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Key Words

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Further Localization of the Gene for Hereditary Parangliomas and Evidence for Linkage in Unrelated Families

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Abstract

Parangliomas 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 paranglioma 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 parangliomas (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 parangliomas. 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

Parangliomas 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 [1]. Familial occurrence has been reported and is consistent with an autosomal dominant mode of inheritance. Penetrance of the clinical manifestations of hereditary paragangliomas is not only age-dependent but was also found to be related to the sex of the transmitting parent. Affected individuals have inherited the disease gene from their father, while expression of the phenotype is not observed in the offspring of an affected female or 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 [2–4], 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 [5].

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 [6]. In affected individuals, the maternal allele might be silenced by the imprinting process while the paternal allele must be inactivated by a physical disruption of the gene sequence such as a point mutation or a deletion. In this model, the imprinting process could either act directly on the gene responsible for the phenotype or alternatively influence the expression of a modifier gene *in trans* [5, 7]. An alternative explanation has been proposed by Van der Mey et al. [3]; 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 remov-

al 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 [7]. However, hereditary paragangliomas is one of the rare examples where the clinically important effect of genomic imprinting at a single locus is absolute and can be studied in large pedigrees.

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

Material and Methods

Family Studies

We examined all available family members from 6 extended Dutch families with head and neck paragangliomas. In total, 111 meioses were studied. Family FGT1 has been described previously [4]. Families FGT3, FGT4, FGT9, FGT10 and FGT18 were recently ascertained (fig. 1–2). Clinical procedures were described elsewhere [4] but briefly for FGT1: diagnoses of family members were based on medical history, physical and otolaryngological examination, and determination of free urinary catecholamine excretion. In a number of cases, whole-body magnetic resonance imaging (MRI) was performed. For confirmation of paragangliomas, contrast-enhanced computed tomography or angiography was performed. When a hormonal active lesion was suspected [¹²³I]MIBG scintigraphy was applied [8]. Diagnoses of members of the other families were based on medical history, physical and otolaryngological examination, and determination of free urinary catecholamine excretion.

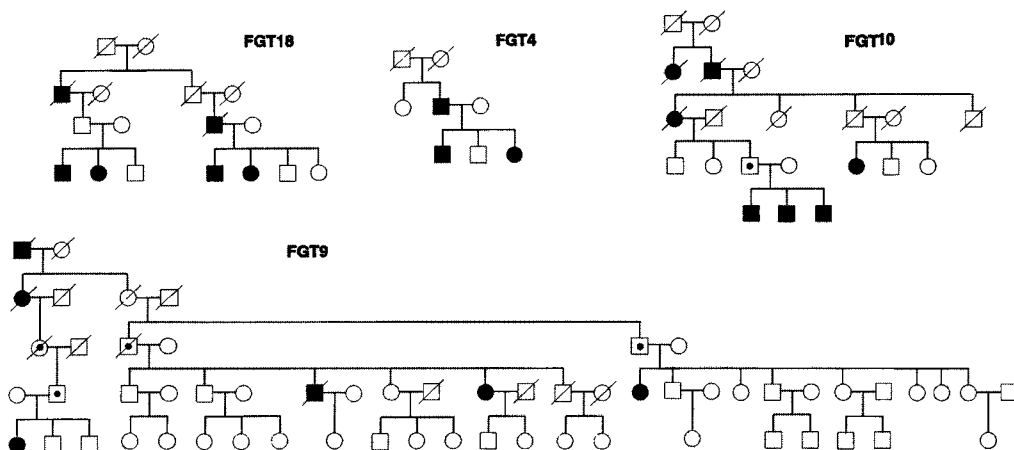


Fig. 1. Pedigrees of five Dutch families with hereditary paragangliomas. Filled boxes indicate affected individuals. Blank symbols indicate individuals that did not show tumor growth or from whom the disease phenotype could not be established. Dotted symbols indicate individuals subject to genomic imprinting.

DNA Studies

Genomic DNA was isolated from peripheral blood as described by Miller et al. [9]. Restriction digestion was carried out according to the manufacturer's recommendations. Gel electrophoresis of 5 μ g DNA samples on 0.7% agarose gels, and DNA immobilization by alkaline blotting onto Nylon membranes (Hybond +, Amersham), were performed according to standard procedures [10]. Hybridization conditions were as described by Maniatis et al. [10] and washing was performed at 65°C to 0.1 \times SSC final stringency. DNA was labelled by primed synthesis according to the protocol of Feinberg and Vogelstein [11]. Information and sources of all polymorphic markers used are described in the Human Genome Database (GDB) [12] and the NIH/CEPH collaborative linkage map [13]. Microsatellite markers were tested in multiplex reactions essentially as described by Weber and May [14] using a Perkin-Elmer-Cetus 9600 Thermocycler. Initial denaturation was 10 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 [14].

Linkage Analyses

Linkage analyses were performed using the LINKAGE program package version 5.1 [15, 16]. In the statistical analyses of linkage, autosomal dominant inheritance was assumed. We allowed for a single copy of the abnormal gene to segregate in this family. In the statistical analyses, genomic imprinting was implicated in that complete absence of penetrance of the phenotype was assumed when the gene was inherited from the mother. To facilitate the analyses, eight liability classes were defined to account for age of onset and the absence of penetrance in children of female gene carriers [4]. 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 independent 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 [13] were analyzed. D11S527 was added to this map based on a microsatellite index map for the long arm of chromosome 11 [17].

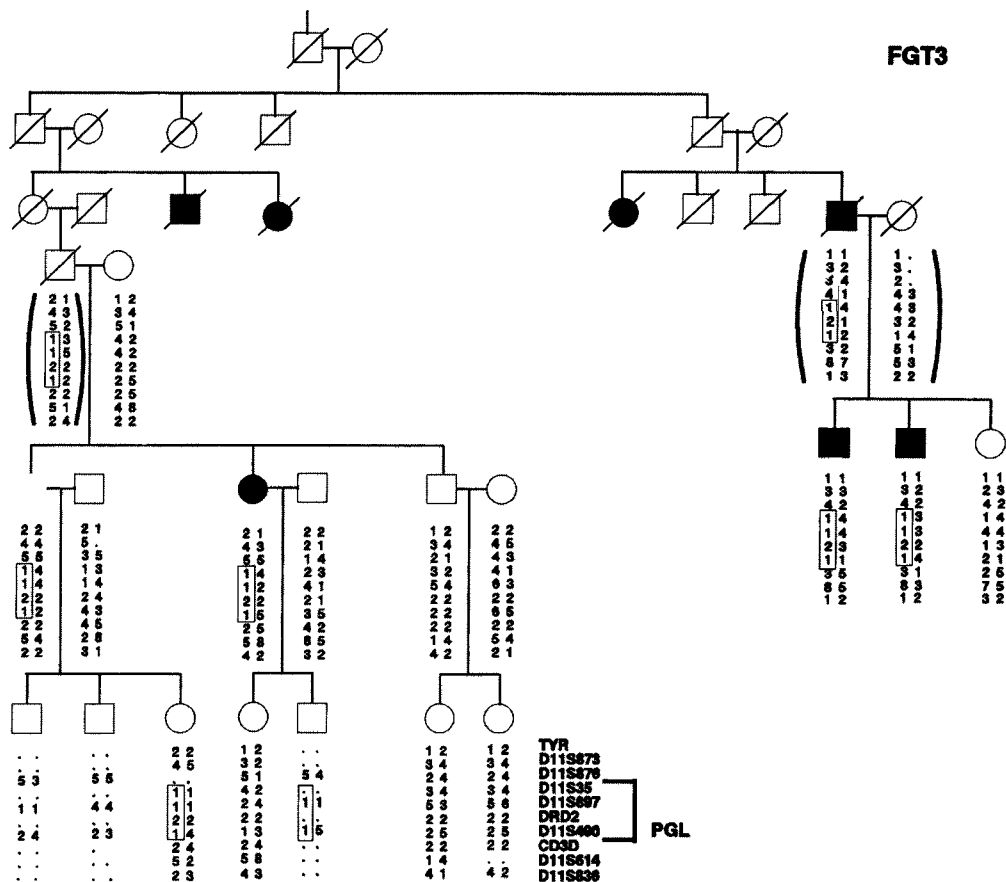


Fig. 2. Haplotype analysis of family FGT3. Markers of chromosome 11q are ordered according to the NIH/CEPH collaborative linkage map [13] from centromere to telomere. The shared haplotype that segregates with PGL in both branches of the family is depicted within boxes.

Results

Twenty polymorphic markers localized on chromosome 11q13-qter were typed in family FGT1 in addition to the five markers that were previously reported [4]. Two-point analyses were performed between all markers and the disease locus (table 1). Several markers gener-

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

A subset of the markers typed in family FGT1 has been typed in five additional families. The segregation pattern of paragangliomas in these families is consistent with genomic imprinting; the disorder is never transmitted by an affected female or female gene carrier (fig. 1, 2). Two-point analysis revealed positive lod scores with markers for 11q13-qter in each of these families. Although none of these families by 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 $\Theta = 0.05$ with marker D11S897 (table 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 3) and multi-point analysis (fig. 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 (fig. 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

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

Locus ¹	Recombination fraction (Θ)						
	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	$-\infty$	-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
CD3C 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 ²	$-\infty$	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 reference [4].

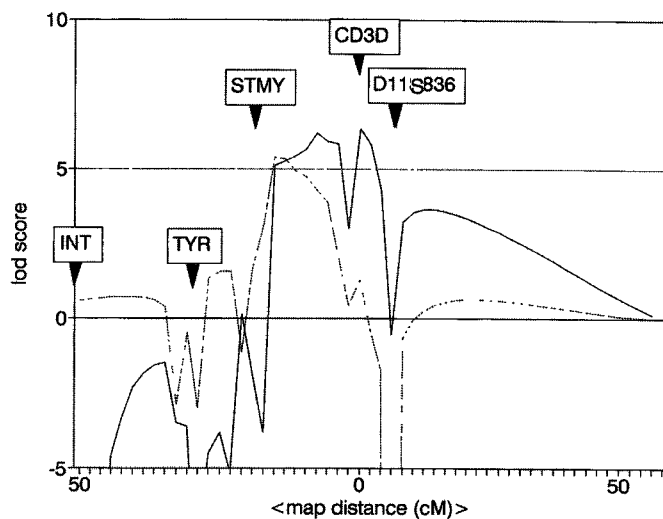


Fig. 3. Multi-point analysis of 16 markers from chromosome 11q on family FGT1 (solid line). Multi-point analysis of 8 markers from chromosome 11q on families FGT3, FGT4, FGT9, FGT10 and FGT18 (dotted line). Inter marker distances and marker order were based on references 13 and 17.

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

Locus ¹	Recombination fraction (Θ)						
	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	$-\infty$	-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 3. Cumulative pairwise lod scores between chromosome 11q13-qter markers in six families with hereditary paragangliomas

Locus ¹	Recombination fraction (Θ)						
	0.000	0.010	0.050	0.100	0.200	0.300	0.400
D11S527	$-\infty$	-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.

STMY and APOC3 in family FGT1 (individuals II-1, III-3) define STMY as the proximal boundary of the candidate region (fig. 4).

In family FGT3, the haplotype linked with the disease locus in one part of the family only segregates in part to the other segment of the family (fig. 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 [16].

Discussion

We recently reported linkage of hereditary paragangliomas to markers on chromosome 11q23-qter in a large five-generation pedigree (FGT1). In this study we report evidence for linkage in five unrelated families with hereditary paragangliomas and a more detailed lo-

calization 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 established in families FGT3, FGT4 and FGT18 (fig. 1, 2). In a number of obligate carrier males, the disease phenotype could not be established, due to either non-penetrance or unretrievable 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. However, the use of a model without imprinting on families FGT1, FGT9 and FGT10 would result in reduced

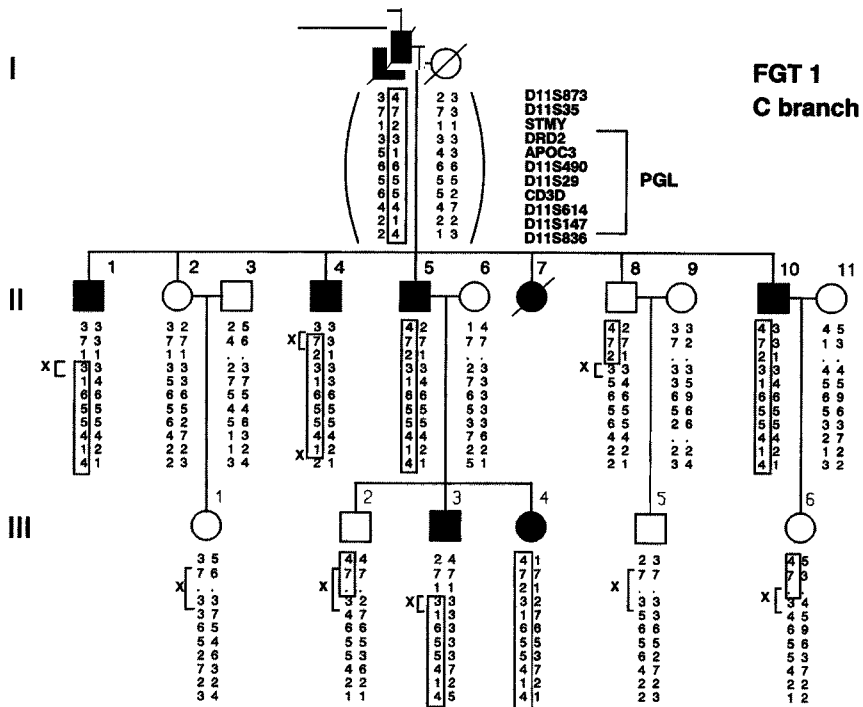


Fig. 4. Haplotype analysis of family FGT1 C branch. Markers of chromosome 11q are ordered according to the NIH/CEPH collaborative linkage map [13] from centromere to telomere. X = recombination events observed. Haplotypes between brackets were constructed using information from all available family members including from branches A and B (data not shown).

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 or female gene carriers 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 [18]. 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 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 [P. Devilee et al., in preparation]. Additional information for the location of PGL may be obtained through detailed 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 [7]. A growing number of human disorders show differences in phenotypes, age of onset and severity that seem to be related to the sex of the parent transmitting the gene. In a number of cancer syndromes, genomic imprinting seems to be involved in disease onset. Sporadic Wilms tumor, rhabdomyosarcoma and os-

teosarcoma show preferential loss of maternal alleles, giving indirect molecular evidence that a tumor suppressor gene might be involved [19–21]. For hereditary paragangliomas, both a dominant (onco)gene and a tumor suppressor gene have been proposed [3, 6]. 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 normal imprint on IGF2 was lost [22, 23]. 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 [24]. 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 [25, 26]. Mutant mice embryos lacking DNA methyltransferase activity do not control differential expression of genes known to be genomically imprinted [27, 28]. 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.

Parangliomas 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 N-CAM is localized within the candidate region for PGL but it lacks a polymorphism that is informative enough to determine its possible role in paranglioma development.

Now that flanking markers have been found for hereditary parangliomas 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 paranglioma development but in addition offers an ideal model system to study the phenomenon of genomic imprinting.

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