

Short Communication

Differential Loss of Chromosome 11q in Familial and Sporadic Parasympathetic Paragangliomas Detected by Comparative Genomic Hybridization

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Parasympathetic paragangliomas (PGLs) represent neuroendocrine tumors arising from chief cells in branchiomeric and intravagal paraganglia, which share several histological features with their sympathetic counterpart sympathoadrenal paragangliomas. In recent years, genetic analyses of the familial form of PGL have attracted considerable interest. However, the majority of paragangliomas occurs sporadically and it remains to be determined whether the pathogenesis of sporadic paraganglioma resembles that of the familial form. Furthermore, data on comparative genetic aberrations are scarce. To provide fundamental cytogenetic data on sporadic and hereditary PGLs, we performed comparative genomic hybridization using directly fluorochrome-conjugated DNA extracted from 12 frozen and 4 paraffin-embedded tumors. The comparative genomic hybridization data were extended by loss of heterozygosity analysis of chromosome 11q. DNA copy number changes were found in 10 (63%) of 16 tumors. The most frequent chromosomal imbalance involved loss of chromosome 11. Six of seven familial tumors and two of nine sporadic tumors showed loss of 11q (86% versus 22%, $P = 0.012$). Deletions of 11p and 5p were found in two of nine sporadic tumors. We conclude that overall DNA copy number changes are infrequent in PGLs compared to sympathetic paragangliomas and that loss of chromosome 11 may be an important event in their

tumorigenesis, particularly in familial paragangliomas. (Am J Pathol 2001, 158:1937–1942)

Parasympathetic paragangliomas (PGLs) are rare, highly vascularized tumors, originating from neural crest-derived chief cells of paraganglia in the head and neck region. They share many histological features with sympathoadrenal PGLs, including pheochromocytomas (PCCs). Metastases of PGLs are uncommon, but may emerge in lymph nodes, lung, and liver. Dependent on the anatomical location the tumor can cause serious symptoms like dysphagia, bradycardia, and hearing loss. Because of this and in view of the risk of progression to malignancy, surgical resection of PGLs is often required.

The carotid body and jugulotympanic paraganglia are the most common sites of origin of parasympathetic PGLs, followed by vagal, laryngeal, and aorticopulmonary paraganglia.^{1,2} Although most parasympathetic PGLs occur sporadically, there is a positive family history in a considerable minority (10 to 50%) of cases.³ Predominance in females, multiple PGLs, and young age of onset are characteristic of familial PGLs, but such features have also been reported in sporadic cases.^{4,5} Co-occurrence of parasympathetic PGLs and PCCs, and occurrence in Carney's syndrome and neurofibromatosis type 1 have been described.^{6–9} Flow cytometric analyses revealed DNA aneuploidy in 21 to 50% of parasympathetic PGLs, which did not predict malignant behavior or decreased survival.^{10–12} A few immunohistochemical studies have suggested a paracrine/autocrine role for IGF-II, *c-myc*, *bcl-2*, and *c-jun* in PGL pathogenesis.^{13–15}

In attempts to clarify the genetic mechanisms underlying the development of parasympathetic PGLs, the in-

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Table 1. Clinical Characteristics and Genetic Findings in Parasympathetic Paragangliomas

Patient	Age of onset/sex	Location	Size (cm)	Horm. activity	Type	Follow-up data (months)	LOH results		CGH results
							D11S134	D11S1986	
1	33/F	VAG	4.0	—	spor.	NED (85)	□	□	5p-, 11p-
2	32/M	CAR	2.5	—	fam.	MUL (85)	■	■	11pq-
3	30/F	CAR	nk	—	fam.	MUL (80)	ni	■	n
4	45/F	CAR	3.0	—	spor.	MUL (68)	ni	□	n
5	45/M	TYMP	0.5	—	spor.	NED (66)	—	—	11pq-
6	42/F	IUG	1.5	—	spor.	REC (127)	—	—	n
7	31/F	CAR	1.5	—	spor.	NED (63)	—	—	9p-, 11pq-
8	29/F	CAR	4.0	—	fam.	MUL (63)	ni	■	1p-, 11pq-
		CAR	1.8	—			ni	■	11pq-, 13q-, 17pq+
9	33/M	CAR	1.5	—	spor.	MUL (61)	□	□	n
		CAR	2.0	—			□	□	n
10	39/M	CAR	4.0	—	fam.	MUL (56)	ni	□	n
11	30/F	CAR	3.5	—	fam.	MUL (248)	■	■	3q-, 11q-
12	33/F	TRACH	2.0	—	spor.	NED (96)	□	□	3pq-
13	55/F	AO-PULM	1.2	+	spor.	NED (96)	□	□	5p-, 11p-
14	38/M	AO-PULM	4.0	+	fam.	MUL* (120)	■	■	2pq-, 11pq-, 18q-

Abbreviations: F, female; M, male; VAG, vagal; CAR, carotid body; TYMP, tympanic; IUG, iugular; TRACH, tracheal; AO-PULM, aorto-pulmonal; nk, not known; fam., familial; spor., sporadic; NED, no evidence of disease; REC, local recurrence; MUL, one or more parasympathetic PGL in other locations including the contralateral carotid body; ni, noninformative; n, no detectable changes; —, no data.

*This patient also presented with carotid body PGL and abdominal masses suggestive of paraganglioma, as detected by computed tomography imaging.

herited form of this tumor has so far been the main subject of investigation. Linkage analysis and loss of heterozygosity (LOH) studies in unrelated families provided evidence for the existence of two distinct PGL susceptibility genes. The putative *PGL1* gene (11q23) and the *PGL2* gene (11q13.1) are both thought to be tumor suppressor genes and maternally imprinted.¹⁶⁻²⁰ Recently, a third, not maternally imprinted gene was demonstrated to cause PGL in a German family (*PGL3*).²¹

Although substantial progress has thus been made in the identification of genetic changes involved in the development of hereditary PGL, comparative data on genomic changes in sporadic and familial parasympathetic PGLs are not available and it remains to be clarified whether these tumors develop along the same genetic pathways. To characterize cytogenetic alterations, we investigated nine sporadic and seven familial parasympathetic PGLs by comparative genomic hybridization (CGH) analysis. In addition, LOH analysis was performed to confirm CGH results.

Materials and Methods

Patients and Tumor Samples

Sixteen benign tumors from 14 patients with parasympathetic PGLs, diagnosed between 1992 and 1996, were studied. The average age of the patients (nine female) at first presentation was 37 years (range, 29 to 55 years) and the mean size of the tumors was 2.5 ± 1.2 cm. Information on family history and other tumors or relevant conditions was obtained by reviewing medical charts and by interviewing all patients, after an average follow-up period of 7.8 years (94 months). Six of 14 patients had a positive family history for parasympathetic PGL and eight

patients had sporadic PGL. A PGL was considered sporadic when there were no first or second degree relatives known with a parasympathetic PGL. Clinical data are summarized in Table 1.

DNA Extraction

Genomic DNA from 12 frozen tumors was isolated using the D-5000 Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). DNA extraction from four formalin-fixed, paraffin-embedded samples was performed by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation, as described elsewhere.²² Only tumors with >80% tumor cell content were included in this study.

CGH and Digital Image Analysis

CGH was performed as described.²² In brief, 1 µg of tumor DNA was labeled with Spectrum Green-dUTPs (Vysis, Downers Grove, IL) by nick translation (BioNick kit; Life Technologies, Basel, Switzerland). The hybridization mixture consisted of 200 ng of Spectrum Green-labeled tumor DNA, 200 ng of Spectrum Red-labeled sex-matched normal reference DNA (Vysis), and 10 to 20 µg human Cot-1 DNA (Life Technologies) dissolved in 10 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 2× standard saline citrate, pH 7.0). Hybridization to normal metaphase spreads (Vysis) took place for 3 days at 37°C. Slides were washed at 45°C three times for 10 minutes in 50% formamide/2× standard saline citrate and two times in 2× standard saline citrate. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole in anti-fade solution for identification.

Digital images were collected from six to seven metaphases using a Photometrics cooled charge-coupled device camera (Microimager 1400; Xillix Technologies, Vancouver, Canada). The QUIPS software program (Vysis) was used to calculate average green-to-red ratio profiles of at least four observations per autosome and two observations per sex chromosome in each analysis. Gains and losses of DNA sequences were defined as chromosomal regions where the mean green-to-red fluorescence ratio was >1.20 and <0.80 , respectively. Over-representations were considered amplifications when the fluorescence ratio values in a subregion of a chromosomal arm exceeded 1.5. Because of some false-positive results at chromosomes 1p32-pter, 16p, 19, and 22 found in normal tissues, gains of these known G-C-rich regions were excluded from all analyses.

LOH Analysis

To validate CGH data independently, 12 PGLs of 11 patients of whom normal DNA was available, were analyzed for allelic imbalances of the 11q23 locus using two microsatellite markers D11S1347 and D11S1986 (Research Genetics, Huntsville, AL).¹⁹ Polymerase chain reaction amplification of tumor and germline DNA was performed in reaction mixtures of 50 μ l. Each reaction contained 50 to 100 ng of template DNA, 0.2 mmol/L dATP, dTTP, dGTP, dCTP, 20 to 50 pmol of each primer, 1.5 mmol/L Mg²⁺, 10 mmol/L Tris-HCl, 50 mmol/L KCl, and 1 U Taq DNA polymerase (Amplitaq Gold; Perkin Elmer, Norwalk, CT). An initial denaturation step at 94°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C (for marker D11S1986 at 52°C) for 60 seconds, and extension at 72°C for 60 seconds. A final extension step was performed at 72°C for 10 minutes. Polymerase chain reaction products of tumor and normal DNA from each patient were diluted 1:1 in 10 μ l of loading buffer (95% formamide, 20 mmol/L ethylenediaminetetraacetic acid, 0.05% xylene cyanol, 0.05% bromophenol blue) and loaded onto a non-denaturing 6% polyacrylamide gel. Electrophoresis was performed at 40 W for 2.5 hours. The DNA was visualized by silver staining and evaluated as described previously.²³ Allelic loss was considered to be present when the intensity of the signal from one allele was significantly reduced in the tumor DNA when compared with normal DNA by direct visualization. Because PGLs are known to contain admixed normal tissue (sustentacular cells and supportive fibrovascular stroma), a weak band of the lost allele was accepted in the determination of LOH.

Statistical Analysis

The chi-square test for nominal variance was used to calculate the statistical significance of differences in genomic changes between familial and sporadic tumors.

Results

Overview of CGH Findings

Figure 1A summarizes all DNA copy number changes identified in 16 parasymphathetic PGLs. Genetic alterations were observed in 10 (63%) of 16 PGLs, and the average number of chromosome arm aberrations per tumor was 1.75 ± 1.69 (range, 0 to 5). With the exception of one gain of chromosome 17, all aberrations implied chromosomal losses and no amplifications were identified (Table 1). The most common chromosome arm copy number change was loss of chromosome 11. Six tumors showed loss of the entire chromosome, one tumor exhibited loss of 11q, and two tumors had a deletion of 11p. Losses of chromosome 11q were strongly associated with familial PGL (86% versus 22% in sporadic PGL) and seemed to be statistically significant ($P = 0.012$). Other genomic imbalances were rare; one gain was found on chromosome 17 (1 of 16), and losses involved 3q and 5p (both 2 of 16), 1p, 3p, 9p, 13q, and 18q (1 of 16 each). Both tumors with loss of 11p also exhibited loss of 5p and seemed to be sporadic tumors. Representative examples of CGH results are shown in Figure 1B.

Comparison of CGH and LOH Results

Twelve tumors of 11 patients of whom normal DNA was available were examined by microsatellite analysis. We could confirm the CGH results of chromosome 11q in all tumors that were analyzed and detected LOH of this region in one additional tumor without apparent loss of 11q in CGH analysis (Table 1). Altogether, most deletions of chromosome 11q were found in familial PGLs (6 of 7 familial PGLs versus 2 of 9 sporadic PGLs). Representative examples of LOH results are shown in Figure 1C.

Discussion

This study represents the first comprehensive, genome-wide analysis of chromosomal aberrations in sporadic and familial parasymphathetic PGLs. Our results indicate that DNA copy number changes are infrequent in these tumors and that tumor suppressor genes on chromosome 11 may play a critical role in the tumorigenesis of familial PGLs. Sporadic PGLs may develop along different genetic pathways.

Cancer, as a genetic disease, is believed to arise from an accumulation of genetic aberrations that promote clonal selection of cells with increasingly aggressive behavior. It is proposed that most cancer cell genotypes demonstrate essential alterations in cell physiology. This is reflected by the finding of multiple chromosomal imbalances in the majority of human cancers. However, our results indicate that DNA copy number changes are infrequent in parasymphathetic PGLs. This is consistent with their characteristically slow growth rate and benign behavior and indicates that some cell-signaling mechanisms, generally disrupted in cancer cells, may still be intact in PGL tumor cells.

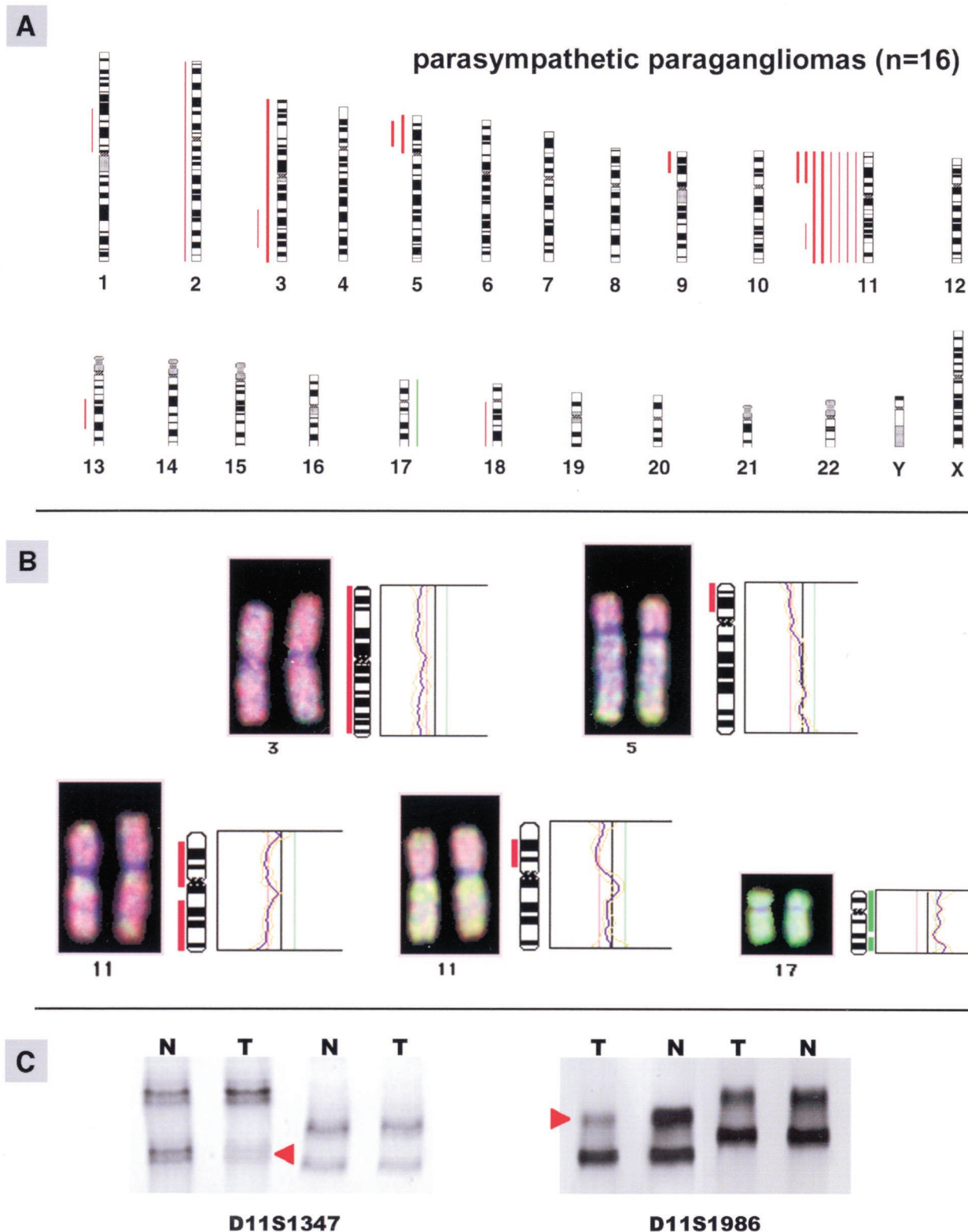


Figure 1. A: Summary of all DNA copy number changes detected by CGH in 16 (seven familial and nine sporadic) parasympathetic PGLs. The vertical **green lines** on the **right** side of the chromosome ideograms indicate gains, the **red lines** on the **left** side indicate losses of the corresponding chromosomal region. Findings in sporadic PGLs are indicated as **solid bars**. **B:** Individual examples of CGH digital images (**left**) and fluorescent ratio profiles (**right**) illustrating genomic alterations of chromosome 3 (loss), 5 (loss of 5p), 11 (loss of the entire chromosome and of 11p), and 17 (gain). **C:** Example of LOH analysis of a familial PGL, showing a band markedly diminished in tumoral (T) DNA in comparison with normal (N) DNA (allelic loss) in two microsatellite markers at 11q23, D11S1986, and D11S1347 (**red arrowheads**).

In a considerable proportion of PGLs, we found a normal DNA copy number profile. To exclude the possibility of false-negative results, we included in our analysis only samples with at least >80% tumor cells. Nonetheless, it should be borne in mind that the lower limit of CGH-based detection is ~10 Mb. Indeed, we found additional loss of 11q by LOH analysis in one tumor.

Only one PGL in this study showed a DNA copy number gain. This gain could not be related to one of the proteins found to be up-regulated in PGLs.¹³⁻¹⁵ Therefore, in parasympathetic PGL, up-regulation of growth stimulating factors is probably caused by other events, such as genetic mutations or rearrangements.

Loss of 11q was the most common chromosomal aberration in our series of PGLs, with a remarkable difference in incidence between familial and sporadic tumors (86% versus 22%, respectively; $P = 0.012$). This may point toward distinct tumorigenic pathways in these subgroups of PGLs.

Co-occurrence of parasympathetic PGLs and sympathoadrenal PGLs (including PCCs) has been described.^{6,8} Because these tumors share many histological characteristics, they may be thought to result from similar genetic changes. However, comparison of CGH data of benign parasympathetic and sympathoadrenal PGLs shows that these types are genetically different. In benign sympathoadrenal PGLs, genetic changes are much more frequent and 11q losses are less frequent compared to the parasympathetic PGLs in this study.^{24,25} Yet, it remains to be tested whether the same tumor suppressor gene(s) on 11q are involved in the tumorigenesis of both parasympathetic PGLs and PCCs.

The long arm of chromosome 11 contains two well-defined critical regions that each harbor a putative PGL disease gene, *PGL1* at 11q23 and *PGL2* at 11q13.^{16,18,26} Recently, Baysal and co-workers²⁷ detected germline mutations of the mitochondrial succinate dehydrogenase complex II subunit d (*SDHD*) gene (11q23) co-segregating with tumor occurrence in PGL families, indicating that this is the putative *PGL1* gene. The mitochondrial complex II is an important enzyme complex in the aerobic respiratory chains of mitochondria. Loss of function of this complex may cause cellular hypoxia and increased superoxide levels. Chronic hypoxia is known to be an important cause of carotid body hyperplasia and conceivably plays a role in tumor initiation and progression of carotid body PGLs. In this respect, it is of interest that two of nine sporadic PGLs showed loss of 5p, the gene locus of another component (the flavoprotein subunit) of the mitochondrial succinate dehydrogenase complex II.²⁸ These tumors also demonstrated loss of 11p, but not of 11q. This may imply that in the pathogenesis of some sporadic PGLs, other components of the mitochondrial complex II are involved in their tumorigenesis.

In conclusion, our study demonstrates that chromosomal imbalances are infrequent in parasympathetic PGLs compared to their sympathetic counterpart, PCCs. We observed a high frequency of 11q loss in familial but not in sporadic PGLs, pointing toward differences in the genetic evolution of familial and sporadic PGLs. Genetic

changes on chromosome 11p and 5p may be involved in sporadic PGLs.

More genetic data are needed to assess the concept and aspects of genetic differences between familial and sporadic parasympathetic PGLs. Further analysis of the candidate tumor suppressor gene *SDHD* and other genes of the mitochondrial complex II is required to determine their involvement in the development of these tumors.

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