Comparative Genomic Hybridization Reveals Frequent Losses of Chromosomes 1p and 3q in Pheochromocytomas and Abdominal Paragangliomas, Suggesting a Common Genetic Etiology

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Pheochromocytomas and abdominal paragangliomas are rare, catecholamine-producing tumors that arise from the chromaffin cells derived from the neural crest. We used comparative genomic hybridization (CGH) to screen for copy number changes in 23 pheochromocytomas and 11 abdominal paragangliomas. The pattern of copy number changes was similar between pheochromocytomas and paragangliomas, with the most consistent finding being loss of 1cen-p31, which was detected in 28/34 tumors (82%). Losses were also found on 3q22–25 (41%), 11p (26%), 3p13–14 (24%), 4q (21%), 2q (15%), and 11q22–23 (15%), and gains were detected on 19p (26%), 19q (24%), 17q24-qter (21%), 11cen-q13 (15%), and 16p (15%). Losses of 1p and 3q were detected in the majority of tumors, whereas gains of 19p and q, 17q, and 16p were seen only in tumors with six or more CGH alterations. This progression of genetic events did not correspond with the conversion to a malignant phenotype. CGH alterations involving chromosome 11 were more frequent in the malignant tumors, compared with the benign tumors (9/12 versus 3/16). In summary, we propose that pheochromocytomas and abdominal paragangliomas, which share many clinical features, also have a common genetic origin and that the loss of 1cen-p31 represents an early and important event in tumor development. (*Am J Pathol* 2000, 156:651–659)

Paraganglia of the sympathoadrenal neuroendocrine system are distributed along the paravertebral and para-aortic axis from the base of the skull to the pelvic floor, and the largest compact collection of paraganglia in this system is the adrenal medulla. Tumors that arise from paraganglia outside the adrenals are named paragangliomas and, if located within the adrenal medulla, pheochromocytomas. Both pheochromocytomas and paragangliomas located within the abdomen are derived from chromaffin cells and produce catecholamines. Histopathological differentiation between benign and malignant lesions is often difficult. Approximately 10% of patients with pheochromocytomas and 15–50% of patients with chromaffin paragangliomas develop metastatic disease, which is the only indisputable proof of malignancy today.^{1,2} Paragangliomas also occur in the head and neck region where they are usually nonchromaffin and rarely metastasize.²

Pheochromocytomas occur either sporadically or, in approximately 10% of the cases, as a part of familial-cancer syndromes, such as multiple endocrine neoplasia type 2 (MEN-2), von Hippel-Lindau's disease (VHL), or neurofibromatosis type 1 (NF1). Pheochromocytomas have also been reported in multiple endocrine neoplasia type 1 (MEN-1) patients.^{3,4,5,6} Recently, loss of the wild-type alleles was demonstrated in two MEN-1-related pheochromocytomas, supporting the underlying role of the *MEN-1* gene in their tumorigenesis.⁷ Although the hereditary forms of pheochromocytoma are fairly well characterized, the genetic background of sporadic pheochromocytomas is still poorly understood. In a minority of sporadic cases, somatic mutations have been found in genes that are involved in the familial forms of the disease, ie, *RET* in 10q11, *VHL* in 3p25, and *NF1* in 17q11.^{8,9}

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Table 1. Clinical and Genetic Data for 34 Pheochromocytomas and Paragangliomas

Case number	Identification number	Diagnosis	Benign/ malignant	Sex	Age at operation (y)	Follow-up (y)	Metastasis	Outcome	Diameter (cm)	Hormonal excretion pattern	MEN-1 mutation analysis
1. Benign tumors											
Sporadic pheochromocytomas											
1	E1657	PHEO	Benign	F	65	3.5	No metastasis	Alive	5.5	NA A DA	D418D
2	E1682	PHEO	Benign	F	52	3.4	No metastasis	Alive	3.5	NA DA	No shift
3	E550	PHEO	Benign	F	52	8.5	No metastasis	Alive	5.5	NA A	D418D
4	E578	PHEO	Benign	F	37	8.4	No metastasis	Alive	4	NA A DA	No shift
5	E600	PHEO	Benign	F	31	7.9	No metastasis	Alive	4.7	NA A	D418D
6	E613	PHEO	Benign	M	58	8.1	No metastasis	Alive	6	NA	D418D, R171Q
7	E81	PHEO	Benign	F	47	12	No metastasis	Alive	9.5	NA A DA	No shift
MEN-2A and NF1-associated											
8	E1899	PHEO-MEN-2A	Benign	F	43	2.5	No metastasis	Alive	2.5	A	ND
9	E1952	PHEO-MEN-2A	Benign	F	34	2.3	No metastasis	Alive	1.9	A	ND
10	E2213	PHEO-MEN-2A	Benign	M	41	1.4	No metastasis	Alive	5.2	NA A DA	D418D
11	E471	PHEO-MEN-2A	Benign	F	45	9	No metastasis	Alive	2.7	NA A	ND
12	E91	PHEO-MEN-2A	Benign	M	53	11.7	No metastasis	Alive	3.5	NA A	D418D
13	E2227	PHEO-NF	Benign	F	70	1.3	No metastasis	Alive	6.2	NA A DA	ND
Sporadic paragangliomas											
14	E2143	PARA	Benign	M	72	1.6	No metastasis	Alive	3.5	NA	D418D
15	E1906	PARA	Benign	M	23	2.5	No metastasis	Alive	4.2	NA	D418D
16	E145	PARA	Benign	M	14	11.3	No metastasis	Alive	4	NA	No shift
2. Malignancy not known											
17	A51300	PHEO*	ND	F	25	2.9	ND	ND	7.5	NA	D418D
18	A41081	PHEO*	ND	F	40	3.3	ND	ND	10	NA DA	No shift
19	A44743A	PHEO*	ND	F	38	3.2	ND	ND	6.2	A	D418D
20	A44741	PHEO*	ND	F	59	3.1	ND	ND	5	A	D418D
21	A43983	PHEO*	ND	M	38	3.2	ND	ND	2.6	NA	R171Q
22	A50444	PHEO*	ND	F	38	3.1	ND	ND	5.5	NA A	D418D
3. Malignant tumors											
Malignant pheochromocytomas											
23	E1553	PHEO	Malignant	M	14	3.8	No metastasis	Alive	5	NA	No shift
24	E1773	PHEO	Malignant	F	77	3	No metastasis	Alive	10	NA A DA	No shift
25	E1971	PHEO	Malignant	F	44	2.3	Met. at diagnosis	Alive	5.7	NA	D418D
26	E2249	PHEO	Malignant	M	40	0.2	Met. at diagnosis	DFD	10.5	NA A DA	No shift
Malignant paragangliomas											
27	E854	PARA	Malignant	M	25	6.8	No metastasis	Alive	3.7	NA	No shift
28	E49	PARA	Malignant	M	43	12.2	Met. after 3 years	DFD	4.7	DA	D418D
29	E370	PARA	Malignant	F	42	9.8	Met. after 3 years	Alive	3	NA DA	D418D
30	E227	PARA	Malignant	F	24	6.6	Met. after 2 years	DFD	10.5	Renin	D418D
31	E92	PARA	Malignant	M	26	0.1	Met. at diagnosis	DFID	3	NA	R171Q
32	E799	PARA	Malignant	M	26	2.2	Met. at diagnosis	DFD	3.5	NA DA	No shift
33	E23	PARA #†	Malignant	M	38	4.1	Met. at diagnosis	DFD	2.2	NA DA	No shift
34	E884	PARA #†	Malignant	F	45	6.7	Met. from no. 29	Alive	7.2	NA DA	D418D

* not available for follow-up; † metastasis; PHEO, pheochromocytoma; PARA, paraganglioma; F, female; M, male; DFD, dead from disease; DFID, dead from intercurrent disease; NA, noradrenaline; A, adrenaline; DA, dopamine; ND, not determined; Met., Metastasis.

However, the involvement of the *MEN-1* gene is yet to be determined. Studies of somatic genetic alterations in primarily benign pheochromocytomas have shown loss of heterozygosity (LOH) in 1p, 3p, 3q, 11p, 17p, and 22q, suggesting the existence of tumor suppressor gene loci in these locations.¹⁰⁻²¹

Most of the patients with head and neck paragangliomas have a familial background related to the *PGL1* and *PGL2* loci, which have been mapped to 11q23 and 11q13, respectively, and are both subjected to maternal genomic imprinting.²²⁻²⁴ Based on the frequent and specific LOH of the 11q23 region in the sporadic and familial forms of these tumors, the *PGL1* gene is anticipated to have a tumor suppressor gene function. Abdominal paragangliomas are most frequently sporadic, although familial forms have also been reported, suggesting that this disease may occur as a distinct genetic entity.²⁵ Available genetic data on abdominal paragangliomas have

been limited and mainly include analysis of single cases or studies of candidate regions in a few selected cases.²⁶

We have used comparative genomic hybridization (CGH) to screen a panel of benign and malignant pheochromocytomas and abdominal paragangliomas for DNA sequence copy number alterations. Furthermore, the involvement of the *MEN-1* gene was determined by mutation analysis.

Materials and Methods

Clinical Cases

The study includes 34 tumors from 33 patients with pheochromocytoma or abdominal paraganglioma. Twenty-eight of the patients were operated on between 1985 and 1997 and then clinically monitored at the Endocrine Sur-

Figure 1. Summary of DNA copy number alterations detected by CGH in 23 pheochromocytomas and 11 abdominal paragangliomas. Each line represents one alteration detected in one tumor with losses illustrated to the left and gains to the right of the ideograms.

gical Unit at the Karolinska Hospital. Six patients were operated on at the Russian Center for Endocrinology, Moscow, Russia, in 1995, but have not been available for follow-up. The study was approved by the local ethics committee at the Karolinska Hospital.

The clinical data for the 23 pheochromocytomas and 11 abdominal paragangliomas are summarized in Table 1. From one patient with a malignant paraganglioma, both the primary tumor and the liver metastasis were analyzed (Table 1, cases 29 and 34). In the pheochromocytoma group, there were four cases of sporadic malignant tumors, 13 sporadic cases without evidence of malignancy, and five MEN-2A-associated cases and one related to NF1. In the paraganglioma group, there were eight sporadic-malignant cases and three sporadic-benign cases. Of the 34 tumors analyzed, 32 were primary tumors (Table 1, cases 1–32). Two of the malignant paragangliomas were metastases. In one of those cases (Table 1, case 34), DNA from the corresponding primary tumor (Table 1, case 29) was also analyzed; in the other case (Table 1, case 33), only metastatic DNA was available. Tumors were classified as malignant, based on histopathological criteria and the presence of metastasis.

Because no World Health Organization classifications covering these tumors have been published, we have adapted the criteria for malignancy published by the Armed Forces Institute of Pathology, ie, extensive local invasion and/or the presence of metastasis.² Of the 12 tumors classified as malignant, 9 had developed metastasis (Table 1).

High-molecular-weight DNA was isolated by standard procedures from fresh-frozen tumor specimens stored at -70°C . To evaluate the proportion of tumor cells, sections were cut from all frozen tumor specimens and subjected to histopathological examination. All tumor samples were shown to contain more than 70% tumor cells.

CGH

All 34 tumors were analyzed by CGH, essentially as described.²⁷ Tumor DNA labeled with fluorescein isothiocyanate-deoxyuridine triphosphate (400 ng; DuPont, Boston, MA) and 400 ng of sex-matched reference DNA labeled with Texas Red-deoxyuridine triphosphate (DuPont) were hybridized together with 10 μg of unlabeled Cot-1-blocking DNA (Boehringer Mannheim, Mannheim, Germany) on normal metaphase preparations (Vysis Inc., Downers Grove, IL). After 48 hours of incubation, the slides were washed and counterstained with 4',6-diamidino-2-phenylindole (Boehringer Mannheim) in an antifade solution. Normal male DNA and female DNA were hybridized together to normal metaphase preparations as a negative control, and, as a positive control, a previously characterized breast cancer cell line (MCF-7) was used.

The ratios of green-to-red fluorescence intensities, representing tumor to normal copy number, were analyzed along each autosome and the X chromosome in a digital image analysis system. In a CGH analysis system, an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan) and a Photometrics Image-Point b/w CCD camera (Photometrics Ltd., Tuscon, AZ) equipped with IPLab software (Signal Analytics Corp., Frederick) were used. Images were further analyzed with the Quips CGH (Vysis) software package. A chromosomal region was considered to be over-represented if the average green-to-red fluorescence ratio exceeded the 1.15 cutoff line (a gain), as amplified if the ratio exceeded the 1.5 cutoff line, and as under-represented if the ratio was below the 0.85 line (a loss).

Mutation Analysis of the MEN-1 Gene

Mutation analysis was performed in 30/34 tumors, using single-stranded conformation analysis (SSCA) and direct sequencing, essentially as previously described.²⁸ The coding exons 2–10 and the untranslated exon 1 of the *MEN-1* gene were amplified using 15 different fragments of 200–300 bp each. Except for primers within exons 2, 3, and 10, the primers originated from the flanking intronic sequences. Genomic DNA (50 ng) was amplified in 15- μL polymerase chain reaction (PCR) reactions containing 50 mmol/L KCl; 10 mmol/L Tris-HCl, pH 9.0; 1.5

Table 2. Losses and Gains on the Most Frequently Involved Chromosome Arms in Relation to the Total Number of Alterations in the Tumor

ID no.	Diagnosis*	No. of changes	Losses					
			1p	3q	3p	11p	11q	4q
E81	PHEO	0						
E1906	PARA	0						
E613	PHEO	1	1cen-p31					
E854	Mal PARA	1	1p					
A44743A	PHEO [†]	1	1p21-22					
E2213	PHEO-MEN-2A	1						
E550	PHEO	2	1cen-p31					
E1657	PHEO	2	1cen-pe1	3q	3p			
E1952	PHEO-MEN-2A	2	1cen-p33					
E145	PARA	2	1p					
A43983	PHEO [†]	2	1p	3q				
E1553	Mal PHEO	2			3p	11p		
E92	Mal PARA	2				11p	11q14-qter	
E578	PHEO	3	1cen-p31					
E91	PHEO-MEN-2A	3	1cen-p31	3q				
E2227	PHEO-NF	3	1p	3q				
E2249	Mal PHEO	3	1p31-35				11q13-q23	
E799	Mal PARA	4	1cen-p31	3q	3p			
E1682	PHEO	4	1cen-p32					
E1899	PHEO-MEN-2A	5	1p			11p		4q21-qter
E1773	Mal PHEO	6	1p	3q				4q
E23	Mal PARA met	6	1p	3q		11p15	11q22-qter	
A51300	PHEO [†]	6	1cen-p32					
E1971	Mal PHEO	6	1cen-p21		3cen-p14	11p	11q13-qter	
E49	Mal PARA	7	1p	3q	3p	11cen-p14		4q13-22
E227	Mal PARA	7	1cen-p31		3p13-21			4q28-qter
A44741	PHEO [†]	7	1cen-p31	3q				
A50444	PHEO [†]	8	1cen-p31	3q				4q
A41081	PHEO [†]	9	1cen-p31			11p12-pter	11q14-qter	
E471	PHEO-MEN-2A	10	1cen-p33	3q	3p			
E370	Mal PARA	12	1cen-p31	3q	3p			
E2143	PARA	12	1cen-p33			11p12-pter		4q
E884	Mal PARA met	13	1cen-p31	3q		11cen-p14		
E600	PHEO	14		3q22-25				4q25-27

*Abbreviations: PHEO, pheochromocytoma; PARA, paraganglioma; NF, neurofibromatosis; Mal, Malignant; met, metastasis.

[†]. Patients not available for follow up.

mmol/L MgCl₂ (Promega, Madison, WI); 0.2 mmol/L each of deoxyribosylthymine triphosphate, deoxyadenosine triphosphate, and deoxyguanosine triphosphate; 0.05 mmol/L deoxycytidine triphosphate; α -³²P-labeled deoxycytidine triphosphate (Amersham Pharmacia Biotech, Stockholm, Sweden) at 1 mCi/reaction, 0.2 mmol/L of each oligonucleotide primer, and 2 U of DNA polymerase (Dynazyme, Finnzyme Oy, Finland). Thermocycling conditions consisted of denaturing at 94°C for 5 minutes and 30 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C, followed by a final extension step for 2 minutes at 72°C. For exons 2.2 and 10.2, the following PCR conditions were used: denaturing at 96°C for 2 minutes and 30 cycles of 96°C for 45 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension step for 2 minutes at 72°C. The PCR products were then electrophoresed in 25% mutation detection enhancement gels (FMC BioProducts, Rockland, ME) at room temperature for 12 hours at 6–8 W, followed by autoradiography. When an SSCA-shifted band was detected, it was excised from the gel and sequenced on a

377 automated fluorescent sequencer (Applied Biosystems, The Perkin Elmer Corp., Foster City, CA).

Statistical Analysis

A correlation between CGH aberrations and clinical features was analyzed using the Mann-Whitney *U* test and Fisher's exact test with the StatView 4.5 software. The six pheochromocytoma cases that were not available for follow-up were excluded from the comparisons between malignant and benign tumors.

Results

CGH Alterations in Pheochromocytomas and Paragangliomas

Chromosomal imbalances were detected in 32/34 tumors (Figure 1 and Table 2). Losses were twice as common as gains. In pheochromocytomas, the most frequent losses

Table 2. Continued

Gains						Other losses	Other gains
11q	19p	19q	16p	17q	17p		
						X 2q 8	8p12-cen-q21
						5cen-q33, 14cen-q23	10 1q
11q						9q21-qter 18 15q22-qter	8q, 15cen-q21 2p12-p23, Xq21-25 15cen-q15, Xp22-pter X
	19p	19q				4p, 2q23-31 15q23-qter 1q32-qter, 17pter-cen-q12	1cen-q13,20q
	19p	19q	16p			2q31-32, 6p21-pter, 6q16-qter 14q, X 6q, 7cen-q31, 8p	
11cen-q13	19p	19q		17q23-qter 17q23-qter		4p13-cen 9p, 15q, 21q	9q32-qter, 22q 18, 20q,13
11cen-q13	19p	19q		17q24-qter 17q23-qter 17q23-qter	17p	21q, X 8, 13q	15q, 16cen-q12, 20 1q, 2p, 4p15-pter, 7cen-q21, 9q31-qter, 16q
	19p	19q	16p	17cen-q12, 17p 17q23-qter	17p12-q12	6cen-q24, 13q21-31	2p21-pter, 12q22-qter
11cen-q13	19p	19q	16p	17q23-qter	17p	2q, 8, 15cen-q21 2q22-33, 6q16-21, 12q15-21, 13q13-31, 14q13-21, 18p	1q, 7, 22q 4p15-pter, 5p14-pter, 15q21-24

were found on chromosome arms 1p (83%), 3q (39%), 11p (17%), 3p (17%), 4q (17%), and 11q (13%). Gains were seen predominantly on 19p (26%), 19q (26%), 17q (17%), and 16p (9%), and in one tumor 20q was amplified. The 6 hereditary and 13 sporadic benign tumors showed similar patterns of CGH alterations. In paragangliomas, the most frequent losses were found on chromosome arms 1p (82%), 3q (45%), 11p (45%), 3p (36%), 4q (27%), and 11q (18%). Gains were seen predominantly on chromosome arms 19p (55%), 11q (36%), 16p (27%), 17q (27%), and 19q (18%). When the results were compared, the gain of 11cen-q13 was found to be significantly more frequent in paragangliomas than in pheochromocytomas ($P < 0.05$, Fischer's exact test). No other major differences in the patterns of copy number changes nor in the minimal regions of involvement between pheochromocytomas and paragangliomas were seen (Figure 1). The minimal regions involved in the most commonly detected losses and gains are listed in Table 3.

The pattern of CGH alterations in the individual tumors varied depending on the total number of detected alterations (Table 2). Loss of 1cen-p31 was detected in 28/32 cases displaying copy number changes and was found

as a single CGH alteration in three samples. Gains of chromosomes 16p, 17q23-24, and 19 were detected only in tumors having a total of six or more CGH changes. Furthermore, gains of 11cen-q13 were seen in tumors having at least four alterations, whereas losses occurred independently of the total number of alterations. There was no correlation between tumor size and number of genetic aberrations, nor between hormonal excretion pattern and chromosomal changes.

CGH Alterations in Benign and Malignant Tumors

The distribution of losses and gains on the different chromosome arms in the benign contra malignant tumors is shown in Figure 2. The main difference was that chromosome 11 was partly lost and/or gained in 9/12 malignant cases *versus* 3/16 benign tumors. Of the nine cases that had developed metastasis, eight showed involvement of chromosome 11 (Table 3). Loss of 11q22-23 was significantly more common in malignant tumors than in benign ones ($P < 0.05$, Fischer's exact test). The mean and

Table 3. Recurrent CGH Alterations Detected in Pheochromocytomas and Paragangliomas

Alteration	Minimal region*	Total† (n = 34)	Benign (n = 16)	Malignant (n = 12)
Losses				
1p	1cen-p31	28	12	10
3q	3q22-25	14	5	6
11p	—	9	2	6
3p	3p13-14	8	2	6
4q	—	7	3	4
11q	11q22-23	5	0	4
2q	2q23-31	5	2	3
6q	6q16-24	4	2	1
8p	8p	4	1	2
15q	—	4	1	2
X	X	4	2	2
8q	8q	3	1	2
13q	13q21-31	3	2	1
14q	14q13-21	3	2	1
Gains				
19p	19p	9	3	3
19q	19q	8	3	2
17q	17q24-qter	7	3	2
11q	11cen-q13	5	1	3
16p	16p	5	2	3
1q	1cen-q31	4	1	2
15q	—	4	3	1
17p	17cen-p12	4	2	1
2p	2p21-23	3	2	1
20q	20q13	3	1	0

*—, No minimal region identified.

†, Six patients were excluded from the analysis.

median values of the total number of genetic aberrations ($P = 0.06$, Mann-Whitney U test) were higher in the malignant tumors (mean 4, median 5.7) than in the benign ones (mean 2.5, median 6), but with a wide range in all groups as illustrated in Table 2 and Figure 2. No specific aberration that correlated to a poor prognosis was identified in the five patients who died from the disease. There was no significant difference in the pattern of CGH alterations between malignant pheochromocytomas and malignant paragangliomas.

Mutation Analysis of the MEN-1 Gene

Mutation analysis of the *MEN-1* gene was performed on 30 of the tumors (Table 1) and did not reveal any mutations, although polymorphisms were readily identified. Twenty SSCA shifts were detected, and by sequencing they were all shown to represent polymorphisms: 17 cases of D418D (57%) and 3 cases of R171Q (10%). Furthermore, the detected frequencies of these polymorphisms were in agreement with those previously reported.²⁹

Discussion

This study represents the first genome-wide screening for copy number changes by CGH in pheochromocytomas and abdominal paragangliomas. CGH changes were detected in 32/34 tumors, which shows that alterations affecting genetic copy number are common in these tumor

types. The most striking observation was the loss of all or parts of chromosome arm 1p, which was detected in 28/34 cases (82%). In previous studies, LOH on 1p has been reported in approximately one half of pheochromocytomas, but no clear, minimal common region has been identified.^{10,11,13-15,26} Some LOH studies have focused on the distal part of 1p, because 1p36 deletions correlate with a poor prognosis in neuroblastomas, a childhood tumor with the same embryonic origin as pheochromocytomas and paragangliomas.³⁰ 1p36 was later found to harbor the p73 tumor suppressor gene.³¹ In this study, the minimal region involved was 1cen-p31, which makes p73 unlikely as a candidate gene (Figure 1). However, the proximal loss of 1p detected by CGH does not exclude additional deletions affecting the p73 locus or other genes in distal 1p. Results from CGH and LOH studies are not always comparable. For example, deletion of one chromosomal region accompanied by duplication of the remaining allele would give the appearance of no loss by CGH, but would be detected by LOH. Nevertheless, the combined results of the present and previous studies suggest the involvement of one or several genes, located within or proximal to 1p31, in the development of pheochromocytomas and abdominal paragangliomas.

Moley et al found LOH on 1p in 9/9 pheochromocytomas from patients with MEN-2, but in only 2/7 sporadic cases, and these authors speculated that 1p could harbor genes that are specifically involved in the tumorigenesis of MEN-2-related tumors.¹⁴ We did not find any significant differences in copy number changes between hereditary and sporadic pheochromocytomas that could confirm this hypothesis.

Losses were more common than gains, indicating that the inactivation of tumor suppressor genes plays a critical role in tumorigenesis. Apart from the loss of 1p, previous LOH studies have reported frequent allele losses on 3p, 3q, and 11p and on 17p and 22q. Mulligan et al allelotyped 16 sporadic and 13 familial pheochromocytomas and found significant LOH on 1p (60%), 3p (55%), 3q (60%), 11p (16%), 13q (10%), 17p (15%) and 22q (40%).¹¹ Khosla et al tested 34 sporadic and 7 familial cases of pheochromocytomas for LOH near a variety of potentially important genetic loci and reported significant allele losses on chromosomes 1p (42%), 3p (16%; *VHL* locus), 17p (24%; *p53* and *NF1* loci), and 22q (31%), but not on 10q (*MEN-2* locus).¹⁰ In a study of 22 pheochromocytomas, 55% of the tumors showed LOH on 1p and 40% on 22q, and the two alterations co-occurred significantly within the same tumors.¹⁵ In the present study, losses of 3q22-25 and 3p13-14 were frequently detected (in 41% and 24%, respectively) in both pheochromocytomas and paragangliomas. The minimal common region on 3p, though, is located proximal to the *VHL* locus. In contrast to previous LOH data, we could not detect copy number changes of 22q in more than two cases. Losses and gains of 17p were detected only in a minority of cases.

With CGH we could not detect any major differences in the pattern of copy number changes between pheochromocytomas and abdominal paragangliomas (Figure 1). The only exception was that a gain of 11cen-q13 was

Figure 2. The number of gains (white bars) and losses (filled bars) on each chromosome arm in the benign (top), and the malignant (bottom) tumors. A dotted line representing two CGH alterations is added to more easily visualize the most frequent gains and losses. The frequency of recurrent alterations is listed in Table 3.

more common in paragangliomas than in pheochromocytomas. This may reflect the higher frequency of malignant tumors in the paraganglioma group, because this chromosomal region is commonly gained in malignant tumors, or it may point toward an involvement of the *PGL2* locus. The general pattern of copy number changes in chromaffin paragangliomas, with the high frequency of 1cen-p13 losses, is quite different from available LOH data on non-chromaffin paragangliomas with specific losses on 11q23.³² Although involvement of the *PGL1* and *PGL2* loci cannot be excluded in the tumorigenesis of chromaffin paragangliomas, it seems likely that alternative molecular pathways are involved in chromaffin and nonchromaffin paragangliomas. Our findings suggest that pheochromocytomas and catecholamine-secreting paragangliomas not only share many clinical features, but also have a similar genetic background.

No mutations in the *MEN-1* gene were detected in any of the tumors, which suggests, despite the sensitivity of our mutation detection strategy (ie, SSCP), that the *MEN-1* gene is rarely involved in the tumorigenesis of

sporadic pheochromocytomas and abdominal paragangliomas. This may explain the very low frequency of these tumors in MEN-1 patients.

The distribution of genetic alterations on the most frequently involved chromosome arms (Table 2) suggests that there may be a progression of genetic events in the development of these tumor types. Because loss of 1cen-p13 was detected in the large majority of tumors, regardless of subtype and degree of malignancy, it is likely that inactivation of a gene located in this region is an important early event. On the other hand, the most common gains detected with CGH were seen predominantly in tumors with a high total number of aberrations. Taken together, losses of 1cen-p13 and 3q22–25 seem to precede gains of 19p and q, 16p, and 17q23–24. However, this genetic progression cannot be directly translated into a clinical progression, which indicates that these are not key events in the development of malignant tumors.

The malignant tumors showed generally a higher number of genetic aberrations than the benign cases, which is a sign of genetic instability. However, as shown in

Table 2, the individual variations in the number of detected alterations fell within a wide range, and, in addition, three benign tumors displayed a large number of alterations. The major difference between benign and malignant tumors (Figure 2) was involvement of chromosome 11, which was partly lost and/or gained in the majority of malignant cases. Loss of 11q22–23 (including the *PGL1* locus) was particularly common in malignant tumors. However, there was no single, minimal region of involvement, which makes these results difficult to interpret.

In conclusion, the results from our study with CGH in pheochromocytomas and abdominal paragangliomas indicate that inactivation of a gene located on the proximal part of 1p is an important and early event in the development of these tumor types and that there may be a progression of genetic events that involves chromosomes 3, 11, 17, and 19. Conversion to a malignant phenotype may be associated with changes on chromosome 11. We further conclude that intra- and extra-adrenal paragangliomas of the sympathoadrenal system share common progression pathways.

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References

1. Tischler AS: The adrenal medulla and extra-adrenal paraganglia: functional endocrine pathology. Edited by K Kovacs, SL Asa. Oxford, UK, Blackwell Scientific, 1991, pp 509–545
2. Lack E: Tumors of the adrenal gland and extra-adrenal paraganglia. Atlas of Tumor Pathology. Washington, DC, Armed Forces Institute of Pathology, 1997
3. Trump D, Farren B, Wooding C, Pang JT, Besser GM, Buchanan KD, Edwards CR, Heath DA, Jackson CE, Jansen S, Lips K, Monson JP, O'Halloran D, Sampson J, Shalet SM, Wheeler MH, Zink A, Thakker RV: Clinical studies of multiple endocrine neoplasia type 1 (MEN1). *Q J Med* 1996, 89:653–669
4. Marx S: Multiple endocrine neoplasia type 1: clinical and genetic topics. *Ann Intern Med* 1998, 129:484–494
5. Carty SE, Helm AK, Amico JA, Clarke MR, Foley TP, Watson CG, Mulvihill JJ: The variable penetrance and spectrum of manifestations of multiple endocrine neoplasia type 1. *Surgery* 1998, 124:1106–1113
6. Teh BT, Kytölä S, Farnebo F, Bergman L, Wong FK, et al: Mutation analysis of the MEN1 gene in multiple endocrine neoplasia type 1, familial acromegaly and familial isolated hyperparathyroidism. *J Clin Endocrinol Metab* 1998, 83:2621–2626
7. Cote GJ, Lee JE, Evans DB: The spectrum of mutations in MEN-1 variant syndromes. Program and Abstracts 80th Annual Meeting of Endocrine Society. New Orleans, LA, Endocrine Society, 1998, pp106–107 (Abstract)
8. Hofstra RMW, Stelwagen T, Stulp RP, Dejong D, Hulsbeek M, Karmsteeg EJ, Vandenberg A, Landsvater RM, Vermey A, Molenaar WM, Lips CJM, Buys CHCM: Extensive mutation screening for ret in sporadic medullary thyroid carcinoma and of ret and vhl in sporadic pheochromocytoma reveals involvement of these genes in only a minority of cases. *J Clin Endocrinol Metab* 1996, 81:2881–2884
9. Gutmann DH, Geist RT, Rose K, Wallin G, Moley JF: Loss of neurofibromatosis type 1 (NF1) gene expression in pheochromocytomas from patients without NF1. *Genes Chromosomes Cancer* 1995, 13: 104–109
10. Khosla S, Patel VM, Hay ID, Schaid DJ, Grant CS, van Heerden JA, Thibodeau SN: Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. *Clin Invest* 1991, 87:1691–1699
11. Mulligan LM, Kwok JBJ, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L, Ponder MA, Telenius H, Tunnacliffe A, Ponder BAJ: Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 1993, 363:458–460
12. Mulligan LM, Gardner E, Smith BA, Mathew CG, Ponder BA: Genetic events in tumour initiation and progression in multiple endocrine neoplasia type 2. *Genes Chromosomes Cancer* 1993, 6:166–177
13. Mathew CGP, Smith BA, Thorpe K, Wong Z, Royle NJ, Jeffreys AJ, Ponder BAJ: Deletion of genes on chromosome 1 in endocrine neoplasia. *Nature* 1987, 328:524–526
14. Moley JF, Brother MB, Fong CT, White PS, Baylin SB, Nelkin B, Wells SA, Brodeur GM: Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. *Cancer Res* 1992, 52:770–774
15. Shin E, Fujita S, Takami K, Kurahashi H, Kurita Y, Kobayashi T, Mori T, Nishisho I, Takai S: Deletion mapping of chromosome 1p and 22q in pheochromocytoma. *Jpn J Cancer Res* 1993, 84:402–408
16. Zeiger MA, Zbar B, Keiser H, Linehan WM, Gnarr JR: Loss of heterozygosity on the short arm of chromosome 3 in sporadic, von Hippel-Lindau disease-associated, and familial pheochromocytoma. *Genes Chromosomes Cancer* 1995, 13:151–156
17. Tanaka N, Nishisho I, Yamamoto M, Miya A, Shin E, Karakawa K, Fujita S, Kobayashi T, Rouleau G, Mori T, Takai S: Loss of heterozygosity on the long arm of chromosome 22 in pheochromocytoma. *Genes Chromosomes Cancer* 1992, 5:399–403
18. Yokogoshi Y, Yoshimoto K, Saito S: Loss of heterozygosity on chromosomes 1 and 11 in sporadic pheochromocytomas. *Jpn J Cancer Res* 1990, 81:632–638
19. Takai SI, Tateishi H, Nishisho I, Miki T, Motomura K, Miyauchi A, Kato M, Ikeuchi T, Yamamoto K, Okazaki M, Yamamoto M, Honjo T, Kumahara Y, Mori T: Loss of genes on chromosome 22 in medullary thyroid carcinoma and pheochromocytoma. *Jpn J Cancer Res* 1987, 78: 894–898
20. Tsutsumi M, Yokota J, Kakizoe T, Koiso K, Sugimura T, Terada M: Loss of heterozygosity on chromosomes 1p and 11p in sporadic pheochromocytoma. *J Natl Cancer Inst* 1989, 81:367–370
21. Xu W, Mulligan LM, Ponder MA, Liu L, Smith BA, Mathew CGP, Ponder BAJ: Loss of NF1 alleles in pheochromocytomas from patients with type 1 neurofibromatosis. *Genes Chromosomes Cancer* 1992, 4:337–342
22. Heutink P, van Schothorst EM, van der Mey AGL, Bardool A, Breedveld G, Pertijs J, Sandkuijl LA, van Ommen GJB, Cornelisse CJ, Oostra BA, Devilee P: Further localization of the gene for hereditary paragangliomas and evidence for linkage in unrelated families. *J Hum Genet* 1994, 2:148–158
23. Baysal BE, Farr JE, Rubinstein WS, Galus RA, Johnson KA, Aston CE, Myers EN, Johnson JT, Carrau R, Kirkpatrick SJ, Myssiorek D, Singh D, Saha S, Gollin SM, Evans GA, James MR, Richard CW III: Fine mapping of an imprinted gene for familial nonchromaffin paragangliomas, on chromosome 11q23. *Am J Hum Genet* 1997, 60:121–132
24. Mariman ECM, van Beersum SEC, Cremers CWRJ, Struycken PM, Ropers HH: Fine mapping of a putatively imprinted gene for familial non-chromaffin paragangliomas to chromosome 11q13.1: evidence for genetic heterogeneity. *Hum Genet* 1995, 95:56–62
25. Sköldeberg F, Grimelius L, Woodward ER, Rorsman F, Van Schorst EW, Wingvist O, Karlsson FA, Åkerström G, Kämpe O, Husebye ES: A family with hereditary extra-adrenal paragangliomas without evidence for mutations in the von Hippel-Lindau disease or the ret genes. *Clin Endocrinol* 1998, 48:11–16
26. Vargas MP, Zhuang Z, Wang C, Vortmeyer A, Linehan WM, Merino MJ: Loss of heterozygosity on the short arm of chromosomes 1 and 3 in sporadic pheochromocytoma and extra-adrenal paraganglioma. *Hum Pathol* 1997, 28:411–415
27. Kallioniemi OP, Kallioniemi A, Piper J, Isola J, Waldman FM, Gray JW, Pinkel D: Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. *Genes Chromosomes Cancer* 1994, 4:231–243
28. The European consortium on MEN1: identification of the multiple endocrine neoplasia type 1 (MEN1) gene. *Hum Mol Genet* 1997, 7:1177–1183
29. Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins

- FS, Emmert-Buck MR, Debelenko LV, Zhuang Z, Lubensky IA, Liotta LA, Crabtree JS, Wang Y, Roe BA, Weisemann J, Boguski MS, Agarwal SK, Kester MB, Kim YS, Heppner C, Dong Q, Spiegel AM, Burns AL, Marx SJ: Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* 1997, 276:404–407
30. Fong C, Dracopoli NC, White PS, Merrill PT, Griffith RC, Honsman DE, Brodeur GM: Loss of heterozygosity of the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci USA* 1989, 86:3753–3757
31. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F, Caput D: Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 1997, 90(4):809–819
32. Devilee P, van Schothorst EM, Berdoel AFJ, Bonsing B, Kuipers-Dijkshoorn NK, James MR, Fleuren G, van der Mey AGL, Cornelisse CJ: Allelotype of head and neck paragangliomas: allelic imbalance is confined to the long arm of chromosome 11, the site of the predisposing locus PGL. *Genes Chromosomes Cancer* 1994, 11:71–78