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Short Communication

Losses of Chromosomes 1p and 3q Are Early Genetic Events in the Development of Sporadic Pheochromocytomas

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Despite several loss of heterozygosity studies, a comprehensive genomic survey of pheochromocytomas is still lacking. To identify DNA copy number changes which might be important in tumor development and progression and which may have diagnostic utility, we evaluated genetic aberrations in 29 sporadic adrenal and extra-adrenal pheochromocytomas (19 clinically benign tumors and 10 malignant lesions). Comparative genomic hybridization was performed using directly fluorochrome-conjugated DNA extracted from frozen (16) and paraffin-embedded (13) tumor tissues. The most frequently observed changes were losses of chromosomes 1p11–p32 (86%), 3q (52%), 6q (34%), 3p, 17p (31% each), 11q (28%), and gains of chromosomes 9q (38%) and 17q (31%). No amplification was identified and no difference between adrenal and extra-adrenal tumors was detected. Progression to malignant tumors was strongly associated with deletions of chromosome 6q (60% *versus* 21% in clinically benign lesions, $P = 0.0368$) and 17p (50% *versus* 21%). Fluorescence *in situ* hybridization confirmed the comparative genomic hybridization data of chromosomes 1p, 3q, and 6q, and revealed aneuploidy in some tumors. Our results suggest that the development of pheochromocytomas is associated with specific genomic aberrations, such as losses of 1p, 3q, and 6q and gains of 9q and 17q. In particular, tumor suppressor genes on chromosomes 1p and 3q may be involved in early tumorigenesis, and deletions of chromosomes 6q and 17p in progression to malignancy. ***(Am J Pathol 2000, 157:353–359)***

Pheochromocytomas (PCCs) represent neuroendocrine tumors derived from pheochromocytes within and outside the adrenal medulla. PCCs usually produce catecholamines and, as a result of inappropriate hormone secretion, can cause life-threatening disorders, such as myocardial infarction and cerebrovascular hemorrhage.

To date no reliable clinical or histopathological markers are available to distinguish benign from malignant PCCs. Metastases, occurring in approximately 10% of the tumors, are the only convincing sign of malignancy. They may already be present at the time of diagnosis or occur only after lag phases as long as 25 to 30 years.[1] Such a situation calls for more accurate markers that can predict the clinical behavior of these tumors.

The genetic mechanisms underlying the tumorigenesis of PCCs are poorly understood. Because of the known association with the inherited tumor syndromes multiple endocrine neoplasia type 2 (MEN2), Von Hippel-Lindau disease (VHL) and neurofibromatosis type 1 (NF1),[2–4] PCCs have been investigated for involvement of the responsible *RET,* VHL, and NF1 genes, respectively.[5–9] However, only a subset of sporadic lesions harbors somatic mutations in these genes, suggesting that they do not play a prominent role in PCC tumorigenesis.[10–13] Furthermore, no mutation has been detected in the c-*mos* proto-oncogene or in the p16 tumor suppressor gene (TSG) in benign and malignant lesions.[14,15] Conflicting results exist with regard to the frequency of *TP53* gene alterations in PCCs as well as for the association of c-*erb*B-2 expression with malignancy.[16–19] Other onco-

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Table 1. Patient Characteristics and Genetic Findings in Sporadic Pheochromocytomas

				CGH results					FISH results			
				Number of changes			Chromosomal changes					
Type/no.	Age/Sex	Size (cm)	Location	All	Gain	Loss	of chromosomes 1, 3, 6, 9, and 17	IHC p53*	1p22†	1p36†	3q26†	6q22†
Benign												
1	63/F	4.7	EA	4	1	3	1p11-31−, 3p−, 9q33-qter+					
2	29/F	18	L	6	3	3	1p11-31−, 3p−, 9q33-qter+, 17pq+					
3	49/M	13	BI	7	2	5	1p−, 3pq−, 17p−, 17q+					
4	34/M	6	L	4	0	4	3pq−, 9q33-qter+		dis/tetras	dis/tetras	monos/dis	dis/tetras
5	75/F	5	EA	6	2	5	1p11-32−, 17pq+		4-2	4-2	tetras	tetras
6	52/F	5	R	9	4	2	9q32-34+					
7	43/F	3	R	3	1	2	1p11-32−, 3q−, 9q32-qter+					
8	42/M	6	L	5	1	4	1p11-32−, 3q−, 6q−, 9q32-qter+		2-1/4-2	dis/tetras	2-1/4-2	2-1/4-2
9	53/M	nk	L	4	0	4	1p11-31−					
10	27/F	11	L	6	2	4	1p−, 3pq−, 9q32-qter+, 17q+					
11	72/F	nk	R	14	2	12	1p11-32−, 3pq−, 6q−, 9q33-qter+, 17q+					
12	43/M	6	R	3	0	3	1p11-31−, 3q21-qter−, 17p−					
13	42/M	4.5	R	3	0	3	1p11-35−, 3q−	−				
14	45/F	2.5	L	7	2	5	1p11-31−, 6q−, 17q22-qter+	−				
15	66/M	3.2	L	6	1	5	1p−, 3pq−, 17p−	−				
16	79/M	5	R	5	1	4	1p11-35−, 3pq−	−				
17	51/F	5	L	3	0	3	1p11-32−, 3q−, 6q−	−				
18	57/M	15	nk	1	0	1		−				
19	47/M	nk	EA	4	0	4	1p−, 3q−, 17pq−	−				
Malignant												
20	49/F	4	Meta., LN	5	2	3	1p11-31−, 3q−, 6q−, 9q33-qter+, 17q22-qter+	+				
21	36/M	nk	nk	1	0	1	1p11-21−					
22	43/F	nk	Meta., LN	5	0	5	1p−, 3q−, 6q−, 17pq−		2-1	2-1	2-1	2-1
23	36/M	21	EA	3	1	2	1p11-31−, 9q32-qter+					
24	70/F	16	L	6	0	6	1p31-pter−, 6q−, 17p−	−				
25	65/F	11	R	13	6	7	1p11-31−, 9pq11-33−, 17p−, 17q23-qter+	+				
26	56/F	8.5	L	21	13	8	1p−, 3p−, 3q+, 6q−, 9pq+, 17pq−	+				
27	23/F	8	nk	4	2	2	1p11-32−, 3q−, 17q+	−				
28	62/F	12	L	16	9	7	6q−	−				
29	49/M	7.5	R	8	0	8	1p−, 6q22-qter−, 17p−	−				

*Data previously published (Ref. 20).
†In combination with the chromosome-specific centromere probe.
Abbreviations: M, male; F, female; L, left adrenal gland; R, right adrenal gland; BI, bilateral adrenal localization; EA, extra-adrenal localization; Meta., metastasis; LN, lymph node; nk, not known; n, no detectable changes; monos, monosomy; dis, disomy; tetras, tetrasomy; 4-2, tetrasomy with only two copies (loss) of the specific region; 2-1, disomy with allelic loss of the specific region.

genes that have been found to be overexpressed in PCCs, include c-*myc*, c-*fos*, and Bcl-2.[19,20]

Several loss of heterozygosity (LOH) studies provided evidence for involvement of TSGs on chromosomes 1p, 3p, 11p, 17p, and 22q.[21–25] However, the impact of these data are limited, since small series of sporadic PCCs were analyzed and often no correlation with clinical data was presented. Moreover, the vast majority of the genome remains unexamined. We performed comparative genomic hybridization (CGH) analysis on 29 apparently sporadic PCCs, including 19 benign and 10 malignant lesions, to identify chromosome alterations which may be important in tumor development and behavior and might have diagnostic utility. Additionally, fluorescence *in situ* hybridization (FISH) experiments were carried out to independently confirm some of the CGH data.

Materials and Methods

Patients and Tumor Samples

Altogether, tumors of 29 patients with an apparently sporadic adrenal ($n = 25$) or extra-adrenal ($n = 4$) PCC were analyzed, including 19 clinically benign and 10 proven malignant lesions (with metastases). A pheochromocytoma was considered sporadic if there was no family history of MEN2, VHL, or its constituent tumors and there were no other signs of MEN 2, VHL, or NF1 recorded by the clinician. Furthermore, previous analyses did not reveal germline mutations in the *RET* and VHL gene in these tumors[10,11] (Dannenberg et al, unpublished data). The average age of the patients was 50.3 years (range: 23–79) and the mean size of the tumors was 7.1 ± 4.6 cm for benign lesions and 11.0 ± 4.7 cm for malignant lesions. Nine of 19 benign and 7 of 10 malignant tumors were from female patients. Except for two lymph node metastases, all specimens were primary tumors. Clinical follow-up was obtained in all benign cases with an average follow-up period of 7.6 years. Relevant characteristics of all patients are listed in Table 1.

DNA Extraction

Genomic DNA from 16 frozen tumors was isolated using the D-5000 Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). DNA extraction from 13 for-

malin-fixed, paraffin-embedded samples was performed by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation, as described elsewhere.[26]

CGH and Digital Image Analysis

CGH was carried out as previously described.[26] One μg of high molecular weight tumor DNA was labeled with Spectrum Green-dUTPs (Vysis, Downers Grove, IL) by nick translation (BioNick kit, Life Technologies, Basel, Switzerland). Archival tumor DNA with fragment sizes of $<$1 kb was labeled with a platinum/D-Green complex (D-Green-ULS) using the Kreatech Universal Linkage System (ULS) D-Green labeling kit (kindly provided by Kreatech Morwell Diagnostics GmbH, Zürich, Switzerland), according to the manufacturer's recommendations. Briefly, 800 ng of tumor DNA and 1.6 μl of D-Green-ULS, adjusted to a total volume of 20 μl, was incubated at 65°C for 20 minutes. To reduce background signals, the ULS-labeled samples were purified using Qiagen nucleotide removal columns (Qiagen GmbH, Hilden, Germany).

The hybridization mixture consisted of 200 ng of Spectrum (or D-) Green-labeled tumor DNA, 200 ng of Spectrum Red-labeled sex matched normal reference DNA (Vysis), and 10 to 20 μg of human Cot-1 DNA (Life Technologies) dissolved in 10 μl of hybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, pH 7.0). Hybridization to normal metaphase spreads (Vysis) took place for 3 days at 37°C.

Digital images were collected from six to seven metaphases using a Photometrics cooled CCD 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 chromosome regions where the mean green-to-red fluorescence ratio was above 1.20 and below 0.80, respectively. Over-representations were considered amplifications when the fluorescence ratio values in a subregion of a chromosome 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.

FISH Analysis

To validate CGH data independently, touch preparations of four frozen PCCs were subjected to fluorescence *in situ* hybridization (FISH) analysis using a chromosome 1-specific centromere probe (pUC1.77) in combination with an 1p22–31-specific YAC probe (929-E-1) (kindly provided by S. Franke, Center for Human Genetics, University of Leuven, Belgium and J.R. Testa, Fox Chase Cancer Center, Philadelphia, PA), or a repeat probe (p179), mapping to the 1p36 region.[27–29] Chromosomes 3 and 6 were examined using a chromosome 3 (pα3.5) and 6 (p308) centromere probe, in combination with a 3q26-specific (*pancpin*) and 6q22-specific (cCI6–44) probe, respectively.[30–33] Cell processing, probe labeling, *in situ* hybridization, and detection of the hybridized signals were performed as recently described.[34] For each tumor, hybridization signals of 100 interphase nuclei were scored. Numerical chromosomal aberrations were considered an aneusomy when the percentage of nuclei showing an abnormal number of centromere signals exceeded 30%. The presence of only half of the locus specific signals compared to the number of centromere signals in more than 30% of the nuclei was interpreted as a deletion.

Statistical Analysis

To calculate the statistical significance of differences in genomic changes between benign and malignant tumors, we used contingency table analysis, which was also used to analyze the relationship between genomic alterations and sex. Regression analysis was carried out to compare the number of chromosomal aberrations with tumor size.

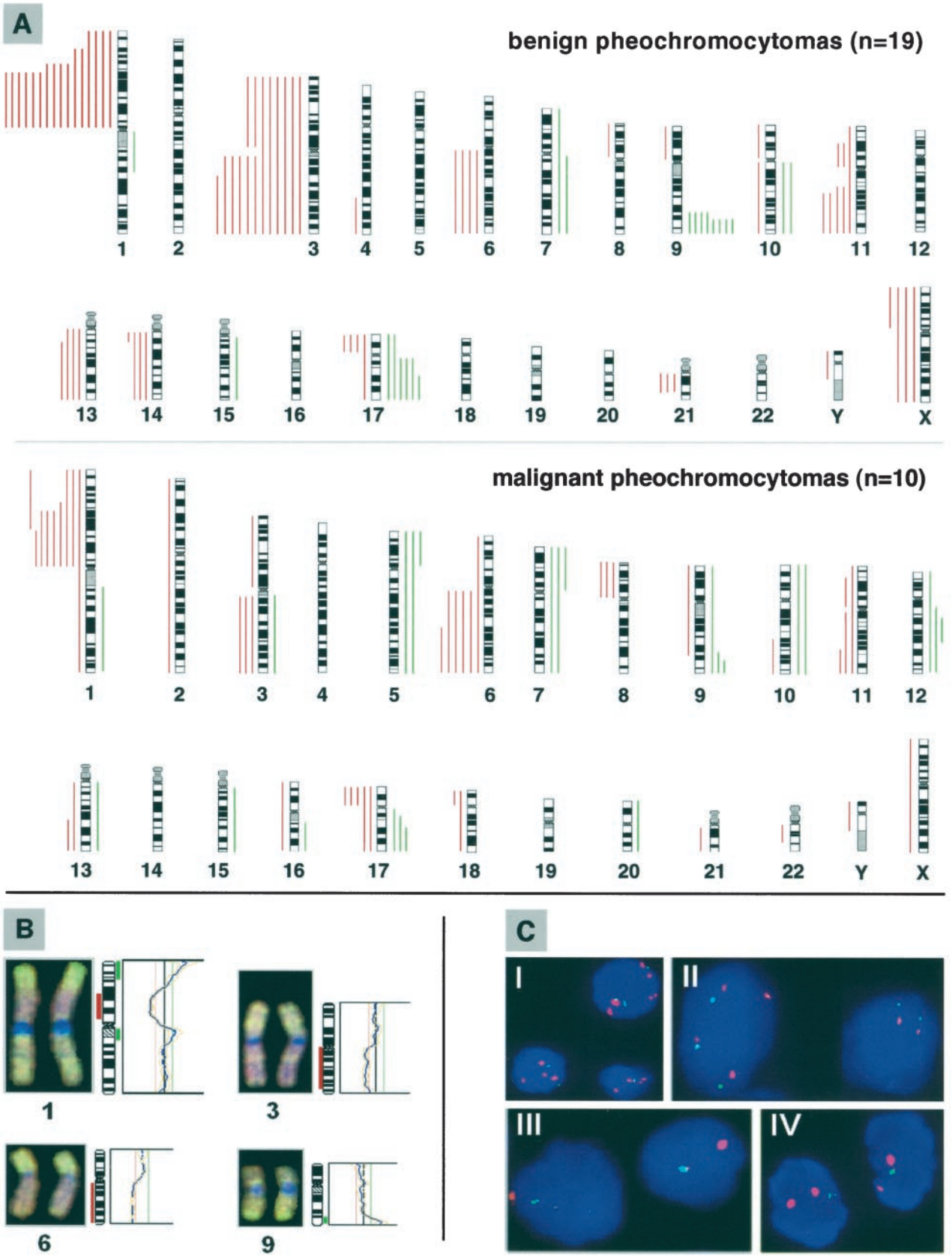
Results

Overview of CGH Findings

Figure 1A summarizes all DNA copy number changes identified in the 29 evaluated sporadic PCCs. Genetic alterations were observed in all PCCs, and the average number of chromosome arm aberrations per tumor was 6.3 $\pm$ 4.4 (range, 1–21). Chromosomal losses (mean, 4.3; range, 1–12) were more frequent than gains (mean, 1.9; range, 0–13) and no amplifications could be detected (Table 1). The average number of chromosomal alterations was marginally associated with malignancy (5.3 $\pm$ 2.7 *versus* 8.2 $\pm$ 6.1), principally due to a higher frequency of gains. The most common chromosome arm copy number changes included losses of chromosomes 1p11-p32 (86%), 3q (52%), 6q (34%), 3p, 17p (31% each), and 11q (28%), and gains of chromosome 9q (38%) and 17q (31%). Representative examples of CGH results are shown in Figure 1B.

Genetic Changes in Relation to Clinical Parameters

Ten of 29 PCCs fell into the category of malignant tumors, since regional or distant metastases occurred in these patients. In these lesions, losses of chromosome 6q were more often observed as compared to clinically benign lesions (60% *versus* 21%, $P = 0.0368$). To a lesser degree, losses of 17p were also related to metastatic disease (50% *versus* 21%), whereas deletions of chromosome 3p and 3q were preferably associated with benign tumors (42% *versus* 10% and 63% *versus* 30%, respectively). Although the malignant tumors were significantly larger in size (11.0 $\pm$ 4.7 cm *versus* 7.1 $\pm$ 4.6 cm, $P = 0.0470$) and demonstrated a slightly higher number of chromosome arm copy number changes, there was no


A
benign pheochromocytomas (n=19)
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X
malignant pheochromocytomas (n=10)
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correlation between the number of genomic aberrations and the tumor size. No differences were seen between adrenal and extra-adrenal PCCs.

Additionally, a statistically significant correlation was found between the number of chromosomal alterations and female gender ($P = 0.0196$), predominantly due to a higher frequency of gains of different genomic regions.

Comparison of CGH and FISH

FISH analysis confirmed the CGH results of chromosomes 1p, 3q, and 6q in 4 tumors that showed chromosomal imbalances as detected by CGH (Table 1). Two of the 4 tumors presented with a partial loss of chromosome 1p (1p11–32). In one case, FISH analysis proved the loss of this region and the retention of 1p36. However, the other tumor appeared to have a complete loss of chromosome 1p, which could be expected, since the 1p32-pter region is known to generate some false positive results in CGH.

In addition, the three benign lesions revealed a tetrasomy in the major population of cells for all chromosomes analyzed. In contrast, the malignant tumor turned out to be diploid. In two of the three aneuploid lesions a subpopulation of diploid tumor cells (as demonstrated by two centromere signals and only one locus-specific signal per nucleus) could also be detected. Examples of FISH results are shown in Figure 1C.

Discussion

Our genome-wide survey of genetic alterations in sporadic benign and malignant PCCs identified specific aberrations associated with tumor initiation and progression, which might be useful in clinical diagnosis. Our results highlight several novel chromosome regions that may harbor genes critically involved in the pathogenesis of sporadic PCCs. A high frequency of losses of chromosomes 1p and 3q in the benign tumors suggests that they are important genetic events in early tumorigenesis. Deletions of chromosome 6q were strongly associated with metastatic disease and thus may help to define the malignant potential of PCCs.

The most commonly encountered chromosomal aberrations in our series of 29 sporadic PCCs involved 1p, 3q, 9q, 6q, 3p, 17p, 17q, and 11q. These findings support and extend the hypothesis of Koshla et al[22] that multiple genes are involved in the pathogenesis of these tumors. Although LOH studies have shown loss of heterozygosity at 1p, 3p, 11q, 17p, and 22q in syndrome-related and sporadic PCCs,[21–25] the here found losses of 3q and 6q and gains of 9q and 17q have not been reported previously. In contrast to a previous LOH analysis,[25] we observed only one tumor with loss of 22q. Possible explanations for this discrepancy are the limited sensitivity of CGH for small losses (<10 Mb) and the fact that G-C-rich chromosome regions, like those in chromosome 22, are known to yield false positive CGH results.

We and others[21–24] found a very high incidence of 1p loss in PCCs, suggesting that it might be an important tumorigenic event. Deletions of chromosome 1p are common in several other human neoplasms, including adrenocortical carcinomas and neurectodermally derived tumors, such as neuroblastomas.[35,36] The smallest common region of overlap as detected by CGH in our study involves the 1p22–32 region. Because of known false positive CGH results at 1p32-pter, we verified the CGH data by FISH using a probe mapping to 1p36. Our observations indicate that some PCCs indeed exhibit a partial loss of chromosome 1p. This correlates well with the results of others,[23,37] who found evidence for a potential PCC tumor suppressor locus in the middle of the short arm of chromosome 1. There are multiple candidate TSGs in this region, including TGFβ-R3, p18INK4C, and PTPRF, none of which has been proven to be relevant in PCC tumorigenesis. One should also consider the possibility that more than a single TSG on 1p contributes to the development of PCCs.

Another interesting finding of our study is the frequent loss of chromosome 3q in PCCs, which is more often encountered in benign than in malignant tumors. This might point to diverging pathways in the development of benign and malignant PCCs, although it is known that in malignant tumors duplications of chromosome arms can occur.[38] In that case, LOH will not be detected by CGH. However, it is certainly conceivable that loss of chromosome 3q is an early genetic event in the pathogenesis of PCCs. Thus far, frequent allelic loss of chromosome 3q has been demonstrated only in osteosarcoma, with a putative TSG localized to a region between 3q26.2 and 3q26.3.[39] The possible relationship of this candidate TSG for PCC development however, is not clear.

Data on the genetic events that determine the malignant potential of PCCs are scarce. We observed a strong association of losses of chromosome 6q with advanced disease stage. Frequent allelic imbalances at 6q have also been reported in other malignancies[40,41] and appear to be related to poor prognosis or metastatic disease in certain tumors.[42,43] Although these data are suggestive of the existence of several TSGs at 6q, none has been identified so far. In addition, underrepresentations of chromosome 17p were often observed in metastasizing lesions. 17p13 contains the *TP53* gene, the TSG most commonly involved in human carcinogenesis. Fourteen of 29 PCCs evaluated in the present study have previously been immunohistochemically investigated for *TP53*

Figure 1. Summary of all DNA copy number changes detected by CGH in 19 benign and 10 malignant sporadic PCCs (**A**). The vertical green lines on the right side of the chromosome ideograms indicate gains, the red on the left losses of the corresponding chromosome region. **B:** Individual representative examples of CGH digital images (**left**) and fluorescent ratio profiles (**right**) illustrating genomic alterations of chromosomes 1 (loss of 1p11–32 with gain of the C-G rich telomeric region), 3 (loss of 3q), 6 (loss of 6q), and 9 (gain of 9q33–34). **C:** Results of FISH analysis. I and II: interphase touch preparations of tumor number 8 analyzed using a centromeric probe for chromosome 1 (red signals) in combination with a 1p22–31-specific probe (green signals) (I) or a 1p36-specific probe (II), demonstrating aneuploidy of this tumor with loss of the 1p22–31 locus (I) but not of the 1p36 locus (II). III: Monosomy of chromosome 3 in case 4. IV: Diploid malignant tumor, showing the two copies of centromere 6 (red signals) and only one copy of the 6q22-specific locus (green signal), indicating loss of 6q.

accumulation.[20] Comparison of these results with our CGH results (Table 1), indicates that *TP53* alterations may play a role in a subset of these tumors. Involvement of another TSG on 17p, however, cannot be ruled out and one candidate gene could be the putative TSG at the medulloblastoma locus 17p13.3.[44] Interestingly, three malignant PCCs exhibited simultaneous gains of chromosomes 5p, 7p and 12q, similar to findings for malignant endocrine pancreatic tumors.[45]

In conclusion, our results indicate that the development of PCCs is associated with specific genetic alterations, of which deletions of chromosome 1p (1p22–32) and 3q are early genetic events. Losses involving chromosome 3q (minimal overlapping region, 3q21-qter) and 6q (6q22-qter) and over-representations of chromosome 9q (9q33–34) and 17q point to new regions that may contain PCC TSGs and oncogenes in addition to the previously reported TSG loci 1p, 3p, 11q, 17p, and 22q.

Further studies are required to narrow down the critical regions in each identified chromosome and to characterize the putative genes involved in tumor initiation and progression of sporadic PCCs.

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