a carotid body tumour, which consisted of >90% tumour cells and an aneuploid fraction with a DNA-index of 1.93. A second carotid body tumour in the opposite side of the neck was detected in this patient about 4 months after surgery. Case VB has a positive family history for HN-paragangliomas. In neither of the three cases selected here did the tumour metastasize to distant organs. The histology of the tumours enabled unequivocal identification of the chief cells.

Total tumour DNA isolation.
Total tumour DNA isolation was performed according to the protocol described earlier (Devilee et al., 1991). DNA extracted from blood leucocytes served as normal control.

DNA Flow Cytometry (FCM) and Sorting.
Procedures for measurement of cellular DNA content of HN-paraganglioma samples using a FACScan flow cytometer (Becton Dickinson) have been described (van der Mey et al., 1991). Sorting, based on Propidium-Iodide content, was performed on a FACStar flow cytometer (Becton Dickinson) and a minimum of 10,000 nuclei were sorted directly in 1.5 ml microfuge tubes and stored on ice as described (Abeln et al., 1994). The concentration of sorted nuclei was adjusted to 50 nuclei per µl by adding 10 mM Tris-Cl pH 8.3, 0.5% Tween20, 1 mM EDTA and DNA isolation was performed as described (Abeln et al., 1994).

Microdissection.
Chief cells could readily be identified on the basis of their nuclear morphology. Unambiguous identification of sustentacular cells was difficult, even in sections that were immunohistochemically stained with the S-100 polyclonal antibody (Kliewer et al., 1989). Individual chief cell nuclei were isolated by microdissection from 5 µm, deparaffinized Haematoxylin and Eosin (HE)-stained sections using an Axiovert 100 inverted microscope (Zeiss) equipped with a micromanipulator (Model 202, Narishige Comp. Ltd.) as described (Abeln et al., 1997), with minor modifications. A total of 50 nuclei were collected in 30 µl buffer (containing 10 mM Tris-Cl pH 8.3, 1 mM EDTA and 0.2% Tween20), 0.1 µl Proteinase K (10mg/ml) was added and this was incubated at 56°C overnight. After 5 minutes inactivation of Proteinase K at 100°C, 10 µl corresponding to about 16 nuclei, was used as input for the polymerase chain reaction (PCR).

Polymorphic markers.
Tumours T143 and T172 showed allelic imbalance for the entire investigated chromosome 11q region (Devilee et al., 1994). This encompasses the 2 centi-Morgan (cM) candidate gene region for PGL1 as observed by haplotype sharing within a large family (van Schothorst et al., 1996). Due to technical limitations for PCR using DNA isolated from paraffin-embedded tumour tissue, markers which amplify products smaller than 200 bp are preferred. We selected two markers flanking the PGL1 region for screening for LOH: markers D11S560 and CD3D, since both markers amplify a small genomic region (±77 and 89 bp, respectively) and are informative for these two tumour samples. Tumour sample VB showed to be informative for marker D11S560 only (data not shown).

PCR and gel-electrophoresis.
A Perkin-Elmer Cetus Thermal Cycler was used for all PCR amplifications. PCR standard conditions were as described (Heutink et al., 1994) with the following minor modifications: a total volume of 50 µl was used with the following cycling program: 5 min denaturation at 94°C, 42 cycles of 30 sec at 94°C, 2 min at 55°C and 1 min at 72°C with a final period of 10 min at 72°C, in the presence of ^{32}P-dCTP.
PCR-products were separated on a 6% polyacrylamide gel (Severn Biotech Ltd.) and after drying the gel, a Konica X-ray film was exposed overnight. PhosphorImaging (Molecular Dynamics) analysis was used to measure the degree of partial LOH -presented by the Allelic Imbalance Factor (AIF)- as described (Devilee et al., 1994). Briefly, this AIF represents the ratio between the ratios of allele intensities in tumour and normal DNA samples.

RESULTS

In a previous LOH-study of HN-paragangliomas, we observed only partial LOH for the PGL1 region on chromosome 11q22-q23 (Devilee et al., 1994) using total tumour DNA. From this set of tumours, we selected two tumours on the basis of the presence of allelic imbalance for the complete 11q-region

(T143 and T172) or the presence of an aneuploid fraction (Tumour VB). DNA histograms of all three glomus tumours show clearly a distinct DNA-aneuploid fraction and a DNA-diploid fraction (Figure 8.1). These fractions were subsequently flow sorted and analyzed by PCR.

Total unfractionated tumour DNA of tumour T143 showed partial loss of the upper allele for marker D11S560 (Figure 8.2, lane 3), resulting in an AIF of 2.0, while the sorted DNA aneuploid fraction showed complete loss of this upper allele (Figure 8.2, lane 1). No loss of heterozygosity was found for the flow-sorted DNA diploid fraction (Figure 8.2, lane 2, AIF 1.1). Similar results were obtained for tumour T172 (Figure 8.2, lanes 5-8; loss of the lower allele) and tumour VB (data not shown): both showed complete LOH in the DNA aneuploid fraction and no LOH in the DNA diploid fraction.

Using the marker CD3D, 15 cM distal to D11S560, exactly the same pattern was found for tumour T143 (Figure 8.3a, lane 1-4) and tumour T172 (Figure 8.3b, lanes 1-5). Patient VB is constitutionally homozygous for this marker. Thus, the DNA-aneuploid tumour cell fractions of three HN-paragangliomas show complete LOH for the candidate gene region (D11S560-CD3D).

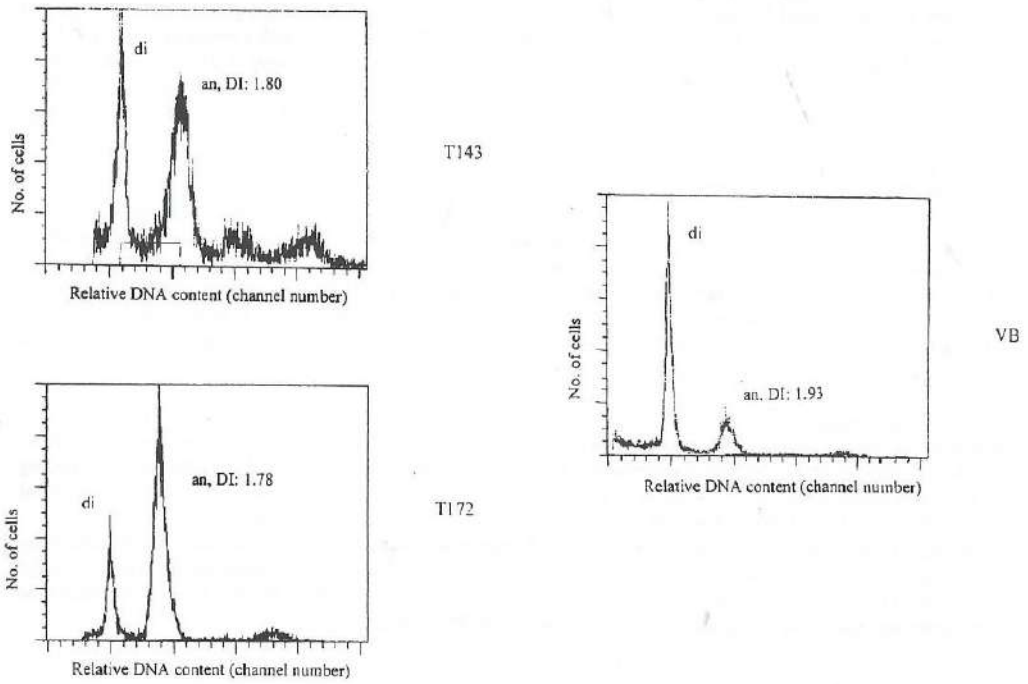
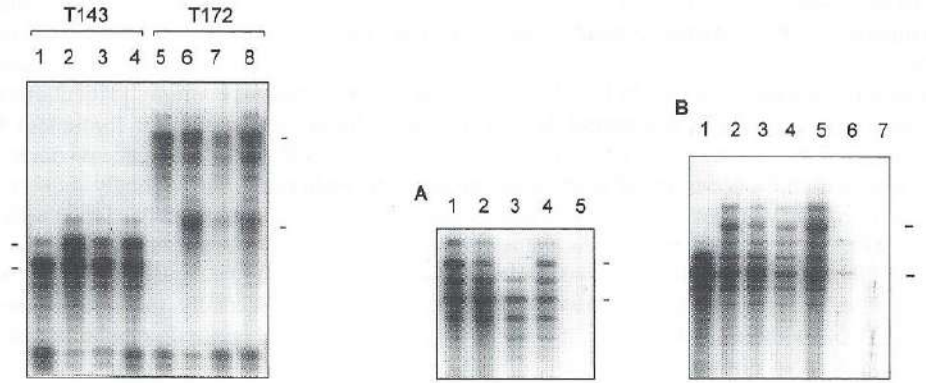


Figure 8.1. DNA histograms of three HN-paragangliomas (T143, T172 and VB).
In each histogram two peaks are visible: the $G_{1,0}$ from the DNA diploid population (di) and the $G_{1,0}$ from the DNA aneuploid population (an). The DNA-index (DI) is given next to the aneuploid peak. The aneuploid peak represents 48% (T143), 79% (T172) and 20% (VB) of the total cell population in the sample, respectively.



Figures 8.2 (left) and 8.3 (right).
Figure 8.2. LOH-analysis using marker D11S560 on 11q22-q23.
Lanes 1-4: tumour T143, lanes 5-8: tumour T172. Lanes 1,5: DNA aneuploid fraction; 2,6: DNA diploid fraction; 3,7: unsorted total tumour DNA; 4,8: normal DNA. Each allele shows one larger shadowband and several smaller ones. Horizontal bars indicate the positions of the alleles (left:T143, right:T172).
Figure 8.3. LOH-analysis using marker CD3D on 11q23.
8.3a. Tumour T143. Lane 1: normal DNA; 2: total tumour DNA; 3:aneuploid fraction; 4:diploid fraction; 5: negative control. Horizontal bars indicate the two alleles.
8.3b. Tumour T172. Lane 1: DNA aneuploid fraction; 2: DNA diploid fraction; 3,4:total tumour DNA; 5: normal DNA; 6: chief cell nuclei; 7: negative control. Horizontal bars indicate the two alleles.

Single-cell microdissection was used to isolate chief cells from tumours T143 and T172. From one HE-stained section of 5 µm, fifty nuclei of chief cells were isolated from at least four distinct areas of the section. An example of a HE-stained section of a HN-paraganglioma is shown in Figure 8.4, in which the chief cells are clearly distinguishable. For marker CD3D, chief cell nuclei of tumour T143 showed complete loss of the upper allele (Figure 8.5a, lane 1), a result identical to that of the sorted DNA-aneuploid fraction (Figure 8.3a, lane 3). The chief cell population of tumour T172 also showed complete LOH (Figure 8.3b, lane 6) for the same allele as did the sorted DNA-aneuploid fraction (lane 1). Marker D11S560 showed similar results for the chief cells of tumour T143 (Figure 8.5b, lane 1),

compared with the sorted DNA-aneuploid fraction (Figure 8.2, lane 1). All these results were confirmed by duplicate PCR analyses (data not shown).

DISCUSSION

In this study, we have demonstrated LOH at the putative PGL1 gene region on 11q22-q23 in purified tumour cell fractions from three different cases. The results show complete LOH for the same allele at two different markers for the 11q22-q23 region in the flow sorted DNA aneuploid fractions as well as in the microdissected chief cells of two HN-paragangliomas. The complete LOH in microdissected chief cells of tumours T143 and T172 indicates that these are monoclonal in origin, particularly since the nuclei were collected from different areas in the section. The monoclonal origin for tumour T143 is supported by the results from the X-inactivation assay (Devilee et al., 1994). The patient with tumour T172 is a male and was thus uninformative for this X-inactivation assay. This strongly suggests, but does not prove, that the aneuploid fractions in these tumours were composed entirely of chief cells. We conclude that the chief cells represent a true neoplastic component of HN-paragangliomas.

The DNA-diploid fractions showed no detectable LOH, which can be explained in three ways. Firstly, all cells within this fraction are non-neoplastic stromal cells. Secondly, this fraction consists of tumour cells without LOH. Thirdly, it consists of cells of polyclonal origin (hyperplasia) in which

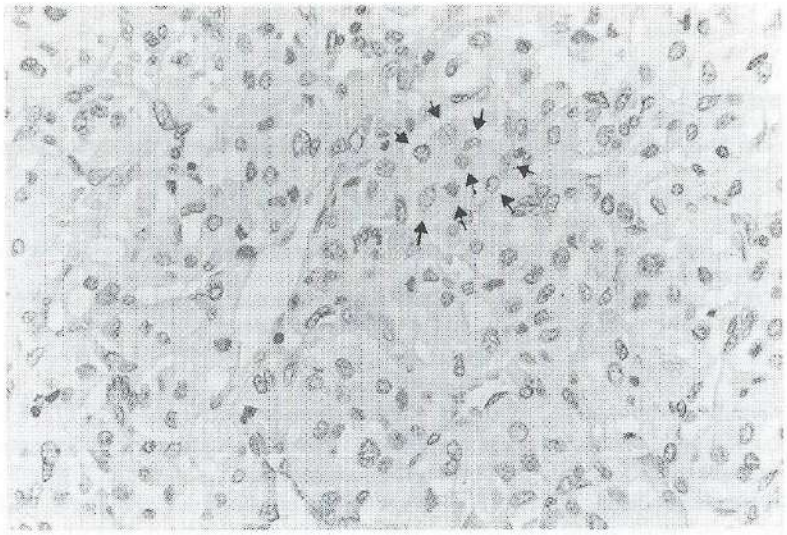


Figure 8.4. A HE-stained section of a HN-paraganglioma.
Arrowheads indicate chief cells forming one cluster, a so called *'Zellball'* (Glenner and Grimley, 1974).

random LOH-events cancel each other out. The exact origin of the cells in this fraction remains unresolved at present, but they are most likely to be the stromal cells, possibly mixed with the sustentacular cells. They are unlikely to be the chief cells, since then we would have detected at least partial LOH events. Random LOH in a polyclonal fraction is also less likely, given the genomic imprinting at the PGL1 locus, and the fact that a substantial proportion of HN-paragangliomas are inherited. These conditions would favour the loss of the same parental allele. LOH-analysis of 13 inherited cases revealed that always the maternal, wild-type allele showed reduced intensity (Baysal et al., 1997a; Devilee et al., 1994).

Our data therefore suggest that, in chief cells, PGL1 is a true tumour suppressor gene according to the two-hit hypothesis of Knudson in which loss of the wild-type allele represents the second, inactivating hit (Knudson, 1989). A possible model to explain the inheritance pattern could be that the mutation at the PGL1 locus generates an imprint-box, which silences the PGL1 gene *in cis* after paternal transmission. Note that such a mutation would not necessarily be in the coding region of the gene. Loss of the remaining active, maternal, allele results in the full inactivation of PGL1, required to ensure chief cell proliferation. After maternal transmission, the gene will revert to an active state (due to imprint erasure) and there will be two active gene copies present. Somatic loss of both copies will be a rare event, and could therefore explain that no patients have been detected yet that have inherited PGL1 from their mother. This model is also in agreement with the, as yet, absence of an endogenously imprinted gene on chromosome 11q or the synteny region on mouse chromosome 9.

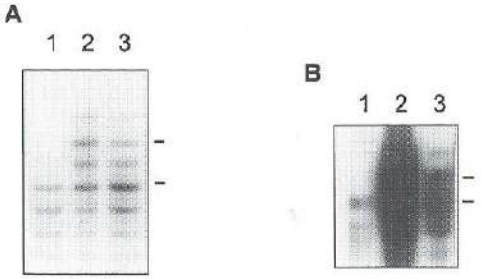


Figure 8.5. LOH-analysis of microdissected chief cells of tumour T143.
8.5a. Marker CD3D; **8.5b.** marker D11S560. Lane 1: chief cell nuclei; 2: normal DNA; 3: total tumour DNA. Horizontal bars indicate the two alleles.

LOH on chromosome 11q has been detected in different kinds of tumours, such as carcinomas of the cervix (Hampton et al., 1994b), or nasopharyngeal (Hui et al., 1996), adenocarcinoma of the stomach (Baffa et al., 1996), ovarian cancer (Davis et al., 1996; Gabra et al., 1996), breast cancer (Koreth et al., 1997), melanoma (Tomlinson et al., 1996), lung cancer (Rasio et al., 1995; Evans et al., 1996) or non-Hodgkin's lymphoma (Meerabux et al., 1994), indicating that at least one tumour suppressor gene is located in this region.

The isolation of sustentacular cells was hampered by technical limitations. Hence we cannot answer the question whether these cells are also of neoplastic origin, which would support the view that HN-paragangliomas are biphasic tumours (Schroder et al., 1986). Also in pulmonary carcinoid tumorlets, a neoplastic origin for S-100 protein positive sustentacular cells has been proposed (Rešl et al., 1996). On the other hand, the exact histogenetic origin and function (providing a growth-regulatory support for the neuro-endocrine chief cells) of HN-paraganglioma sustentacular cells remains obscure. Analysis of carotid body tumours showed that mainly the chief cells proliferate, in agreement with our findings (Glenner and Grimley, 1974).

In conclusion, the data presented here support the view that the chief cells are a major, if not the only, neoplastic component of HN-paragangliomas. Furthermore, they suggest that PGL1 is a true tumour suppressor gene, whose inactivation represents a crucial event in the genesis of these tumours.

Acknowledgements

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9

A High Resolution STS, EST, and Gene-Based Physical Map of the Hereditary Paraganglioma Region on Chromosome 11q23

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The genes responsible for hereditary paragangliomas (glomus tumors, MIM 168000) have been mapped to two distinct loci on the long arm of chromosome 11. Most of the informative families appear to be linked to the distal locus on chromosome band 11q23 (PGL1), which has been previously confined to a 2 cM interval by haplotype analysis in an extended Dutch pedigree. To facilitate the identification of the PGL1 disease gene, we constructed an approximately 4 Mb ordered clone contig map of STSs, ESTs, and known genes which spans the PGL1 critical region on chromosome band 11q23. Among 29 new positional candidate ESTs, only two (EST100999 and EST241777) mapped within the PGL1 critical region. We further characterized the genomic organization of promyelocytic leukemia zinc finger (PLZF) gene which maps within the PGL1 critical region, and physically excluded the serotonin receptor type 3 (5HT3R) gene. Finally, we identified a common, silent, single base substitution polymorphism in the 5HT3R gene, and characterized the allele sets of two new highly polymorphic microsatellite repeats within the PGL1 critical region.

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Introduction

Hereditary non-chromaffin paragangliomas (PGL, glomus tumors, MIM#168000) are mostly benign, slow growing tumors of the head and neck region which are inherited from carrier fathers in an age-dependent, autosomal dominant fashion subject to genomic imprinting. Genetic linkage analysis in two large, unrelated Dutch families assigned putative PGL loci to two different regions of the long arm of chromosome 11 at 11q23.1 (PGL1) (Heutink et al., 1994) and 11q13.1 (PGL2) (Mariman et al., 1993). We previously showed that most of the informative PGL families map to the PGL1 locus at chromosome band 11q23, and narrowed the critical region to a 7 cM region between D11S1647 and D11S622, by meiotic recombinant analysis of affected individuals (Baysal et al., 1997a). We further confined PGL1 locus to a 2 cM interval between D11S938 and D11S1885 by haplotype analysis of several nuclear Dutch families, all of which were distantly related to a common ancestor (van Schothorst et al., 1996).

As a first step in the identification of the PGL1 disease gene, we constructed an ordered clone contig map of sequence tagged sites (STSs) spanning an approximate 4 Mb region which encompasses the critical region of PGL1 locus on chromosome band 11q23. We tested 7 functionally characterized genes, 29 expressed sequence tags (ESTs), 19 short tandem repeat polymorphisms (STRPs), and 29 non-polymorphic STSs by PCR-based STS content mapping on 28 YACs, 2 PACs, and a completely sequenced cosmid. We analyzed a set of overlapping YACs and PACs by pulsed field gel electrophoresis (PFGE) and Southern hybridization, and identified a single NotI site within the PGL1 critical region. During our evaluation of the functionally characterized genes in the region, we further elucidated the genomic organization of the promyelocytic leukemia zinc finger (PLZF) gene, identified a new BfaI/NheI restriction fragment length polymorphism (RFLP) within the coding region of the serotonin receptor 3 (5HT3R) gene, and characterized the allele sets of two new highly polymorphic STRPs within the PGL1 critical region.

Materials and Methods

Individual mega-YAC clones of Centre d'etude du Polymorphisme Humain (CEPH), ESTs, STRPs, and other custom-designed PCR primers were obtained from Research Genetics (Huntsville, AL). Small insert, non-chimeric YAC clones were obtained from a chromosome 11-specific YAC library provided by Tom Shows at the Rosewell Park Cancer Institute(RPCI) (Qin et al., 1993). PAC clones were obtained by screening DNA pools of a human PAC library constructed by Pieter de Jong at RPCI. The single cosmid clone in the contig, cSRL17e5, was completely sequenced and this DNA sequence as well as primer sequences for cSRL-172g9 and cSRL-17e5 cosmid ends were available at web site http://medermott.swmed.edu/ of the Genome Sequence and Technology Center of the Eugene McDermott Center for Human Growth and Development at the University of Texas Southwestern Center at Dallas, Texas. YAC and PAC clones were grown in selective media and DNA was isolated either in liquid media for STS content mapping or in agarose blocks for PFGE analysis, using standard procedures. PCR Primer sequence information of most ESTs and STSs used in this study is available at The Genome Database at http://gdbwww.gdb.org//. PCR primer sequences and their optimum annealing temperatures for other regional genes, ESTs and new STRPs are listed in Table 9.1.

STS content mapping was based on at least two confirmatory PCR amplification results. PCR amplification was performed using either 60 ng of cloned template DNA or 120 ng of total genomic DNA. The final concentrations of reagents in the 25 µl PCR reaction volume were as follows: 1X PCR reaction buffer (1.5 mM $MgCl_2$);1 mM PCR primers; 0.20-0.25 mM each dNTP; and 1 U Taq Polymerase (Boehringer-Mannheim, Indianapolis, IN). The mixture was heated

to 94° C for 3 min in a Techne PHC3 thermal cycler. The usual cycling conditions consisted of 35 cycles of 94° C for 45 s, 55° C for 45 s, and 72° C for 45 s, followed by a final extension of 72° C for 10 min. The annealing temperature was modified depending on the T_M of a primer set. A final primer concentration of 0.3 µM was used when amplification was performed using ^{32}P-end labeled primers for SSCP and STRP allelic analyses.

The PCR amplification products were analyzed in 1-2% agarose gel after ethidium bromide (0.1 mg/ml) staining for STS content mapping, in 6% denaturing (7 M urea) polyacrylamide gel for STRP polymorphism analysis using M13 sequencing ladder as a size standard, or in non-denaturing polyacrylamide gel (MDE gel, AT Biochem, Malvern, PA) with or without 5% glycerol for SSCP analysis. Sequencing of the 5HT3R conformers was performed by cycle sequencing (fmol sequencing, Promega, Madison, WI) of the gel extracted/ re-amplified SSCP bands using the same amplification primers. BfaI and NheI (NEB) restriction enzyme reactions were performed by directly digesting an aliquot of amplification product, and analyzing the digestion products on 2% agarose gels. Allele frequencies of cSRL17e5 CA and CTTT STRPs were calculated using an unrelated set of 79 Caucasian chromosomes chosen from the previously reported PGL families (Baysal et al., 1997a).

The undigested and Not I restriction enzyme-digested high molecular weight DNAs of a set of YAC/PAC clones were separated by PFGE using LKB Pulsaphor Electrophoresis Unit. Electrophoresis was performed in 1.5% agarose gels in 1XTBE buffer at 10° C, using pulse times between 60 and 120 sec at 180 V for 40 hours. Saccharomyces cerevisiae chromosomal DNA and lambda DNA digests were used as size standards. The products were transferred to nylon membrane (Hybond N+, Amersham, Arlington Heights, IL) in alkaline conditions (0.4 N NaOH), and hybridized with various ^{32}P-labeled probes, using standard techniques (Sambrook et al., 1989).

Table 9.1: PCR primers used for genomic mapping in 11q23.

Locus	PCR Primer Sequences	Primer Location	Amplicon size (bp)/T_a
NNMT	Forward: 5'-TCTAACTGCCAAGTCATGTGCTG-3'	3'-flanking region	260/59 °C
	Reverse: 5'-ACACTTCTCTGTAGGCAGGTAC-3'	3'- flanking region	
5HT3R	Forward: 5'-CAGAGGATTTCTGCTTAGGCCC-3'	cDNA, 3'-UTR	199/59 °C
	Reverse: 5'-TAAAGCCATGGTGAGCTGCTGG-3'	cDNA, 3'-UTR	
HSPA8	Forward: 5'- TTCGTAGCAAATTCTGTGGCAG-3'	cDNA, 3'-UTR	623/60 °C
	Reverse: 5'-CTCAGAAGAAATGGAAGCCCA-3'	cDNA, 3'-UTR	
OBF1	Forward: 5'- AGGCTTAGTGCTACCATGTGGGTT-3'	cDNA, 3'-UTR	193/65 °C
	Reverse: 5'-TCCAGCCTGGGCAATGGAGTGA-3'	cDNA, 3'-UTR	
EST241777	Forward: 5'-TCGCCCTCCTGGCATGTGATTG-3'	cDNA	111/60 °C
	Reverse; 5'-AAAGGAGGTGGGCTGGCGGCA-3'	cDNA	
PLZF Exon 1	Forward: 5'-CAGGGAACTCTTCAGCCACCTG-3'	cDNA 643-664	280bp/60°C
	Reverse: 5'-GTGGCACCTGCTCGGCACTCT-3'	cDNA 902-922	
PLZF Exon 2	Forward: 5'-AACCTCTCTTTTCCTTTCCCTTAC-3'	5' flanking intron	147bp/64°C
	Reverse: 5'-GCCACTAAGGCGAACTTGCCT-3'	3' flanking intron	
PLZF Exon 3	Forward: 5'-CGGGTGCCAAAGCCTTTGTCTG-3'	cDNA 1367-1388	83bp/66°C
	Reverse: 5'-ATGGGTCTGCCTGTGTGTCTC-3'	cDNA 1429-1449	
PLZF Exon 4	Forward: 5'-TTTCTGAGGCACCCCCTCTCCT-3'	5' flanking intron	230bp/64°C
	Reverse: 5'-TCCACCCATCAGCTGGACTGAC-3'	3' flanking intron	
PLZF Exon 5	Forward: 5'-AGAGGCAAAGGCCTGATCCAGC-3'	5' flanking intron	245bp/64°C
	Reverse: 5'-GTCCCAGAGCCAGCTCAGGC-3'	3' flanking intron	
PLZF Exon 6	Forward: 5'-CCCTTTGAGTGTAAGCTCTGCCA-3'	cDNA 1800-1823	301bp/55°C
	Reverse: 5'-TGTCATAGTCCTTCCTTCATCTCA-3'	cDNA 2077-2100	
(CTTT)n	Forward: 5'-TTCTGCCTACTATTCCACCCTG-3'	cSRL17e5	182-222/60 °C
	Reverse: 5'-GACTACAGGGCATGTATGACTC-3'	cSRL17e5	
(CA)n	Forward: 5'-ATACCTCTCCGGATTTACTTTCC-3'	cSRL17e5	189-219/60 °C
	Reverse; 5'-TTTCCAGTCCAGTGGCTGGAC-3'	cSRL17e5	

Results

A framework map of CEPH mega-YACs was initially constructed by STS-content mapping of CEPH mega-YAC DNA pools. In the PGL1 critical region bounded by D11S938 centromerically and D11S1885 telomerically, groups of RPCI YACs were incorporated to the map when anchored by known 11q23 markers (Qin et al., 1996) or when predicted to map to 11q23 by Inner Product Mapping (Perlin et al., 1995). The region was further saturated by testing new STSs derived from mega-YAC ends (Arai et al., 1996) and cSRL cosmids ends which mapped to the largely overlapping tumor suppressor critical region at chromosome band 11q23 (Evans et al., 1996). Two PACs and a completely sequenced cosmid, cSRL17e5 were also incorporated into the contig. Candidate ESTs and genes were selected based on regional mapping data from a chromosome 11 radiation hybrid map at WTCHG (James et al., 1994) or whole genome EST mapping (Schuler et al., 1996) and from fluorescent in situ hybridization (FISH) and somatic cell hybrid mapping of individual genes (5HT3R, NNMT).

This 11q23 clone contig is composed of 31 clones, and spans approximately 4 Mb (Figure 9.1). A total of 65 STSs, ESTs, and gene exons were mapped to intervals defined by clone overlaps in this contig. CEPH mega-YACs y888-h-11 and y981-d-12 demonstrated positive hit patterns for discontinuous STSs, implying the presence of internal deletions or rearrangements in these YACs. No evidence for deletion or rearrangement was found for other YAC or PAC clones in this contig. Insert sizes of selected YACs and of the two PACs were checked by CHEF Pulsed Field Gel Electrophoresis (PFGE). A NotI digestion was performed on the same set of clones which covered the PGL1 critical region (Figure 9.2). A single NotI site was identified within the region. This unique NotI site was further localized in PAC28 (130 kb) between D11S938, proximally, and first exon of PLZF gene, distally, which detected 45 kb and 85 kb fragments, respectively, by Southern hybridization (data not shown). Fine mapping of this NotI site within PAC28 clone helped to define the maximum length of the interval between D11S938 and distal end of y957-e-4 as 815 kb, i.e. the sum of 45 kb proximal band of PAC28 and 770 kb distal fragment of y957-e-4. After combining 1000 kb insert size of distally overlapping y771-d-4, the maximum length of the PGL1 critical region was therefore estimated to be 1.8 Mb (Figure 9.2).

Twenty eight available positional candidate ESTs from the transcript mapping consortium (Schuler et al., 1996) sequence were localized inside or outside of this clone contig mapping panel (Figure 9.1). Surprisingly, only one new expressed sequence EST100999 (D11S2276E) mapped within the ~1.8 Mb PGL1 critical region. Seven other ESTs mapped within the 4 Mb contig, but outside of the PGL1 critical region. The remaining twenty EST clones did not map within this contig. EST241777 was identified through sequencing of cSRL-17e5, and was confirmed by PCR analysis to reside within the PGL1 critical region. The dopamine receptor type 2 (DRD2), and neural cell adhesion molecule (NCAM) genes were mapped to the proximal region of the contig. Two other genes, heat shock protein A8 (HSPA8), and octamer binding factor type 1 (OBF1), which were previously localized to 11q23 by FISH analyses (Tavaria et al., 1995; Junker et al., 1996) and might be considered as plausible PGL1 candidate genes, did not map to this contig.

PLZF, NNMT and 5HT3R are three functionally characterized genes previously mapped to chromosome band 11q23.1 by FISH (Chen et al., 1993b; Aksoy et al., 1995; Weiss et al., 1995) or RH mapping (James et al., 1994). We further localized each of these genes within or very close to the PGL1

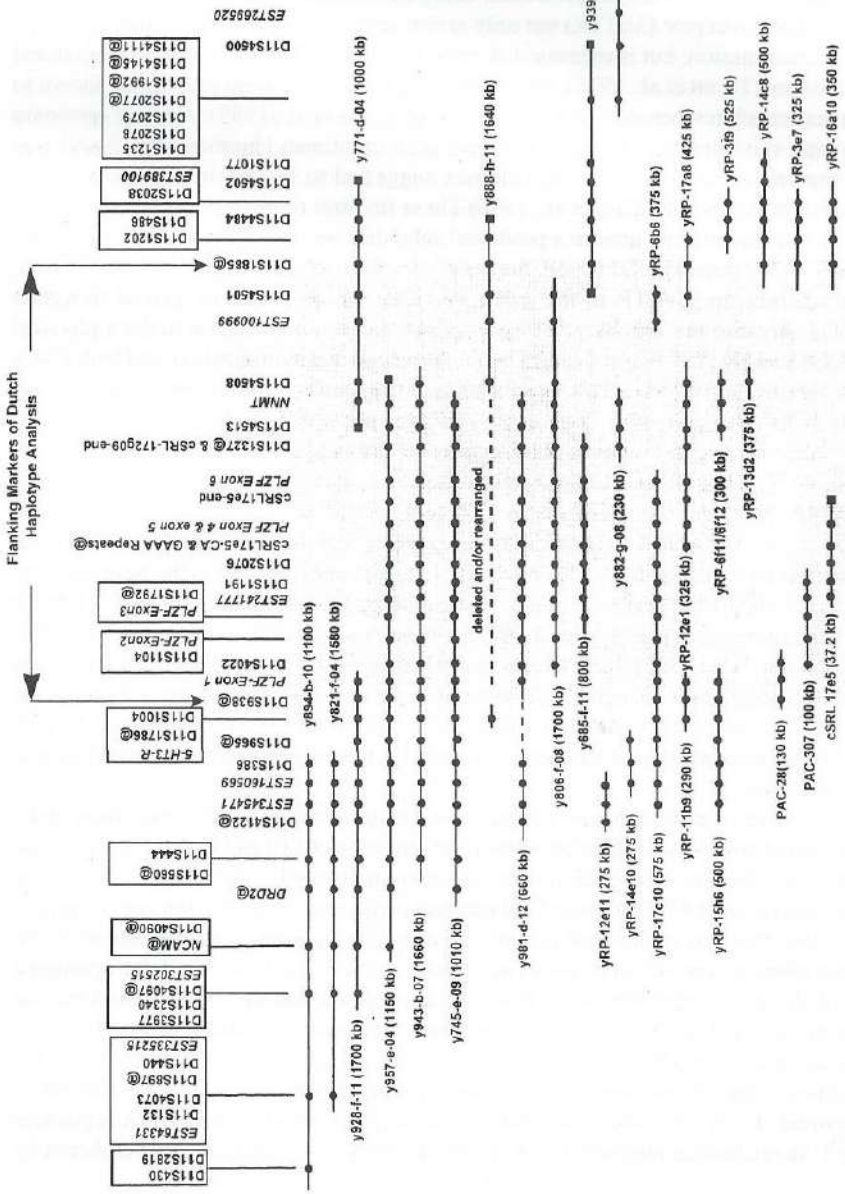

Figure 9.1. A physical contig of the YAC/PAC/cosmid clones spanning the PGL1 critical region and regionally mapped STSs/ ESTs and genes are shown. Positive hits are depicted by dark circles. Intermarker distances are not to scale. The squares at the ends of some CEPH megaYACs represent YAC-end derived STSs. Clone names and their sizes in parenthesis are depicted on one side. If several markers map to the same interval, i.e. when their order is unknown, they are clustered within a rectangle. Polymorphic markers are tagged with a '@' sign. ESTs and genes which mapped to this contig are shown in italics. ESTs and genes not mapping to this contig include: OBF1, HSPA8, EST394772, EST231080, EST270040, EST169097, EST165406, EST198329, EST53061, EST50770, EST156263, EST42542, EST220739, EST41285, EST224689, EST42572, EST64550, EST70435, EST64292, EST183057, D11S262146, D11S2284E.

critical region. NNMT, nicotinamide N-methyltransferase gene encodes an enzyme that is involved in drug and xenobiotic metabolism (Aksoy et al., 1994) and does not appear to be a good candidate gene for PGL1. NNMT maps distally within the PGL1 interval into the overlapping region of y957-e-4 and y771-d-4, using PCR primers derived from the 3'-flanking region of the gene. We also placed all three exons of the gene to this interval by PCR amplification using exon specific primers (data not shown).

The serotonin type 3 receptor (5HT3R) not only serves as a ligand-gated ion channel receptor in serotonergic neurotransmission, but is expressed at important developmental stages in central and peripheral neuronal tissues (Tecott et al., 1995). Serotonin 5HT1c receptors were previously shown to induce malignant transformation when expressed in fibroblasts (Julius et al., 1989). Another serotonin receptor, 5HT2R maps very close to the retinoblastoma gene on human chromosome 13 and was hypothesized as a mediator of cell growth. 5HT2R was suggested to be imprinted because of its exclusive maternal allelic expression (Kato et al., 1996).These findings prompted us to further refine the physical localization of the 5HT3R gene as a positional candidate for the imprinted PGL gene. We initially co-localized 5HT3R gene with D11S938, the proximal boundary of the PGL1 critical region, using primers derived from the 3'-UTR of the gene. However, we subsequently placed this gene proximal to D11S938, because the two overlapping PACs in the proximal region make a physical contig between D11S938 and D11S1792 as detected by PCR analysis and hybridization, and both PACs are negative for this 5HT3R derived STS. This new location of the gene to chromosome band 11q23.1 excludes it from the PGL1 critical region. Before this gene was physically excluded from the PGL1 critical region, however, we began mutation screening in the coding region of the gene by SSCP analysis of PCR amplified products. This search resulted in identification of a new RFLP polymorphism using forward primer 5'-ATG GGA GGA CCC CAG GAC TTC G -3' and reverse primer 5'-CTC CTC CAC TGG GCT GTA CCC A-3' . Nucleotide sequence analysis of 299- bp fragment showed a single base change from adenine (allele 1) to guanine (allele 2) in the position 1596 of 5HT3R cDNA sequence (Miyake et al., 1995). This silent nucleotide substitution disrupts a BfaI or NheI restriction enzyme recognition site. Digestion of the 299 bp product with either enzyme identifies two alleles: allele 1 has two bands of 215 and 84 bps, and allele 2 has a single band of 299 bp. Allele 1 and allele 2 have frequencies of 0.75 and 0.25, respectively, in a sample of 79 unrelated Caucasian chromosomes. Identification of this new common, RFLP polymorphism in the coding region of 5HT3R allows for testing of biallelic expression of this gene and a polymorphism useful for association studies in neuropsychiatric diseases.

PLZF, Promyelocytic Leukemia Zinc Finger, is a *Kruppel* related C_2-H_2 zinc finger gene identified through a patient with variant acute promyelocytic leukemia (APL) and a t(11;17) reciprocal translocation (Chen et al., 1993a). The PLZF protein has been implicated in functions as distinct as early hematopoiesis (Reid et al., 1995) and early forebrain and peripheral nervous system development (Cook et al., 1995). Since previous studies that partially unraveled the genomic organization of PLZF implied that the gene spans at least 40 kb (Chen et al., 1993b; Licht et al., 1995), we have attempted to map each exon of the gene separately by PCR on the assumption that the exons would map to different intervals in the contig (Figure 9.1). First, we designed PCR primer pairs from various locations in the cDNA sequence and compared the amplification products of genomic clones with that of the PLZF(B) cDNA clone to identify exon-exon boundaries and the number of exons. We identified 6 exons within the reported cDNA sequence. The junctions between the exons on the cDNA sequences are predicted to lie 3' to nucleotide numbers 1,268, 1,366, 1,449, 1,624, and 1,792, as numbered by

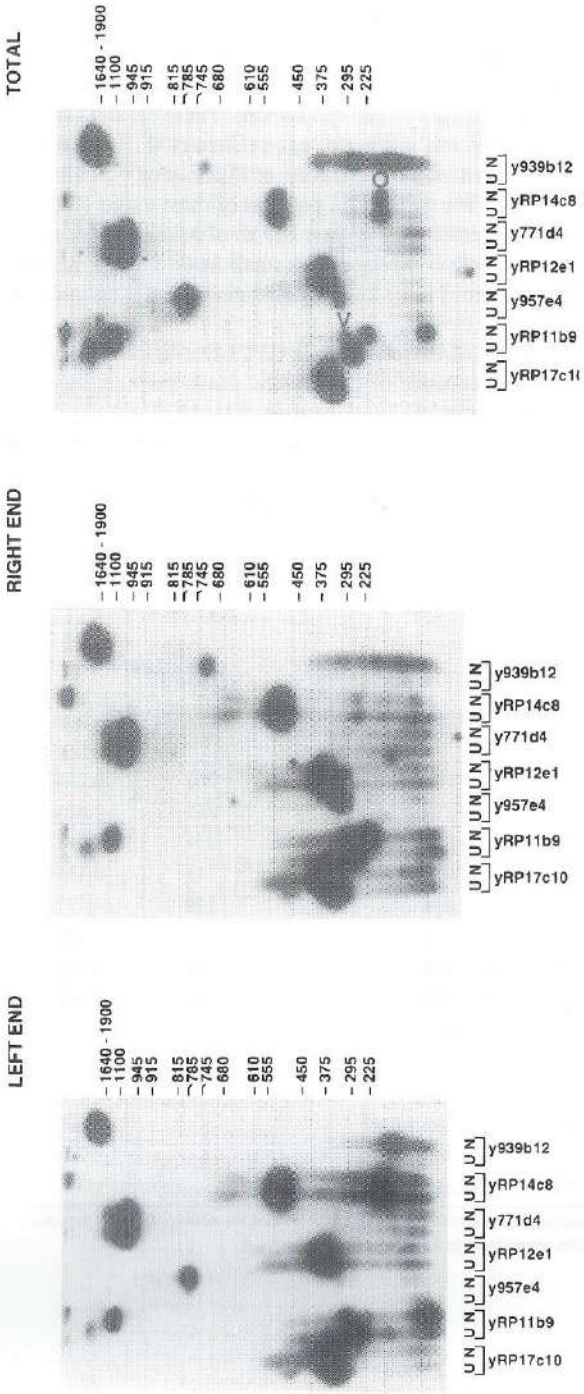

Figure 9.2. A subset of overlapping YAC DNAs were separated by PFGE before (U) and after NotI (N) restriction enzyme digestion. The gel was blotted and consecutively probed by YAC left, right end specific probes and human C_0t-1 DNA after stripping the previous probe to check insert sizes and NotI sites. A molecular weight standard is given on the right. A single NotI site has been located proximally within the PGL1 critical region as evidenced by the release of two bands from y957e4 and from completely overlapping yRP11b9. Telomeric YACs y771d4 and yRP14c8 do not contain any NotI sites. The bands denoted by an arrowhead and a circle have been interpreted as incomplete digestion and YAC rearrangement products, respectively. The yRP17c10 clone used in this particular experiment failed to give the expected STS hit pattern obtained in several previous preparations of yRP17c10 DNA (data not shown), and thus the absence of expected NotI site is due to clonal heterogeneity of this YAC.

Chen et al (1993b). We discovered the presence of a single large exon with a size of at least 1,288 bp at the 5'-end of the gene. We designed several PCR primer pairs from overlapping segments from this large first exon and confirmed its genomic integrity (data not shown). These results also show that the previously-reported, alternatively spliced region within this exon (Chen et al., 1993b) does not cause an interruption at the genomic level. This genomic organization of PLZF gene was also confirmed by a separate cosmid contig (data not shown).The distinct hit patterns of most exons (Figure 9.1) imply that they are widely separated and that the gene covers at least 120 kb of genomic region. Nevertheless, the whole gene was captured within a contig comprised of 4 small insert clones, namely, PAC28 , PAC307, cSRL17e5, and yRP12e1. All exons have been located distal to the unique NotI site, and within the PGL1 critical region.

We also analyzed the polymorphism characteristics of a CTTT tetra nucleotide STRP and a CA dinucleotide STRP identified within the sequence of cSRL17e5. Both markers have much higher heterozygosity values than the previously identified STRP markers (D11S938, D11S1327, D11S1792, and D11S1885) within the PGL1 critical region (Table 9.2). These new highly polymorphic markers will be useful for identification of unique PGL haplotypes for linkage disequilibrium studies and loss-of-heterozygosity studies in defining the smallest region of overlap in LOH studies of PGL1 tumors.

Table 9.2: Allelic analysis of the two new STRPs within the PGL1 critical region.

Marker	cSRL17e5 CA repeat[a]		cSRL17e5 CTTT repeat	
Expected Heterozygosity	0.90		0.89	
Allele No	Size (bp)	Frequency	Size (bp)	Frequency
1	189	0.038	263	0.038
2	191	0.179	267	0.025
3	193	0.090	271	0.152
4	199	0.013	275	0.114
5	201	0.038	279	0.177
6	203	0.115	283	0.228
7	205	0.064	287	0.190
8	207	0.038	291	0.051
9	209	0.038	299	0.013
10	211	0.103	303	0.013
11	213	0.064	-	-
12	215	0.103	-	-
13	217	0.090	-	-
14	219	0.013	-	-

[a] Unequal intensity of allelic amplification products has been observed for cSRL17e5 CA repeat, and it should be used with caution especially in LOH studies.

DISCUSSION

Although the ongoing large scale physical and genetic mapping projects provide initial skeleton physical maps of a region of interest for positional cloning projects, these maps still require considerable refinements of marker order and distance estimations. A physical map of 11q23 using large-insert, mega-YAC clones has been published but the marker and clone density within the PGL-1 critical region is poor (Arai et al., 1996). Physical maps based exclusively on CEPH mega-YACs are often unreliable for subsequent steps including subcloning and restriction enzyme analysis, because of high rates of chimerism and deletions. Likewise, the cDNA radiation hybrid mapping consortium provides a large bin of imprecisely mapped ESTs. For example, out of 15 ESTs from the same chromosome 11 radiation hybrid bin position 531.0 in the Whitehead database at http://www-genome.wi.mit.edu/, only 6 mapped within the 4 Mb contig. The remaining 9 ESTs did not hit any clone in the contig, and thus are localized outside of this contig. Similarly, the physical order of four STRPs spanning the PGL-1 critical region, D11S938-D11S1792-D11S1327-D11S1885 (Figure 9.1) does not match either with the genetic map of Genethon where the order is D11S1327-D11S938-D11S1885-D11S1792 or with the physical map of WICGR database where the order is D11S1327-D11S938-D11S1885. The critical region for PGL-1, defined by haplotype sharing among affected individuals between D11S938 and D11S1885, is covered by small insert, non-chimeric PACs and RPCI YACs. The only gap within this small-insert contig is covered by a previously characterized non-chimeric mega-YAC y-771-d-4 (Arai et al., 1996). This contig provides 3 to 9 fold coverage by genomic clones and allows for unequivocal ordering of most of the ESTs, STRPs and STSs in the PGL1 critical region.

We mapped a total of 9 ESTs, 19 STRPs, 29 non-polymorphic STSs, and 5 genes, including 6 exons of PLZF gene, to distinct intervals in the contig. EST100999 and EST241777 are localized within the PGL1 critical region, and thus can be considered positional candidates for the PGL1 gene. A total of 20 ESTs, and 2 genes have been physically excluded from this contig. A single NotI site has been mapped proximally in the contig. The identification of only one NotI site does not seem to support the supposition that imprinted genes reside in CpG-rich domains in the genome (Barlow, 1995).

This clone contig may also be helpful in identification of the tumor suppressor gene(s) postulated to reside in this region of chromosome band 11q23. This region has been observed to undergo loss of heterozygosity (LOH), as demonstrated by allelic imbalances, in many common types of solid tumors, including breast (Hampton et al., 1994a; Negrini et al., 1995; Tomlinson et al., 1995), cervical (Hampton et al., 1994b), colorectal (Keldysh et al., 1993), bladder (Shaw and Knowles, 1995), lung (Rasio et al., 1995), ovarian (Gabra et al., 1996) and nasopharyngeal carcinomas (Hui et al., 1996), and in melanomas (Tomlinson et al., 1993; Herbst et al., 1995). Interestingly, similar observations have also been made in paraganglioma tumors, in which the lost allele is exclusively maternal. This parent specific allelic loss within the PGL1 critical region is probably a consequence of imprinting phenomenon displayed by the PGL gene and has been discussed elsewhere (Devilee et al., 1994; Baysal et al., 1997a). Although the genomic extent of the reported imbalances are generally too large to define the precise location or the number of tumor suppressor gene(s), the genomic region covered by the clone contig in this study is partly (Negrini et al., 1995) or totally (Hampton et al., 1994a; Hampton et al., 1994b; Herbst et al., 1995; Rasio et al., 1995; Shaw and Knowles, 1995; Evans et al., 1996; Gabra et al., 1996; Hui et al., 1996) contained within the described deletion domains of chromosome band

11q23. Hence, this clone contig with its newly characterized highly polymorphic markers and mapped ESTs/genes will help in the fine mapping of allelic imbalances and the elucidation of PGL1 and other putative tumor suppressor genes in this 11q23 region.

Acknowledgments

We thank Dr. Jonathan Licht for the gift of PLZF(B) cDNA clone, Dr. Tom Shows for providing the chromosome 11-specific RPCI YAC library and Dr. Michael Higgins for help in the screening of RPCI YACs for marker D11S1327. This work was supported by grants from the American Cancer Society (VM-180 and IRG58-34) to C.W.R.

10

GENOMIC STRUCTURE OF THE HUMAN PLZF GENE AND EXCLUSION AS THE CAUSATIVE GENE FOR HEREDITARY HN-PARAGANGLIOMAS.

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The human PLZF (Promyelocytic Leukaemia Zinc Finger) gene encodes a *Krüppel*-like zinc finger protein, which was identified via the reciprocal translocation t(11;17)(q23;q21) fusing it to the RARα gene in promyelocytic leukaemia. Because of its segmental and tissue specific expression pattern, plus the co-localization on 11q23 with the PGL1 locus for hereditary head and neck paragangliomas, we determined its genomic organisation to enable mutation analysis in patients. The PLZF gene has 7 exons varying in size from 87 to 1358 bp spanning at least 120 kb. The ATG initiation codon resides in exon 2, while the TGA stop codon and the 3'-polyadenylation site locate in exon 7. Flanking intronic sequences were identified and all splice acceptor and donor sites conformed to the gt/ag rule. Five polymorphic markers could be fine located in its vicinity. Not a single aberration could be detected in 12 paraganglioma patients from different families using (Fiber)-FISH, single stranded conformation polymorphism analysis, Southern blot analysis or direct sequencing of the gene, thereby excluding PLZF as a candidate gene in the etiology of HN-paragangliomas.

An abbreviated version of this chapter has been submitted.

Introduction

The PLZF (Promyelocytic Leukaemia Zinc Finger) gene was identified as being fused to the RARα gene through a t(11;17) translocation in a patient with acute promyelocytic leukaemia (Chen et al., 1993a). The 2019 nt PLZF coding sequence encodes a 673 amino acid protein of Mr 74340, which contains 9 zinc-fingers, a proline rich region and several phosphorylation target sites. Its expression pattern is highly tissue and developmental specific: during murine development *Plzf* is expressed in neural tissue (especially at the branchial arches) and the AGM (aorta, gonad, mesonephros) region which give rise to haematopoietic progenitors (Cook et al., 1995). In human its expression is detected in bone marrow, early myeloid cell lines, peripheral blood mononuclear cells, ovary and to a lesser extent in kidney and lung (Chen et al., 1993b). The murine expression at the rhombomeric boundaries suggested that *Plzf* might play an important functional role in the segmentation of the vertebrate hindbrain, potentially regulating boundary cell interactions (Cook et al., 1995; Avantaggiato et al., 1995). The Krüppel like Zn-finger protein was located in the nucleus in a distinct speckled pattern, suggesting its compartmentalization in the nucleus (Reid et al., 1995) and function as transcription factor (Sitterlin et al., 1997; Li et al., 1997b).

Human PLZF has been mapped to 11q22-q23 by radiation hybrid mapping (James et al., 1994) and we located the gene in more detail between the markers D11S938 and D11S1327 (Baysal et al., 1997b). This locus is contained within the region harbouring the PGL1 gene for hereditary head and neck paragangliomas (HN-paragangliomas; van Schothorst et al., 1996). These rare tumours of the parasympathic paraganglion system are slow growing, mostly benign tumours. In a substantial proportion of the cases familial inheritance is observed which is most consistent with an autosomal dominant model with incomplete penetrance; only after paternal transmission do tumours occur, which could be explained by genomic imprinting (van der Mey et al., 1989; Heutink et al., 1992; McCaffrey et al., 1994). The role of PLZF in tumorigenesis and its segmental and tissue-specific expression during embryogenesis, make it an attractive candidate gene. We therefore decided to deduce its genomic structure to enable mutation screening in patients.

Results and discussion

Previously, we identified a 3-marker haplotype of about 2 cM, defined by the markers D11S1792-D11S1327-D11S908 (cen→tel), which was conserved among all patients from a multibranch family (van Schothorst et al., 1996). PLZF lies very close to D11S1327 (Baysal et al., 1997b).

Two YACs from the CEPH-YAC library (y745-E-09 (1010 kb) and y685-F-11 (800 kb)) cover most of the PGL1 candidate gene region (van Schothorst et al., 1996; Baysal et al., 1997b), and contain D11S1327 and D11S1792. These 2 YACs were subcloned in a sCOGH1 cosmid vector (Datson et al., 1996). Screening of the cosmid library with various probes resulted in a tiling path covering most of the PLZF gene and physically located the polymorphic markers D11S938, D11S1792 and D11S1327 with respect to the PLZF gene (Figure 10.1). From cosmid cIB6, 291 bases of end-sequence were identified and a 245 bp STS was developed (forward primer TAGAGCATGAGCTTGCAGTCA and reverse primer ACAGGAAGGAGACAGCGAAA) enabling its mapping within intron 2 (Figure 10.1).

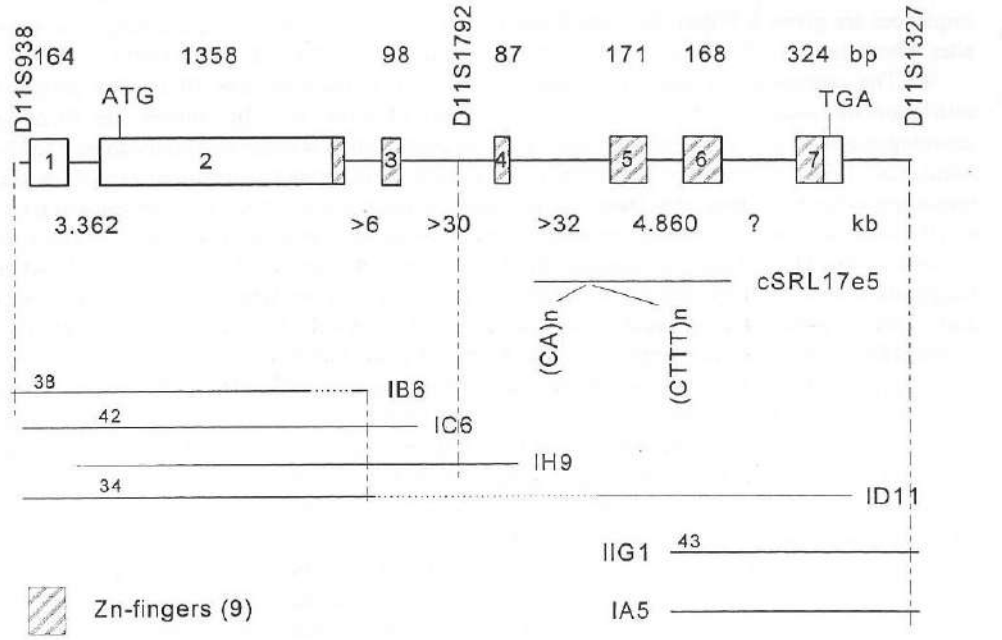

Figure 10.1. Genomic structure of the human PLZF gene and markers located nearby. The centromere is on the left side. Cosmids are represented by bars with identification and sizes given besides, and gaps by dotted lines. Exons are represented by boxes with their sizes in bp above, and intervening intron sizes are given below (kb). The functional protein domain containing the 9 zinc fingers is shaded. The exact size of intron 6 is unknown.

The extent of overlap between the cosmids was approximated by Southern blotting and fluorescence *in situ* hybridisation (Fiber-FISH; Florijn et al., 1995), allowing intervening gaps to be deduced. A gap within the tiling path, located in intron 4, could apparently not be subcloned in cosmids.

To determine the unknown exon/intron boundaries, either cosmids or YACs were directly sequenced and sequences were compared with the published cDNA sequence (Chen et al., 1993b; GenBank Z19002). This resulted in a total of 7 exons varying in size from 87-1358 bp. Exon 1 had been omitted in a previous publication (Baysal et al., 1997b) and was identified by 5' RACE, although the unambiqously assignment of the transcription start site was not undertaken. The completely sequenced cosmid cSRL17e5 (GenBank U73639) was shown to contain a part of the PLZF gene: 31,693 bp of intron 4, exon 5, intron 5, exon 6, and 95 bp of intron 6. The 3' UTR sequence has been extended and the last 6 bases of the published cDNA sequence turned out to be identical to the EcoRI palindrome and was not present in the genomic sequences we obtained. The ATG start-site is located within exon 2 (nt 91), corresponding with position 1 in the published (partial) cDNA sequence (Chen et al., 1993b).

Instead of the reported A at their position -52, we found a T, which was confirmed by sequencing a set of human DNA samples. The sizes of the different exons and introns and the splice acceptor/donor sequences are given in Figure 10.1 and Table 10.1. The two large introns surrounding exon 4 are the sites where the t(11;17)(q23;q12) translocation breakpoints reside (Licht et al., 1995).

The untranslated exon 1 is located within a CpG island (Figure 10.2). One unique *Not*I restriction-site was identified (326 bases upstream of exon 1). This places the PLZF gene unambiguously on the 1200-2000 kb fragment of the *Not*I restriction-map of chromosome 11q22-q23 (Arai et al., 1996). Furthermore, several SP1 sites (gggcgg) are located upstream of exon 1, indicating transcription factor binding sites. Based on YAC/PAC contigs in this region the gene spans at least 120 kb (Baysal et al., 1997b). Database searches revealed 9 expressed sequence tags (ESTs): 3 sense PLZF sequences (GenBank # F00118 contains 304 bp of exon 2, # AA428940 contains exon 3 and small fragments of exons 2 and 4, and # AA296151 contains exon 4 and small fragments of exons 3 and 5), and 6 clones containing antisense exon 7 sequences (# AA468119, AA614232, AA603431, and AA642184) of which 2 extend into exon 6 (# AA845269 and AA296152).

Primers were designed within the introns flanking each exon (Table 10.1) for sequencing probands from 12 HN-paraganglioma families; these patients were selected on the basis of different disease-linked haplotypes for the markers D11S938-D11S1792-D11S1327-D11S1885 to increase the chance of screening different mutations. In addition to families from the Netherlands (e.g. FGT4, FGT2, FGT189), they included probands from Italy (FGT37), India (FGT34), French-Canada (FGT26), Germany/Luxembourg (FGT15) and France (FGT21).
No sequence changes were detected in these samples relative to controls; not in the coding region, nor in the splice sites, nor in the total flanking 978 bp intronic sequences. SSCP-analysis of the 3' UTR region on a set of unrelated individuals did not reveal any band-shifts (data not shown).

The characteristics of the PLZF gene found so far is in contrast with a characteristic of imprinted genes: they tend to contain small and few introns (Hurst et al., 1996). Furthermore, since mutations within the 5' untranslated exons of the imprinted gene SNRPN were shown to be related with Prader-Willi and Angelman Syndrome (Dittrich et al., 1996), we investigated this region of the PLZF gene carefully. Amplified cDNA, particularly the 5' part using specific primers, from tumour tissue as well as leucocytes, and subsequent sequencing showed only the wild-type sequence (data not shown).

The absence of polymorphisms (expected rate of 1 per 1000 bp, Cooper et al., 1985) might suggest hemizygosity due to large deletions, but the markers D11S938, D11S1327 and D11S1792 flanking and within the PLZF gene were heterozygous for 7/9, 6/11 and 2/11 cases, respectively. Two newly identified markers within intron 4 (Figure 10.1; Baysal et al., 1997b) were heterozygous in 3/3 (CA) and 7/10 (CTTT) cases, making hemizygosity very unlikely.

We genotyped all the patients of the large Dutch HN-paraganglioma kindred which had shown complete haplotype sharing at the PLZF-flanking markers D11S1792, D11S1327 and D11S908 (Van Schothorst et al., 1996), with these two newly identified markers. These markers did not share an allele among all patients of this family (data not shown). This strongly suggests that the previously observed allele-sharing occurred by chance, instead of identity-by-descent, which is supported by the fact that the frequency of the shared haplotype in a control population was 18% (Van Schothorst et al., 1996).

In conclusion, the complete genomic structure of the human PLZF gene has been elucidated and mapped physically in relation to three Genethon markers and two new polymorphic markers, but a role for PLZF in the development of hereditary HN-paragangliomas could not be demonstrated. In fact,

 Text continues on page 122

Table 10.1. Splice acceptor/donor sites and primer sequences used for amplifying exons.

forwardprimer	splice acceptor site[1] ..	exon	..splice donor site[1]	reverseprimer	size (T_a °C)
*CCGTGGTGATTTGCTAACCT		exon 1 (164 bp)	..G-C-G-g-t-g-a-g-t	AAAAAAGGCACAAGCCCAG	337 (61)
*CCTCTTGCTAAGGGCTTGG	c-c-t-a-g-C-C-T..	exon 2 (1358 bp)		GCTGTCCACCATGATGACC[2]	373 (57)
TCTTCCGTGACCTCTGG[3]			..C-A-G-g-t-a-g-g-c	AGCTAGTCCTACATCAGTCGGG	256 (55)
GATGTGTTTCCATCGGTGC	t-a-c-a-g-G-A-A..	exon 3 (98 bp)	..C-A-G-g-t-a-g-g-c	ACCTTCCATCTAGTGTGTCCG	503 (55)
CCGCTCACCAGTGGACTATT	t-t-c-a-g-C-G-G..	exon 4 (87 bp)	..C-T-G-g-t-g-a-g-t	TGGAAAACCTGGAGAACAGG	177 (55)
ATGCACCTTGTATTCCCACC	c-a-c-a-g-G-C-A..	exon 5 (171 bp)	..C-A-G-g-t-a-g-g-t	GCTCCCAGAGACCCAGATC	267 (55)
GAGAGGCAAAGGCCTGATC	c-a-c-a-g-G-C-G..	exon 6 (168 bp)	..C-A-G-g-t-a-c-c-g	GTCCCAGAGCCAGCTCAG	246 (55)
CAGCTTCCCTGGCACCGCC	c-t-c-a-g-G-T-G..	exon 7 (324 bp)		TCCTTCCTTCATCTCACTCCA[4]	391 (55)

[1] Capital letters represent exonic sequences, small letters intronic sequences. Canonical splice site sequences are underlined. * denotes the M13-sequence which is added for sequencing using the ALF sequencing protocol (CGACGTTGTAAAACGACGGCCAGT). Numbering of cDNA primers is according to Chen et al., 1993b: [2] 126-108, [3] 1048-1065, [4] 2092-2072. Exon 2 fragments amplified using only cDNA primers are as follows: 2afor (-46- -27) rev (741-722), 2bfor (680-697) rev (845-828) and 2cfor (781-800) rev (1116-1097).

Figure 10.2. Intronic sequences (lower case) flanking the PLZF exons (upper case).

Sequence: 1068 bp + untranslated exon 1 (164 bp) + intron 1 (3203 bp) + exon 2 (1358 bp) + partial intron 2 (72 bp). CpGs within 5' sequence are represented in bold. The unique *NotI* site is underlined; the ATG start site is shown in bold and underlined. **GenBank AF076613**

```
   1 ctgcagtcag aggaagagta cattttcttt ggctgctcta ccctctgaag tagggcggcc
  61 agctgaagga ggacacactt ttgaggggcc cagaggttgt ccaagcttcc ccctgccccc
 121 tgaagactgt gcactgagct gggcgcagtt ctcgggaact gtttcaccag attgctgggg
 181 gcgggggtag gataggcagc cgagaggctg tccaaggttt tgggagagag aaaagtttct
 241 cccaggactc gaccttggcc tccagcaatc gcgacagcta aaaacgggtg tctcgcttcg
 301 acatagatcc ccgcggacct tctggcacct ggttcactag cgcccgcgaa ctctgcctcg
 361 ggagacttat tgaaatccgg atgctcaagc cgggaggcgc gcagtaacca ggaggatgag
 421 agggccgggt ttgggctagg aaagcggcct tttggggcag atgtcagggg gactgcagcc
 481 ccgagccatg agaaaaaagt taaaggcgag atgacacgca ctgaattggg gcaaacattg
 541 gaagaggaga caaaactgcg tgcttgagca ccggggtgcg gggaggggag gacaaaaccc
 601 gtatccagtg caaattaaaa tcttgggagt aggtgggggc tgctgcgcgc ccttcaccct
 661 cagttcccct attaaggatt ctgagtcccc atgcactcct gtcctctggc tccttcctcc
 721 ctctccgctc ggccggtgag aggcggccgc cggcgcccca gcagcagcta gatgtcaggt
 781 cgaagcctgg agcgcagagc gcggggaagg gaggggaagg gaggggtggg gaaggagggg
 841 tggggcgggg agcgcgcgcg ggctcggcca gtgcggggtg gtgggcgggc tgagcccggg
 901 gacgcgcggg aggggggagg gaaggggggc agctgtgggc agggagccgg gctcggcgcc
 961 agcactaaag atggagaggc gccggggcct tgcaggggag ggggctcggc gttgacgtgg
1021 gacgcggcgg aggcagcagc cgtggtgatt tgctaacctc gcagcagaGA GGAGTTGAGG
1081 GCGATAGAGC GGGTACTGCA ACTGCCGGGC GATGCTGTCG CTGCCGCCGT GATACGGAGA
1141 GCAACAGTTC CCCAGCAACA CCCCTCCCCG ACACAGGCAC ACACCCCCCG ACAGGCACGC
1201 ACACCCACCC CACAGTGCCC GGCTCGGCTG CGgtgagtga ggggccggga agacggcacc
1261 gcccagcgac gccgcgcgcg gggcttcccg gggggagagg tctgggggcc gcgcgctcgc
1321 ttcggccact cggctgggct tgtgcctttt ttatttggcg tgttccttc ctgccgctgc
1381 agccgtcgcc gccaccggtt ggaggtgggg ggcgtagcgg tgtgcggggg ctgagggcgt
1441 gagcagaggg ctcgggggcg gatccggagc tttgtaccgc cagggctggg cagcaacaga
1501 gcccgtgggt gctcttatgt atgcggaccg gtgcgcgggc agagataagg ttgtggttta
1561 tttatttgtg tgtttattcg ccggccggct gggaagctag aatcggagga gctgacgagt
1621 agatctgggg gcggagggga gcaggactgg gactgcttac gttttgtttc tctttgagaa
1681 aacgtggtgg gctttttctt gattggactt gatccccacc ccccttttgc aggggaggga
1741 gggaagctcc agagggtctg cagcgctgcg ggccctcctc ggctctcggc gggaccggcg
1801 gtgacaccga gctgccgag cgctcccggc gtctcggtgg gtgccggtct ctgtacctga
1861 tgcgttcggg atgccttccc accctggtgg cccgccgtta gctcgaacag cgcctcgtac
1921 actcccgcac gcgcttgaat gcgacggtcc cgcggccgag gaacaacgga cgaacggagc
1981 gctccgcacc gatcgtcccg cctcaccgag acgctcgcac cgtgcttggg ccgggcgcgc
2041 tgcctggcc cgctggccag aggcctggga cccagccggt cgctcccagg gggtcacggc
2101 cctgggtcgg agagggaggg cgggcagacc ccttctcgcc tttcctccca caactcgctc
2161 ggggcttttg tgcttccct tcccgcgggg cgggtccgcc tccctgccg ctctcgccgc
2221 ggagtccagc cgcaccggac tgtcgccgtt cctcccgtc tctttcgctt tccctcgtcc
2281 ctagctcagc tctccttctt tcaggagtct agctcctcgg gaaaagttgc ttccccaagt
2341 ttgctgaagt cgtctccaag tctcggtggg ggtcgctggg aactgggggg gtgtgagagc
2401 gcggtcgatc ccggagctcg ggcgggttat cgccggctct tcccgcgcc gcctctggga
2461 gcctgcagcc agcgccagcc ggatcgcggg ccgagtggtg ggtgggggag acgctgcccg
2521 tgggcgccca gatttccctc aggacccaaa aggcttgttt cagggcgcca agcttccatt
2581 cttttggctg ttcctcctа gttctcagaa accccgatgt ctgcgggaaa gcacgcggtg
2641 gctcggctcg gctgaatgct catcccaacc ttgacgcctc cgtcccaccc ggggcgggca
2701 gcaaaaatgt cttgaccacc gtcctgtgaa tcctttctgc gtagcttggt ggctcctgaa...
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2761 ...ctgatagagc tccttctggg ggtggaggca agcaagcagt gttcgaagag tgagggagag
2821 accttgctgt tccttgctct cagtgcattc actgcaatat tggtcgctgt aagacagaca
2881 cggggagggt cctgggcctg cgcggtcttt ctaacactcc tttaagtgag gttttggagtc
2941 taggagccct cttattgatc ccggacaggc tctgggagag gcctttgttt cccagccctg
3001 tcttcccttc gtactggttg taactcaacc cacaggtgct ggtgccaata tcacttccag
3061 cgcctctgag ccatgtccag catcccttgc tgcccctggt cacaggactt gcagactcct
3121 tggagcctgt gggacacacg tggagttcac acacccagcc cagcatgagc actcctggct
3181 gtaggggacc atatttaact atggtctctc tccatacctt aatgacccat ccttcttcgt
3241 tttttagatc ctcctgtcca ccgggcagtt gtgggcagga gggtaggtac gacaccttac
3301 gtaagtgtgt ggtgtgtaaa cacacacaca ctttttttt ttttttctga gacggagttt
3361 tgctcctgtt gcccaggctg gagtgcagtg gcgcaatctc ggctcacggc aacctccgcc
3421 tcccggtttc aagtgattct cctgcctcag cctcccgagt agctgggatt acaggcgggt
3481 gccaccgcgc ctggctaatt tgtatttttg tagagacggg cttctccatg ttggtcaggc
3541 tggtctcgaa ctcccgacct caggtgattc cgcctgcctg ggcctcccaa agtgctggga
3601 ttagaggcgt gagccaccgc gccctgcccc acacactctt tctgtctgtc tctggctggt
3661 gtacatacac acagttcaga ttttatatta tacatacaat aactttgagg cttccgtggg
3721 tagaaatgac ccaagttagt gtgtgggttg tgtgtgttcc tgttagtacc tagggatggg
3781 gacaggcctt gggtctgcca gcgtttgcag ccctgagaac agtagaaggc cagaggccaa
3841 ggctgatggt gagaggatag cctttgcgga ggagagccca gtgtccatag atttctgtgg
3901 ccatggctaa caggctcctg gctccaatga gtttgactct gcttctgggg ctcctgtagt
3961 gaggcagagt ttggaaaaac tgtttaaaga taaggcttga ccaatccagg ggctggcgtg
4021 aagctgacaa ggaaggggct aaagtcttgc taattttaag taatgcgcat tcatcataat
4081 gaatgttgcc tagcctaggg agcccctact ctcagacaca gagaaagctg aggacttata
4141 ggacttcact acaattttca tttcacttca tctaacagat gtcatagagt agtgaattag
4201 gcatctgctg tggcagaacc ttcttttтac tctcagcatc cctggcttga aaggcagatt
4261 agtagatttc taggaagcta cttcaattaa aatctctaag atcctcttgc taagggcttg
4321 gcaacaggga ggaggggcca tgttgtagtg gttgaattct tacttttagg gacactgatg
4381 aatttgtctt ttgttctctc atctcttttg cttcttcccc tcttctttct cctagCCTCC
4441 TCTATTGGCC CAGGAAGCCC ACCCAGCCCC GCCTCGCAGA GCCCAGAAGG AAAGAAAGCC
4501 TCATGCCTGA GCCGAGGGGA GCACCATGGA TCTGACAAAA ATGGGCATGA TCCAGCTGCA
4561 GAACCCTAGC CACCCCACGG GGCTACTGTG CAAGGCCAAC CAGATGCGGC TGGCCGGGAC
4621 TTTGTGCGAT GTGGTCATCA TGGTGGACAG CCAGGAGTTC CACGCCCACC GGACGGTGCT
4681 GGCCTGCACC AGCAAGATGT TTGAGATCCT CTTCCACCGC AATAGTCAAC ACTATACTTT
4741 GGACTTCCTC TCGCCAAAGA CCTTCCAGCA GATTCTGGAG TATGCATATA CAGCCACGCT
4801 GCAAGCCAAG GCGGAGGACC TGGATGACCT GCTGTATGCG GCCGAGATCC TGGAGATCGA
4861 GTACCTGGAG GAACAGTGCC TGAAGATGCT GGAGACCATC CAGGCCTCAG ACGACAATGA
4921 CACGGAGGCC ACCATGGCCG ATGGCGGGGC CGAGGAAGAA GAGGACCGCA AGGCTCGGTA
4981 CCTCAAGAAC ATCTTCATCT CGAAGCATTC CAGCGAGGAG AGTGGGTATG CCAGTGTGGC
5041 TGGACAGAGC CTCCCTGGGC CCATGGTGGA CCAGAGCCCT TCAGTCTCCA CTTCATTTGG
5101 TCTTTCAGCC ATGAGTCCCA CCAAGGCTGC AGTGGACAGT TTGATGACCA TAGGACAGTC
5161 TCTCCTGCAG GGAACTCTTC AGCCACCTGC AGGGCCCGAG GAGCCAACTC TGGCTGGGGG
5221 TGGGCGGCAC CCTGGGGTGG CTGAGGTGAA GACGGAGATG ATGCAGGTGG ATGAGGTGCC
5281 CAGCCAGGAC AGCCCTGGGG CAGCCGAGTC CAGCATCTCA GGAGGGATGG GGGACAAGGT
5341 TGAGGAAAGA GGCAAAGAGG GGCCTGGGAC CCCGACTCGA AGCAGCGTCA TCACCAGTGC
5401 TAGGGAGCTA CACTATGGGC GAGAGGAGAG TGCCGAGCAG GTGCCACCCC CAGCTGAGGC
5461 TGGCCAGGCC CCCACTGGCC GACCTGAGCA CCCAGCACCC CCGCCTGAGA AGCATCTGGG
5521 CATCTACTCC GTGTTGCCCA ACCACAAGGC TGACGCTGTA TTGAGCATGC CGTCTTCCGT
5581 GACCTCTGGC CTCCACGTGC AGCCTGCCCT GGCTGTCTCC ATGGACTTCA GCACCTATGG
5641 GGGGCTGCTG CCCCAGGGCT TCATCCAGAG GGAGCTGTTC AGCAAGCTGG GGGAGCTGGC
5701 TGTGGGCATG AAGTCAGAGA GCCGGACCAT CGGAGAGCAG TGCAGCGTGT GTGGGGTCGA
5761 GCTTCCTGAT AACGAGGCTG TGGAGCAGCA CAGgtaggcc ccgctccagc cccgcacctg
5821 atgtaggact agctacacct ctacactgtt cataggctgt ccttt
```

Sequence: clB633B (intron 2). **GenBank AF076614**

```
   1 taaagcctcg tctctcatct attcactaga gctcatatct agagtagagc atgagcttgc
  61 agtcagaatc gtctgattaa gttcagctct gatgtttaat agatctacag tctgcagtaa
 121 ggcacttaat ctctctgaac ctcagtttca agtccacaga aattgggaaa ataatagcac
 181 ctgcattaat aggattgtta tgtggattaa ataaaacaac ataggtaatg tgttgagttt
 241 cgtagtcagt gctcagaatg agttattatt ttcgctgtct ccttcctgta t
```

Sequence: partial intron 3 (145 bp) + exon 4 (85 bp) + partial intron 4 (196 bp). **GenBank AF076615**

```
   1 tctgagccat ggatgatccc tcactcagat ttttgcgggt gtccagtccc cttccctagt
  61 gtctacctgc acagttgtgg cccaggacct ccccgctcac cagtggacta ttgctctagt
 121 ggtaactgcc tctcctttct ttcagCGGGT GCCAAAGCCT TTGTCTGTGA TCAGTGCGGT
 181 GCACAGTTTT CGAAGGAGGA TGCCCTGGAG ACACACAGGC AGACCCATAC TGgtgagttg
 241 acttggattc ctgttctcca ggtttttccac agtgttggta gcacatggac atgaactgtc
 301 tgggtggcaa cctcttttta gcaaatgtga catccagtaa tttcctgggc gagctgtgag
 361 taggatgggc ttctgccacg ttcagctact cttgtctgtg tggctggcca gcaggagtta
 421 caagtatg
```

Sequence: partial intron 6 (124 bp) + exon 7 (324 bp) + 3'UTR (127 bp). Translational stop is represented in bold and underlined; the poly-A signal is underlined. **GenBank AF076616**

```
   1 aagttctgtt ggagcaagcc cagctggagg aacccagctt ccctggcacg cctgagggtg
  61 acatggtgcc ctcaccgcct tctgtctgtc ctcactttct cctgccctgt ccctccgccc
 121 tcagGTGAGA AGCCCTTTGA GTGTAAGCTC TGCCACCAGC GCTCCCGGGA CTACTCGGCC
 181 ATGATCAAGC ACCTGAGAAC GCACAACGGC GCCTCGCCCT ACCAGTGCAC CATCTGCACA
 241 GAGTACTGCC CCAGCCTCTC CTCCATGCAG AAGCACATGA AGGGCCACAA GCCCGAGGAG
 301 ATCCCGCCCG ACTGGAGGAT AGAGAAGACG TACCTCTACC TGTGCTATGT GTGAAGGGAG
 361 GCCCGCGGCG GTGGAGCCGA GCGGGGAGCC AGGAAAGAAG AGTTGGAGTG AGATGAAGGA
 421 AGGACTATGA CAAATAAAAA AGGAAAAGaa aaaaaaaaaa ggaaggaaaa ggaaaacctg
 481 gtagcaaaaa cggccttggt ttctctctgg gctccagatg ccctggctgc caagccactg
 541 cccctcctct gggctnncct ccacctcctc tcctgg
```

genotyping of the two intron 4 markers indicated that PGL1 does not map in the immediate vicinity of PLZF, and that the haplotype sharing in this region in family FGT189 was a false-positive signal. Recombinant-mapping in two HN-paraganglioma families places PGL1 in a ~10 cM candidate region, defined by D11S1647 and D11S908 (van Schothorst et al., 1996; Baysal et al., 1997a). Haplotyping within this region with additional markers at a high-resolution spacing should be performed in order to find more significant evidence for the location of PGL1.

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11

DISCUSSION

Within this thesis, the efforts to clone the disease gene PGL1, which is linked with hereditary HN-paragangliomas, and the first steps to unravel the tumor biology are described. Although the actual cloning of this gene and detection of mutations has not been achieved, several positive results with their implications have been accomplished. A number of aspects have been discussed elsewhere, some of which needing extra attention, will be further discussed here. Finally, I will describe different models which we believe might explain the inheritance pattern and growth of these tumours.

The discovery of a founder effect for hereditary paragangliomas of the head and neck, has a great advantage for the families which are too small on their own to offer genetic counselling based on lod scores. The haplotype sharing as observed for the candidate gene region is now not only applicable for individuals belonging to family FGT189 seeking counselling (Oosterwijk et al., 1996), but also for the individuals from the other Dutch HN-paraganglioma families from South-West Holland (chapter 5). The frequency of this haplotype is so low in the general population that counselling solely based on haplotyping is acceptable. However, the definitive cloning of the disease gene PGL1 and its mutation will most likely circumvent the laborious workload for identifying the disease-linked haplotype (with its possible recombinations) and offer the possibility to determine the presence of the mutation unambiguously.

Positional cloning of a disease gene, like PGL1, highly depends on several aspects to succeed. Some, which influence the outcome, are given facts: the age-dependent penetrance of the disease for example. In our case, if all mutation-carriers would suffer from HN-paragangliomas, unambiguously assignment of the disease-haplotype is possible. However, several individuals were identified which can either be non-penetrant gene carriers or non-affected individuals. This duality clearly hampers identification of recombination sites refining the candidate gene region, since an individual with a recombination within the previous candidate gene region might develop these tumours after another 10 or 20 years of life or not at all. Unambiguous assignment of tumour growth (affected) clearly has its impact as well, although use of techniques as MRI and CT greatly facilitated this. As an example, a 75 year old man had no complaints, although the family pedigree indicated this person to be a paternal gene carrier having affected offspring. Using MRI, bilateral carotid body tumours were identified.
Another aspect is the incidence of the disease. With a rare disease, it is difficult to ascertain enough families to detect recombinations within the candidate gene region to refine the position of the disease gene. We were largely supported by the genealogical discovery of a shared ancestral origin for three families (FGT189, chapter 3). However, even with a reasonable number of families, the occurrence of recombinations within the candidate gene region in patients is still a matter of chance. In chapter 6, we conclude that the possibility of recombination-suppression for the interval D11S1647-D11S2077 seems unlikely, although we do not see any recombinant in patients for the disease chromosome. However, since it seems that we do see recombinational differences for the chromosomal region 11q22-q23, other possibilities have been analysed.

Recombination suppression in PGL1 chromosomes

Thus far, by recombinant analysis the candidate gene region could finally be refined to a 6 cM region between the markers D11S1647 and D11S2077 (Baysal et al., 1997a, chapter 9). Further refinement was achieved by haplotype sharing in family FGT189 to an approximately 2 cM region (D11S1986-

D11S897, chapter 6), although initial haplotype sharing analysis, due to insufficient marker density, led to a slight mis-location of the PGL1 region (see below). During the search for the minimal candidate gene region, as described in chapters 2, 3 and 6, a striking observation was made: meiotic recombination of the 11q22-q23 region on the disease-chromosome seems to be suppressed. Within a region of 16 cM (as defined by the markers STMY and CD3D, chapter 2), approximately 16 recombinations per 100 meioses should be observed, while we observed only two 'hard' recombinants per 246 meioses. The haplotype-sharing of only a small number of markers between all patients of family FGT189 indicates that several recombinations have occurred, based on the assumption of only one ancestral haplotype and mutation, but unfortunately this only could be inferred to have happened in previous generations (chapter 3). To investigate whether this recombination suppression is sex-specific, disease-specific, or both, marker data from 19 families were analysed.

In general, the male recombination map for chromosome 11 is smaller than that for the female (110.8 vs 179.6 cM, sex-average 145.3 cM; Litt et al. 1995), and for the 11q22-q23 region between the markers D11S927 and CD3D this is 4.9 (male) vs 19.6 (female) cM. Nevertheless, recombinations in male meioses are observed in this region. Even for the specific candidate gene region as defined by recombinant analysis, which corresponds approximately with D11S927-D11S908, these numbers are 3.5 cM vs 8.6 cM.

All meioses were counted disease-associated when the specified parent carries a disease-chromosome, according to the family-branch, phenotype or haplotype-sharing. This chromosome can be either paternal (affected or non-penetrant) or maternal (imprinted). Within the 16 cM chromosomal region, a complete homozygous genotype has never been observed in families, indicating the presence of only one founder chromosome (data not shown). The statistical analysis (χ^2-test) revealed statistical significant suppression of recombination on the disease-chromosome in the interval D11S927-CD3D as compared with recombination in male or female non-disease chromosome meioses ($p<0.002$) in family FGT189 (Table 11.1). Between male and female disease-chromosome recombination a significant difference could be detected ($p<0.006$), suggesting that this phenomenon is gender-dependent i.e. a higher suppression in males.
Unfortunately, this significance drops ($p>0.09$) when the other families are analysed, mainly due to the relative low number of female disease meioses and male non-disease meioses. If the families showing a founder effect are analysed separately, since they are assumed to be due to the same mutation, a

Table 11.1. Recombinant analysis between D11S927 and CD3D.

		FGT189			all families			founder families		
		rec[1]	non-rec[1]	tot meioses[2]	rec	non-rec	tot meioses	rec	non-rec	tot meioses
female	ndis[3]	33	99	132 (25)	54	220	274 (19)	37	183	220 (17)
	dis[3]	3	22	25 (12)	5	28	33 (15)	5	26	31 (16)
male	ndis	17	46	63 (27)	21	80	101 (20)	17	77	94 (18)
	dis	9	85	94 (9)	28	185	213 (13)	16	145	161 (10)
total		62	252	314 (19)	108	513	621 (17)	75	407	482 (15)

[1] (non-)rec: (no) recombinations observed; [2] total number of meioses with percentage in brackets; [3] ndis/dis: non-disease/disease meiosis. Founder families are described in chapter 5.

statistically significant difference is observed ($p<0.050$) in suppression of recombination in chromosomal region D11S527-D11S836 (11q13-q24), although no significance is reached ($p>0.14$) for the smaller candidate region D11S927-CD3D. This suggests that other phenomena, outside the candidate region, might play a role in recombination and analysis of more pedigrees and thus more meioses should lend support to the described observations. In that way, smaller candidate regions might provide evidence for specific suppression of meiotic recombination.

An explanation for recombination suppression could be a (small) chromosomal inversion, since this affected region pairs difficult with its homolog during meiosis (Hartl, 1975). We therefore analysed karyotypes of 13 probands and 16 sporadic patients by chromosomal G-banding, but could not detect any aberration (data not shown). This is in agreement with the results found in an independent analysis of three patient samples (Zaslav et al., 1995). Fluorescence in situ hybridisation, using YACs and cosmids from the candidate gene region plus flanking regions, did neither indicate any aberration. Induction for fragile sites by folic acid, to map fragile sites due to e.g. triplet instability, did not show any fragile site within this chromosomal fragment.

Another possibility for suppression of recombination could be the loss of a recombination hot-spot (Purandare and Patel, 1997), influencing the probability of recombination within a large flanking region. Although this has not been confirmed or excluded, other, as yet unknown, phenomena might play a role here.

Important for the assignment of recombinants is the density of the marker-maps. For chromosomal region 11q22-q23, almost all known markers have been analysed to identify concordant/discordant haplotypes in the families. These markers are spaced on average by approximately 1.5 cM (Fain et al., 1996), but for refined localisation, usage of point-polymorphisms in a PCR set-up (Kwok et al., 1996; Kruglyak, 1997) might attribute largely to identify the smallest region for PGL1.

The haplotype sharing in family FGT189, as initially observed for the markers D11S1792 and D11S1327, has been misleading, which was partly attributed by the fact that its population frequency is quite high with 48.6%. Therefore, we initiated a search to find more markers to support this location and reduce the specific disease linked haplotype-frequency. Two new markers within the PLZF gene (chapters 9, 10), however, do not co-segregate with the disease in family FGT189, thereby creating a zebra-pattern with the two markers D11S1792 and D11S1327. Since these two markers have a high frequency for the disease-linked allele, this represents coincidental co-segregation. This is supported by the results as reported in chapter 6, where we report on the finding of an 8 marker haplotype segregating in family FGT189, and this interval is more proximally located. This new location of the PGL1 disease gene is still within the region comprised by the markers analysed in the genetic heterogeneity analysis, namely between D11S927 and D11S1327 and it will most likely not influence the observed genetic heterogeneity.

Family FGT2 which showed linkage to 11q13 does not show linkage to this new proximal gene region either and the question whether there are two genes or only one gene involved with HN-paragangliomas remains unanswered. The possibility remains that somewhere within this new interval there is a very small interval in which the disease mutation resides which is shared between all families, including family FGT2 showing linkage to 11q13. Such a small shared interval, at most 0.010 cM (10 kb), has

been observed flanking the D13S232 marker co-localizing with the γ-sarcoglycan gene involved in severe childhood autosomal recessive muscular dystrophy (Piccolo et al., 1996). A second gene involved in hereditary HN-paragangliomas might be possible, although more families are needed to support this localization. It remains, however, curious that such a rare disease can be caused by two loci of which one locus is assigned on the basis of only one kindred.
Analysis of candidate genes within the region will eventually reveal the true disease gene(s); for the 11q13 region the recently published sequence containing several genes near marker PYGM will be helpful (Kedra et al., 1997).

Models for tumorigenesis

The function of the putative disease gene has been partly elucidated by the LOH analyses as described in chapters 7 and 8. The complete LOH as observed in the sorted aneuploid DNA fractions and the micro dissected chief cells in a small subset of tumours might indicate that the PGL1 gene acts as a tumour suppressor gene as explained by the model of Knudson for tumorigenesis (Knudson 1971). Analysis of hereditary tumours not showing aneuploidy should reveal, once the candidate gene is identified, whether the wildtype allele is inactivated or silenced by, for instance, point mutations or epigenetic silencing.

To propose a model for tumorigenesis for PGL1, several conditions have to be fulfilled. First, tumours only arise after paternal transmission, and never after maternal transmission; the mono-allelic expression of the gene should be reversible as explained by genomic imprinting; a grandmaternal disease gene will only give rise to tumorigenesis after paternal transmission (van der Mey et al., 1989). Secondly, the inheritance pattern is autosomal dominant. Thirdly, the disease is rare, with an incidence in the Netherlands between 1 in 100,000 and 1 in 1,000,000 (Oosterwijk et al., 1996). Fourthly, most tumours showed only partial LOH for 11q22-q23 (chapter 7; Baysal et al., 1997a), but this was shown in a set of tumours to be complete LOH in the chief cells, suggesting a loss-of-function (chapter 8). Also, fifthly, the allele with reduced intensity (and thus likely loss) was always of maternal origin, as observed in a total of 13 tumours (chapter 7; Baysal et al. 1997a).

In the past few years several models have been postulated to explain the inheritance pattern (van der Mey et al., 1989; Hulsebos et al., 1990; Heutink et al., 1992; Mariman et al., 1995; Baysal et al., 1997a). Of these, the models as described by Mariman et al. (1995) and Baysal et al. (1997a) will be briefly discussed since they also include the specific parental LOH, whereafter we will postulate our views and probabilities.

An interesting model has been proposed by Mariman et al (1995), in which they take into account the two genes PGL1 and PGL2 for tumorigenesis. In a normal situation, the two maternal copies are inactive due to genomic imprinting, while both paternal copies are active. If a mutation leading to inactivation of the gene is inherited maternally, this will not lead to tumour growth due to the inactivation by the imprint. However, why should it not be possible to loose the paternal 11q13-q23 arm thus leading to complete silencing of all four gene copies? This loss can be due to either LOH or UPD mat (2 maternally inactive copies). However, UPD or loss of this large chromosomal interval might be intolerable due to the loss of other, specific control genes within this chromosomal segment. This region is not indicated on the human UPD map, although only regions with UPD leading to a different phenotype are mentioned (Ledbetter and Engel 1995).

In the case of an inherited paternal mutation in the PGL2 gene, a second mutation in the remaining active PGL1 gene is necessary. To account for the observed maternal LOH, they suggest that loss of imprint (LOI) of the maternal PGL1 allele is necessary, followed by LOH inactivating this gene copy. A main disadvantage of this model is obvious: if the active paternal PGL1 gene is inactivated by a mutation or LOH (paternal), no active tumour suppressor gene remains and tumour growth should be possible. Both conditions have thus far not been observed. LOI has been observed for IGF2 in tumorigenesis (Feinberg 1993), but this is more compatible with a proto-oncogene with a function as growth factor than with a tumour suppressor gene for which loss-of-function is necessary.

To account for this LOI and a proto-oncogene, Baysal et al. (1997a) proposed two possible models. Their first model deals with one imprinted oncogene PGL1 (due to the mutation), which is only paternally active. An active, non-imprinted tumour suppressor gene is located nearby. Loss of the maternal active tumour suppressor gene is necessary and should not involve the loss of the proto-oncogene PGL1. However, two genes are necessary to explain tumorigenesis and the paternal active tumour suppressor gene remains.

The second model proposes that the secondarily deleted tumour suppressor gene might be imprinted reciprocally (i.e., the maternal allele would be transcriptionally active) to the PGL oncogene that is mutated in the (paternal) germline. This would imply that always one inactive maternal tumour suppressor gene is present, which would be equivalent to the first hit in tumorigenesis and a single somatic mutation is sufficient for (sporadic) tumours to develop. This might suggest that the incidence of HN-paragangliomas would probably be higher than observed, although the number of cells within the specific paraganglia in the head and neck region could be that low that chances of inactivating one copy are very low.

We therefore propose the following model: a normally non-imprinted tumour suppressor gene becomes subjected to imprinting due to a mutation within the regulatory sequence. Two inactivating mutations are needed for sporadic tumours to develop, based on the Knudson model and the low tumor incidence. If the mutation is inherited paternally, the tumour suppressor gene PGL1 is proposed to be inactivated and the second loss will occur by maternal LOH. Oppositely, when the mutation is inherited maternally, the gene is normally active and two somatic mutations are needed for tumorigenesis, thus explaining that no tumour growth is yet observed after maternal transmission. This, if true, would be the first example of a mutation rendering a non-imprinted gene into an imprinted copy. It would also imply that a candidate gene should not only be screened in the coding region, but also in the regulatory regions like the promoter and introns. The importance of intronic sequences has been underestimated and should be a warrant for all positional cloning efforts, especially when mutational screening of candidate genes is undertaken (Engelberg-Kulka et al. 1993; Moore 1996; Tycowski et al. 1996).

Moreover, this model implies that sporadic patients are very rare. At least 50% of the cases are believed to be inherited (van der Mey et al., 1989). Skipping of generations after maternal transmission might be the main reason for not knowing the familial inheritance pattern. Haplotyping of 31 'sporadic' Dutch patients without known familial inheritance for markers on chromosome 11q22-q23 indicated that most, if not all, patients do show some identical alleles as observed in the founder families for markers NCAM to D11S1885, but no shared region can be defined (data not shown). Since the phase is unknown in most cases (no parents available), this number might be lower. Typing of markers in the new candidate gene region (D11S1986-D11S897) should reveal whether there are one or more marker-alleles identical in all cases, which might point to the region of interest. A strong founder effect will

also account for the low general incidence, next to the possibility of several mutations causing imprinting at this gene.

Another model implies that the specific mutation could induce a function as an imprinting centre comparable with mutations at the imprinting centre on chromosome 15q13 influencing imprinting of genes at distances up to 1 Mb (Ferguson-Smith 1996). This would imply that genes located 'far' away from this imprinting centre, like NCAM, DRD2 and PLZF, might thus be affected in regulation of expression in tumour tissues, while no involvement is assumed according to discordance of polymorphic markers near these genes. This is also in agreement with the non-imprinting status at the moment of this chromosomal region, but seems less likely compared with the previous model. Expression analysis of these genes in tumours could be done to analyse this possibility.

An alternative model, but less likely, assumes an imprinted tumour suppressor gene being maternally active. Normally, this imprint is lost during/after embryogenesis, leading to two active gene copies in the adult. Due to the mutation at the PGL1 gene, paternal LOI is impossible thus keeping this copy inactive. Maternal LOH will be the second necessary step to inactivate the tumour suppressor gene function completely. A disadvantage of this model is the fact that a tumour suppressor gene will be inactive due to the imprinting during embryogenesis and LOH of this region within this timespan might result in tumorigenesis. This is in contradiction to the age-of-onset seen in patients. Secondly, LOI should occur in all individuals after embryogenesis and such an event has not been observed thus far.

As soon as the putative tumour suppressor gene PGL1 is cloned, the validity of the different models can be tested, and this will significantly contribute to the insight in the mechanism of genomic imprinting and how this can explain the particular hereditary transmission of HN-paraganglioma.

ABBREVIATIONS

AI(F)	allelic imbalance (factor)
AS	Angelman syndrome
bp	basepair(s)
BWS	Beckwith-Wiedemann syndrome
cDNA	complementary DNA
cen	centromere
cM	centiMorgan
CT	computed tomography
DNA	deoxyribonucleic acid
DRD2	dopamine receptor D2 gene
EST	expressed sequence tag
FGT	familial glomus tumor
HN-paraganglioma	head and neck paraganglioma (familial)
IC	imprinting centre
IGF2(R)	insulin-like growth factor 2 (receptor) gene
kb	kilobase (1000 bp)
LD	linkage disequilibrium
lod	likelyhood of odds-ratio
LOH	loss of heterozygosity
LOI	loss of imprinting
mat	maternal
Mb	megabase (1000 kb)
MMR	mismatch repair
MRI	magnetic resonance imaging
mRNA	messenger RNA
NCAM	neural cell adhesion molecule gene
(O)MIM	(online) Mendelian inheritance in man
pat	paternal
PCR	polymerase chain reaction
PGL1	paraganglioma gene 1; located on chromosome 11q22-q23
PGL2	paraganglioma gene 2; located on chromosome 11q13
PLZF	promyelocytic leukaemia zinc finger gene
PWS	Prader-Willi syndrome
RNA	ribonucleic acid
s.r.o.	smallest region of overlap
STS	sequence tagged site
tel	telomere
UPD	uniparental disomy
VHL	Von Hippel Lindau gene
YAC	yeast artificial chromosome

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Summary

Paragangliomas are rare, usually benign slow growing tumours. They originate from the tiny glomus bodies present throughout the body as part of the parasympathic nervous system. The most common types in the head and neck, denoted as HN-paragangliomas, are the carotid body tumour, the glomus jugulo-tympanic tumour and the vagal body tumour. The inheritance pattern in familial cases shows a non-Mendelian inheritance due to genomic imprinting: only after paternal transmission do tumours arise. Although these tumours are mostly benign, their location and growth pattern might be a reason for surgery. A main disadvantage is the neurologic damage being done. Early detection of tumour growth and identification of carrier status (those at risk) might help in the medical guidance. Identification of the underlying disease gene will be very helpful in this regard. As no clue of whatsoever about the function of PGL1 is known, the strategy of positional cloning was undertaken. Within this thesis, the results of linkage analyses to map the disease gene PGL1 and the subcloning of the candidate gene region with its genes are discussed. Besides, results of analyses of tumour DNA to reveal the putative function of the PGL1 gene are discussed.

Previously, the disease gene PGL1 has been mapped proximal of marker D11S836 on chromosome 11q22-q23 within one Dutch family (FGT1; Heutink et al. 1992). Recombinant analysis to reduce the candidate gene region resulted finally in a 2 cM region (**Chapters 2, 3**). Important in this assignment was the genealogy study showing that two Dutch families of Roman-Catholic origin living in the same village as FGT1 were in fact related to each other, resulting in a large 7 generation family (FGT189). Since no new hard recombinants were detected, we had to rely on haplotype sharing between all patients in the different branches to map the PGL1 gene. In this way, all patients are considered to be due to the same ancestral mutation (identical by descent) and non-sharing of markers resulted from ancestral recombinations only. This resulted in a 2 cM region between D11S938 and D11S1885, thus excluding the genes neural-cell-adhesion-molecule (NCAM) and the dopamine-receptor D2 (DRD2) as possible candidate genes (**Chapter 3**). The haplotype frequency defined by the three co-segregating markers was, however, 18% and could therefore be shared by chance.

At the same time, another Dutch family (FGT2) was shown to be linked to a separate region on 11q13 (Mariman et al. 1993, 1995) and this family excluded the PGL1 region. We initiated a genetic heterogeneity analysis for the two possible candidate loci PGL1 on 11q23 and PGL2 on 11q13; both two loci are separated by a genetic distance of at least 35 cM. Seventeen families were included in the analysis and there was significant evidence for heterogeneity with 93% of the families linked to PGL1. However, when the single kindred linked to PGL2 was excluded from the dataset, all other 16 families were shown to be linked to PGL1 (**Chapter 4**). We therefore concentrated on the chromosomal region 11q22-q23 only.

Haplotype analysis indicated that a set of Dutch families shares a large part of the chromosomal 11q22-q23 region, indicating that they share most likely a common ancestor. However, genealogical studies back to 1800 did not reveal any familial connection, although all these families originated in South-West Holland (**Chapter 5**).

Physical mapping of the chromosome 11q22-q23 region encompassing the 2 cM PGL1 locus identified several markers and excluded possible candidate genes/expressed sequence tags (ESTs) (**Chapter 9**). It also resulted in a "zebra-pattern" of segregating and non-segregating markers for family FGT189.

Since now only two neighbouring markers showed haplotype sharing in family FGT189, we concentrated on the region around the segregating markers D11S1327 and D11S1792. Within this region, the promyelocytic zinc finger (PLZF) gene was mapped by radiation hybrid mapping (James et al. 1994). This PLZF gene functions as a transcription factor and its expression is observed at the branchial arches during murine embryonal development, which coincides with the location of the HN-paragangliomas found. Elucidation of the genomic structure and subsequently mutational analysis of HN-paraganglioma patients indicated no aberration at all (**Chapter 10**). Two new polymorphic markers were located within intron 4, PLZF-CA and PLZF-CTTT, which both turned out to be discordant within family FGT189, most likely excluding this region as the PGL1 locus.

We therefore analysed other markers located between the two markers which defined the candidate gene region by recombinant analysis: marker D11S1647 at the proximal side and marker D11S2077 at the distal side. Finally, indeed, 8 segregating markers were identified in a 2 cM region. This region is more proximally located. The identified 8 marker disease-haplotype was never seen in unrelated individuals, supporting this new localisation. Detailed analysis, including linkage disequilibrium analysis, of a set of 25 families indicated that a founder effect might be influencing a large part of all Dutch HN-paraganglioma families (**Chapter 6**). Before subcloning of this region and identification and screening of candidate genes to identify the PGL1 gene is undertaken, careful examination and extended analysis of all family marker data (linkage disequilibrium analysis) should be performed. This analysis should reveal the smallest candidate gene region, excluding the labourious work of subcloning a too large region.

Since the tumour biology is intriguing behind those slow growing HN-paragangliomas, we started analysis of total tumour DNA for loss of heterozygosity (LOH). In general, non-random LOH as observed in total tumour DNA compared with the patient lymphocyte DNA indicate the presence of a tumour suppressor gene. LOH of 11q22-q23 will thus only be detected in the case the PGL1 gene acts as a tumour suppressor gene.
This analysis resulted in the observation of detectable losses for predominantly chromosome 11q22-q23. There were also tumours showing a repetitive pattern of loss *vs* non-loss, a so-called zebra-pattern in which case no exact location can be deduced. In all cases was, however, only partial LOH observed, which hampered the conclusion whether we were dealing with a tumour suppressor gene or not. In a total of 8 tumours where the parental origin of the 'lost' allele could be assigned, it was always the maternal (wildtype) allele that had reduced intensity. Furthermore, of 3 tumours informative for the X-inactivation analysis, 2 were shown to be polyclonal of origin (**Chapter 7**). These results did not answer the question whether these tumours are monoclonal of origin with a possible subpopulation showing polyclonal growth. We therefore extended the LOH analysis to the sorted aneuploid fractions of three tumours and these fractions showed complete LOH, while the diploid fractions -as expected- did not (**Chapter 8**). Single-cell microdissection was applied to obtain chief cells (one of the two cell types in a HN-paraganglioma) and LOH-analysis indicated that these cells also showed complete LOH. Thus, PGL1 acts as a true tumour suppressor gene and the chief cells are a major, if not the only neoplastic component of HN-paragangliomas. Furthermore, the complete LOH in the chief cells indicate that these cells are monoclonal in origin. Examination of sustentacular cells should reveal their genetic origin and elucidate the scientific discussion about these tumours and their origin and growth patterns.

Finally, in the general discussion, we propose a model to explain the characteristics of these

tumours, including the specific losses and the observed genomic imprinting, in which the PGL1 gene is rendered by the mutation into an imprinted tumour suppressor gene. This will result in (wildtype) expression only after maternal transmission. But, if this model is true, it will imply that screening of candidate genes should be mainly performed within promoter and intron regions to localize this imprint-box.

SAMENVATTING VOOR LEKEN

Het in dit proefschrift beschreven onderzoek heeft als doel om de erfelijke factor voor paragangliomen in het hoofd/hals gebied te identificeren.

Paragangliomen zijn tumoren van het parasympatische zenuwstelsel, dat onder andere betrokken is bij het bewaken van de zuurstofconcentratie in het bloed. Deze tumoren kunnen door het gehele lichaam voorkomen, maar de erfelijke vorm betreft vooral tumoren die in het hoofd/hals gebied voorkomen, de HN-paragangliomen. HN-paragangliomen zijn onder andere te vinden bij de splitsing van de halsslagader (de carotid body tumor), in het middenoor (de glomus vagale tumor) of aan de schedelbasis (de glomus jugularis tumor). De meerderheid van deze tumoren is goedaardig en groeit langzaam. Er wordt geschat dat de jaarlijkse incidentie van deze tumoren tussen de 1 op de 100.000 tot 1 op de 1.000.000 personen is (Oosterwijk et al., 1996).

Van een aantal HN-paraganglioom patiënten is bekend dat ook andere familieleden deze tumoren hebben wat duidt op een erfelijke factor. Het interessante aan de overerving is dat de tumoren alleen ontstaan als deze erfelijke factor door de vader wordt doorgegeven. Als een moeder de factor doorgeeft ontstaan nooit tumoren maar haar erfelijk belaste zoon kan wel weer aangedane kinderen hebben. Zo'n ouder-afhankelijk effect wordt genomische imprinting genoemd.

Elk individu bestaat uit een zeer grote collectie van uiteenlopende typen cellen. Om te kunnen functioneren moet elke cel beschikken over de informatie die nodig is om al zijn moleculaire onderdelen te kunnen maken. In plaats van dat cellen nu alleen de informatie bevatten die voor hun specifieke functie nodig is b.v. voor het maken van specifieke spiereiwitten in spiercellen en lenseiwit in cellen van de ooglens, bevatten alle cellen de informatie voor alle celtypen. Echter alleen de voor hun specifieke functie noodzakelijke informatie is toegankelijk en kan worden gebruikt voor de productie van eiwitmoleculen die de bouw en functie van het celtype bepalen.

Deze informatie ligt opgeslagen in het DNA, dat de basen (letters) A, G, C en T bevat. Lange strengen basen (in wenteltrapvorm) vormen chromosomen, waarvan ieder individu 23 paren heeft: van chromosoom 1 t/m 22 één van de moeder en één van de vader, en twee geslachts-chromosomen (XX of XY). Tijdens de vorming van de geslachtscellen, de eicellen en zaadcellen, wordt het aantal chromosomen in tweeën gedeeld, zodat na samensmelting van de twee geslachtscellen elke cel in de embryo weer in totaal 46 chromosomen heeft. Tijdens de vorming van de geslachtscellen kunnen er stukken DNA uitgewisseld worden tussen de identieke chromosoomparen; dit wordt recombinatie genoemd. Het DNA codeert voor alle macromoleculen (eiwitten) die het lichaam nodig heeft, waarbij iedere opeenvolging van drie basen één onderdeel hiervan, een aminozuur, representeren. Het gehele stuk DNA dat codeert voor één zo'n eiwit heet een gen. Hoewel de overeenkomstige chromosomen in elk van de 21 paren lichaamschromosomen in principe dezelfde erfelijke informatie bevatten zijn er toch geringe verschillen die zorgen voor de variatie in erfelijke eigenschappen. Ook in de niet-coderende stukken DNA van de chromosomen komen verschillen voor b.v. variaties in de lengte van gerepeteerde base sequenties zoals CACACA... of TATTATTAT..... die ons in staat stellen de vaderlijke en moederlijke chromosomen van een paar te onderscheiden. Deze gerepeteerde DNA fragmenten liggen verspreid over alle chromosomen en zijn daarom zeer bruikbare markeringspunten ("markers") om een chromosomale positie (locus) aan te duiden. De lengte van deze markers varieert

met het aantal keren dat het base sequentie CA of TAT gerepeteerd is. De varianten van een bepaalde marker worden "allelen" genoemd. Elke persoon heeft dus voor iedere marker twee allelen: één van de vader en één van de moeder. Elk chromosoom bevat vele markers die onregelmatig verspreid liggen. Door dat bandenpatroon van meerdere markers (een soort streepjescode, aangeduid als "haplotype") van alle individuen van een grote familie met behulp van statistische programma's te vergelijken met het voorkomen van HN-paragangliomen kan een specifieke marker gevonden worden dat dichtbij het niet goed functionerende gen ligt dat deze tumoren veroorzaakt. Elk gen is uniek; als het gen dat als celgroeiremmer werkt echter kapot is, kan het niet goed meer, of helemaal niet meer functioneren en kunnen er bijvoorbeeld tumoren ontstaan. Een beschadiging van het DNA heet een mutatie; een mutatie kan bijvoorbeeld het veranderen van één base zijn of het missen (een deletie) van vele duizenden basen. Dit kan per aangedane familie verschillen. Het gemuteerde gen dat groei van HN-paragangliomen toestaat, is PGL1 genoemd (voor paragangliomen). Analyse van een Nederlandse familie (FGT1; Familiaire Glomus Tumoren) toonde aan dat het ergens gelokaliseerd is op de lange arm van chromosoom 11, 11q (Heutink et al., 1992). Mijn taak was nu om het PGL1 gen te vinden met de specifieke mutaties; de resultaten heb ik in dit proefschrift beschreven.

Hoofdstuk 1 bevat de algemene inleiding, waarvan een klein gedeelte hierboven beschreven is.

In **hoofdstuk 2** beschrijven we de analyse van 5 andere Nederlandse families met HN-paragangliomen. Recombinant-onderzoek plaatste het PGL1 gen in een interval van 16 centiMorgan (cM) op chromosoom 11q22-q23; een genetische afstand van 1 cM komt ruwweg overeen met 1 miljoen basen. Gezien het feit dat een enkel gen zo klein kan zijn als enkele honderden basen, is dit gebied dus nog veel te groot om het PGL1 gen te gaan zoeken.

In **hoofdstuk 3** beschrijven we de analyse van een zeer grote HN-paraganglioma familie, waardoor veel patiënten met elkaar vergeleken konden worden. Deze nieuwe familie (FGT189) bestaat uit de 3 verschillende families FGT1, FGT8 en FGT9 die door genealogisch of stamboom- onderzoek aan elkaar gekoppeld werden door een vrouw geboren in 1776. Alle patiënten vertoonden voor 3 markers (met de namen D11S1327, D11S1792 en D11S908) dezelfde allelen, wat het gen PGL1 in een interval van ongeveer 2 cM rondom deze markers plaatste. Een nadeel van deze 3 markers is het feit dat het allel dat gekoppeld is met de ziekte veel voorkomt (64%, 76% en 38%, respectievelijk), waardoor de kans vergroot is dat deze koppeling een toevalswaarneming is.

Indertijd werd een andere Nederlandse HN-paraganglioma familie (FGT2) beschreven door Mariman en collega's (1993,1995), maar zij lokaliseerden het gen (PGL2) in een ander gebied op hetzelfde chromosoom (11q13). Een mogelijke oorzaak zou kunnen zijn dat die familie een ander defect gen heeft dat de tumorgroei veroorzaakt. Het feit dat er meer dan één gen verantwoordelijk is voor een specifieke ziekte, wordt genetische heterogeniteit genoemd. Om deze genetische heterogeniteit te onderzoeken, hebben we in een samenwerkingsverband 17 families onderzocht met zowel markers in het gebied 11q22-q23 als in het gebied 11q13. Hieruit bleek onder andere dat alleen familie FGT2 genetische koppeling vertoonde met 11q13, terwijl alle andere 16 families met 11q22-q23 gekoppeld bleken (**hoofdstuk 4**).

Door het genealogisch onderzoek zoals vermeld in hoofdstuk 2, kregen we de indruk dat misschien wel meer families met HN-paragangliomen eenzelfde (verre) voorouder zouden kunnen hebben. Aangezien familie FGT189 uit de buurt van Leiden komt, hebben we alle 10 beschikbare HN-paraganglioma families uit Zuid-Holland geanalyseerd (**hoofdstuk 5**). Enerzijds met

stamboomonderzoek met behulp van burgerregisters en kerkregisters, en anderzijds met haplotype analyse. Het stamboomonderzoek terugvoerend tot omstreeks 1800 leidde niet tot koppeling van verschillende families, maar de genetische data liet zien dat al deze families hetzelfde haplotype hebben op het ziektechromosoom in de buurt van het PGL1 gen. Dit is een zeer sterke aanwijzing dat deze families toch een verre gemeenschappelijke voorouder hebben en dus ook allemaal dezelfde mutatie in het PGL1 gen hebben.

Het gebied van zo'n 2 miljoen basen waar het PGL1 gen zich zou moeten bevinden, is vervolgens uitgebreid bestudeerd. Om dit te doen is er gebruik gemaakt van stukjes DNA die in gist en bacteriën zijn gekloneerd, zodat er veel uitgangsmateriaal beschikbaar is. Vervolgens konden we verschillende bekende markers en genen precies lokaliseren (**hoofdstuk 9**). Eén gen trok onze aandacht, aangezien dit gen in de muis een belangrijke functie heeft in de embryonale ontwikkeling van hersenstructuren in het hoofd/hals gebied en tevens omdat het gemuteerde gen in de mens kan leiden tot kanker van bloedcellen (leukemie). Dit gen, PLZF voor promyelocytic leukaemia zinc finger gene, hebben we intensief onderzocht (**hoofdstuk 10**). Allereerst hebben we de precieze samenstelling van het gen bepaald; de stukken die coderen voor een deel van het eiwit (een exon) zijn namelijk gescheiden door stukken die niet coderen (de intronen). Elke base van het gen (in totaal meer dan 3000 basen) hebben we geanalyseerd, maar er is geen verandering gevonden in de patiënten. Dit zou erop kunnen duiden dat dit gen niet het PGL1 ziektegen is. In één van de intronen van het PLZF gen vonden we twee nieuwe markers (PLZF-CA en PLZF-CTTT). Analyse van de markers in familie FGT189 liet echter zien dat beide markers niet gekoppeld zijn met de ziekte, waardoor er een soort van zebra-patroon ontstond van gekoppelde en niet-gekoppelde markers. De koppeling van de allelen van de 3 markers D11S1792, D11S1327 en D11S908 was dus inderdaad een toevalstreffer, zodat we moesten concluderen dat het PGL1 gen dus in een ander klein gebied op chromosoom 11q22-q23 moet liggen.

Analyse van nieuwe markers in het grotere gebied waar PGL1 zou kunnen liggen (tussen markers D11S1647 en D11S2077, zo'n 6 cM) toonde aan dat er inderdaad een nieuw gebied van ongeveer 2 cM is waar PGL1 hoogstwaarschijnlijk gelokaliseerd is (**hoofdstuk 6**). In dit gebied zijn tot nu toe al 8 verschillende markers gevonden die in familie FGT189 gekoppeld zijn, waardoor de statistische significantie veel hoger is. Genetisch onderzoek van de andere HN-paraganglioma families toonde aan dat vrijwel alle Nederlandse families een verre voorouder delen, alhoewel een drietal families een andere voorouder lijken te delen. Families die uit het buitenland komen, o.a. België, India en USA, lijken inderdaad niet verwant te zijn aan deze specifieke Nederlandse voorouder en zouden dus een andere mutatie in het PGL1 gen kunnen hebben.

Zoals hierboven beschreven, zou PGL1 een functie als celgroeiremmer kunnen hebben en fungeren als zogenaamd tumor suppressor gen. Om tumorgroei te initiëren, zullen beide genkopieën uitgeschakeld moeten worden. De eerste mutatie (hit) is reeds via de geslachtscellen overgedragen, en is bij een erfelijke belaste persoon aanwezig in alle cellen. De 2e hit vindt plaats in een lichaamscel en kan als deze door 'mitotische recombinatie' veroorzaakt wordt gepaard gaan met verlies van het "wild type" gen met flankerende DNA sequenties. Hierdoor zal van de markers die in de buurt liggen ook één allel verloren gaan. Dit fenomeen wordt LOH (loss of heterozygosity) genoemd. Analyse van HN-paragangliomen op LOH duidde erop dat er inderdaad een regio lijkt te zijn op chromosoom 11q22-q23 waar één van de twee allelen verloren is gegaan (**hoofdstuk 7**). Echter, er werd nooit een compleet verlies van een allel waargenomen, waardoor ook andere mogelijkheden tumorgroei kunnen

veroorzaken. In die gevallen waar we de ouder konden vaststellen van het allel dat een zwakker bandje gaf, was het altijd het maternale allel. Dit ondersteunt ons vermoeden dat PGL1 een tumor suppressor gen is, aangezien de eerste hit overgeërfd is via de vader en de 2e hit verlies van het maternale allel (met een nog functioneel PGL1-gen) zou moeten zijn. Door alle tumorsamples met elkaar te vergelijken, zou er een gemeenschappelijke regio gevonden moeten kunnen worden waar alle tumoren een verlies te zien geven en waar het desbetreffende gen zou moeten liggen. In ons geval zou het PGL1 gen ten opzichte van marker D11S560 meer naar het uiteinde van chromosoom 11q kunnen liggen, maar het zebrapatroon van wel en geen verlies, zoals waargenomen in een aantal samples, geeft aan dat dit nog geen eenduidige lokalisatie is.

Om het eventuele verlies beter te kunnen analyseren, hebben we uit tumorcoupes specifieke cellen geïsoleerd. Een HN-paraganglioom bestaat uit twee typen cellen: de chief (type I) cellen die geklusterd liggen en omgeven worden door de zogenaamde sustentacular (type II) cellen. De chief cellen vertonen inderdaad compleet verlies van het wildtype allel, een indicatie dat PGL1 inderdaad een tumor suppressor gen is. Indien een tumor ontstaat uit een enkele voorouder cel die klonaal uitgroeit, zullen alle cellen hetzelfde defect bevatten. Indien in de tumorcellen het aantal chromosomen door verstoring van de normale celdelingsregulatie verandert (aneuploidie), zal dit dan ook in alle tumorcellen waargenomen worden. Dit is inderdaad het geval in de tumoren waar we gekeken hebben naar deze gesorteerde fracties. In de aneuploide fracties werd compleet verlies waargenomen van de regio rondom PGL1, een indicatie dat de chief cellen deze fractie vormen en dat de tumoren inderdaad een klonale nieuwvorming zijn (**hoofdstuk 8**).

Het proefschrift wordt afgesloten met een algemene discussie (**hoofdstuk 11**), waarin ik naast het fenomeen van mogelijke recombinatie-onderdrukking op het ziektechromosoom, ook mogelijke modellen bespreek waarmee alle karakteristieken van deze tumoren verklaard zouden kunnen worden. In elk geval lijken deze tumoren een zeer specifieke groep te vormen en zouden ze een belangrijke bijdrage kunnen leveren aan het begrijpen van het mechanisme van genomische imprinting en hoe dit verband houdt met het ontwikkelen van tumoren.

CURRICULUM VITAE (Nederlands)

De auteur van dit proefschrift, Evert Matthijs van Schothorst, is geboren op 1 april 1968 te Nairobi, Kenya. Na het behalen van zijn Atheneum diploma aan het Baudartius College te Zutphen in 1986, studeerde hij Moleculaire Wetenschappen aan de Landbouw Universiteit te Wageningen. Tijdens het eerste 6-maands afstuurdeervak bij de vakgroep Moleculaire Genetica (Erfelijkheidsleer) onder leiding van Dr.Ir. Jaap Visser werden de eerste stappen in de moleculaire genetica gezet. Doel van het onderzoek was het karakteriseren van de regulatie van het phosphofructokinase gen in de schimmel *Aspergillus nidulans*. Het tweede 6-maands vak werd bij de vakgroep Experimentele Diermorphologie en Celbiologie gevolgd, onder leiding van Dr. René Stet en Prof.Dr. Wim van Muiswinkel. Hier werd onderzoek verricht aan het immuunsysteem van de karper m.b.v. cDNA clones. Tenslotte liep hij in 1991 een 9-maands stage bij Dr. Clive Roberts en Prof.Dr. Alec Jeffreys te Leicester, Engeland. Hier werd de single strand conformational polymorphism (SSCP) methode opgezet en toegepast om mutaties in een regulator gen van de schimmel *Aspergillus niger* te onderzoeken. In 1992 studeerde hij af in Wageningen. Een kort project werd bij de groep van Dr. Hans de Jong, Cytogenetica (Erfelijkheidsleer), gedaan waar fluorescentiemicroscopie en image-processing werden toegepast voor cytogenetisch onderzoek.
In 1993 begon hij als onderzoeker in opleiding (OIO) bij de vakgroepen Anthropogenetica (Prof.Dr. Gert-Jan B. van Ommen) en Pathologie (Prof.Dr. Cees J. Cornelisse) aan de Rijks Universiteit te Leiden onder leiding van Dr. Peter Devilee. Dat onderzoek is in dit proefschrift beschreven.
Naast actief bezig te zijn met sport, met name Ultimate frisbee en kanoën, is hij sinds 1995 ook actief lid binnen de landelijke werkgroep 'Sport en bewegen' van de Diabetes Vereniging Nederland.
Momenteel werkt hij als postdoc onderzoeker bij het Nederlands instituut voor ontwikkelingsbiologie (NIOB, Hubrecht laboratorium) te Utrecht (Dr. Paul T. van der Saag) in samenwerking met het laboratorium voor Experimentele Patho-Oncologie van de Daniel den Hoed kliniek, Rotterdam (Dr. Leendert H.J. Looijenga).

CURRICULUM VITAE (English)

Evert Matthijs van Schothorst was born on 1 April 1968 in Nairobi, Kenya. He finished high school (Baudartius College te Zutphen, The Netherlands) in 1986 and started the same year his university study Molecular Sciences at the Agricultural University of Wageningen. His first 6-months project was to characterise the expression regulation of phosphofructokinase in the fungus *Aspergillus nidulans*, under supervision of Dr.Ir. Jaap Visser at the department of Molecular Genetics (Erfelijkheidsleer). His second project under supervision of Dr. René Stet and Prof.Dr. Wim van Muiswinkel focussed on the immune system of the common carp at the department of experimental animal morphology and cell biology. In 1991 he started a 9-months project at the department of Human Genetics, Leicester University, UK under supervision of Prof.Dr. Alec Jeffreys and Dr. Clive Roberts. He set up the SSCP-analysis in order to identify mutations in a regulator gene in *Aspergillus niger*. He graduated in 1992 in Wageningen. After a project in the group of Dr. Hans de Jong, Cytogenetics (Erfelijkheidsleer), he started his PhD project in 1993 at the departments of Human Genetics (Prof.Dr. Gert-Jan B. van Ommen) and Pathology (Prof.Dr. Cees J. Cornelisse) of Leiden University, under supervision of Dr. Peter Devilee. The results are discussed in this thesis.
He is not only active in sports, especially Ultimate frisbee and kayaking, but he also became in 1995 member of the Dutch workgroup 'Sports and exercises' of the Dutch Diabetic organisation (DVN).
At the moment he is a post-doc researcher at the Dutch institute of developmental biology (Hubrecht laboratorium, Utrecht, Dr. Paul T. van der Saag) in collaboration with the Laboratorium of Experimental Patho-Oncology at the Daniel den Hoed kliniek, Rotterdam (Dr. Leendert H.J. Looijenga).

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van Schothorst EM, Prins DEM, Baysal BE, Beekman M, Licht J, Cornelisse CJ, van Ommen GJB, Richard III CW, Devilee P: Genomic structure of the human PLZF gene and exclusion as the causative gene for hereditary HN-paragangliomas. (submitted)

Stellingen behorende bij het proefschrift **'Genetics of hereditary head and neck paragangliomas (glomus tumours)'** van Evert M. van Schothorst:

1. Het syndroom waarbij zowel paragangliomen in het hoofd/hals gebied als hypothyroïdisme voorkomen, zoals beschreven door Hart and Maartense (Clin. Endo. 1992; 36: 295-296), lijkt niet als zodanig te bestaan.
   Dit proefschrift (hoofdstuk 5)

2. Het gebruik van statistische analyses en uitkomsten, waaronder de lod-score, moet met zeer veel wantrouwen gebeuren.
   Dit proefschrift (hoofdstukken 3 en 6)

3. HN-paraganglioma patiënten met een functionele tumor kunnen deze wellicht in de toekomst met veel winst van de hand doen aan Parkinson patiënten.
   Espejo EF, et al. 1998, Neuron 20: 197-206

4. Eén founder mutatie in PGL1 die de meeste, zo niet alle, (familiaire) HN-paragangliomen veroorzaakt heeft als groot voordeel dat de klinische genetica straks met één simpele test alle risicodragers met grote betrouwbaarheid kan screenen.
   Dit proefschrift (hoofdstuk 6)

5. Founder effecten bij erfelijke ziekten (o.a.) in Nederland worden onderschat, aangezien hier te weinig aandacht aan wordt besteed bij mutatie-analyse van een nieuw gekloneerd ziektegen.

6. Een niet-coderend RNA (H19, Xist, IPW) kan nog wel een boodschap bevatten.

7. Het 'non-transmitted allele' bepaalt mede het fenotype van de nakomeling.
   Bennett et al. 1997, Nat Genet 17: 350-352

8. Indien wetenschappelijke groepen hun rendement willen verhogen is ondersteuning van research management een belangrijk aandachtsgebied.

9. Het is opmerkelijk te noemen dat diabeten bij strenge controles op vliegvelden nog nooit hun insulinepen (een effectief moordwapen) hoefden in te leveren.

10. Het feit dat de combinatie trein en bus vaak veel te wensen overlaat, wordt (niet alleen) duidelijk uit de mededeling "de NS zet bussen in".

11. De term kleurling zou beter passen bij de (emotionele) kleurveranderingen van een zogenaamde blanke.

12. Er is maar één succes: te kunnen leven zoals je wilt.
    Christopher Morley

Evert M. van Schothorst

Dankwoord.

Na een periode van meer dan 4 jaar werken aan een uitdagend project, is het duidelijk moeilijk om er een einde aan te breien. Zeker als er niet datgene uit gekomen is waar je je op gericht had (en gehoopt), maar dat hangt nu eenmaal nauw samen met het doen van wetenschap. Toch zijn er meer dan genoeg interessante aspecten gevonden wat het allemaal meer dan goed maakt. Dat was nooit en te nimmer allemaal mogelijk geweest zonder hulp van, en samenwerking met, anderen. Een woord van dank is dan ook zeker op z'n plaats.

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De medewerking van alle HN-paraganglioma patiënten en hun familieleden was onmisbaar voor het project. Mijn dank is dan ook groot. Het initiëren van genetische counseling, in samenwerking met het Klinisch Genetisch Centrum Leiden, onder leiding van Jan en later Annet, en met Bert B. en Yvonne van de afdeling DNA-diagnostiek was een grote stimulans tijdens het traject. Dit zal zeker alleen maar toenemen met alle founder families.

During the last year, I enjoyed the collaboration with the group of Charly Richard and Bora Baysal in Pittsburgh enormously. Not only practically, but also for our hugh amount of discussions we had by e-mail and during several meetings. I hope this collaboration will lead to the elucidation of the PGL1 gene in the very near future. Good luck!

Ook de mensen die in Leiden doorgaan met dit project, Peter T. en Anne: heel veel succes! Hopelijk is het gen snel gevonden, zodat jullie het leuke onderzoek aan de imprinting kunnen gaan doen.

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Een belangrijke uitlaatklep was en is voor mij sport; bij deze wil ik dan ook graag zowel labgenoten (zaalvoetbal en squash), als teamgenoten van het Ultimate Frisbee (eerst HULK, tegenwoordig Force Elektro) hartelijk danken voor de vele uurtjes. Force Elektro: dit jaar worden wij kampioen, oke? Dankzij het vele water rondom Leiden kwam ik in contact met veel mensen bij de kanovereniging Levitas, waar ik ontzettend goede herinneringen aan heb. Ik zal de vele dagjes/weekendjes zee kanoën, wildwater varen en toertochten met jullie zeker missen.

De affiniteit en enthousiasme voor sport heb ik, sinds ik in de landelijke werkgroep Sport en Bewegen van de Diabetes Vereniging Nederland zitting kon nemen, kunnen uitdragen aan veel lotgenoten tijdens sportweekenden en voorlichtingsavonden: Bart, Erik, Leo, Rypke, Jan, Ellen, Jeanette, Nico, Jolande, Augustine, Okke, Joris, Koen (en de anderen van de Vlaamse afdeling) en alle anderen: we zitten op de goede weg en hopelijk overtuigen we iedereen er van: Ontspanning door inspanning (maar wel met, of juist dankzij, een goede diabetes regulatie).

Mijn huisgenoten Jan en Barbara wil ik bij deze hartelijk danken voor de gezelligheid afgelopen jaren.

En last, but surely not least: pappa, mamma, Wijnand en Babette: hartelijk dank voor jullie ondersteuning, begrip, en de gezellige avonden/weekendjes samen voor zover mogelijk gedurende al die jaren. Hopelijk krijg ik nu weer meer tijd voor jullie allemaal.

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Evert.