Array-comparative genomic hybridization in sporadic benign pheochromocytomas

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Abstract

Pheochromocytomas (PCC) are catecholamine-producing tumors arising from the adrenal medulla that occur either sporadically or in the context of hereditary cancer syndromes, such as multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau disease (VHL), neurofibromatosis type 1, and the PCC-paraganglioma syndrome. Conventional comparative genomic hybridization studies have shown loss of 1p and 3q in the majority of sporadic and MEN2-related PCC, and 3p and 11p loss in VHL-related PCC. The development of a submegabase tiling resolution array enabled us to perform a genome-wide high-resolution analysis of 36 sporadic benign PCC. The results show that there are two distinct patterns of abnormalities in these sporadic PCC, one consisting of loss of 1p with or without concomitant 3q loss in 20/36 cases (56%), the other characterized by loss of 3p with or without concomitant 11p loss in 11/36 (31%). In addition, we found loss of chromosome 22q at high frequency (35%), as well as the novel finding of high frequency chromosome 21q loss (21%). We conclude that there appear to be two subgroups of benign sporadic PCC, one of which has a pattern of chromosomal abnormalities that is comparable with PCC from patients with MEN2 and the other that is comparable with the PCC that arise in patients with VHL disease. In addition, genes on 21q and 22q might play a more important role in PCC pathogenesis than had been assumed thus far.

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Introduction

Pheochromocytomas (PCC) are rare neuro-endocrine tumors arising from the adrenal medulla. Similar tumors arise from extra-adrenal chromaffin tissues, and are now referred to as sympathetic paragangliomas (PGLs; Baguet *et al.* 2004). These tumors produce catecholamines, causing paroxysmal or sustained hypertension in the majority of patients. The elevated blood pressure can cause myocardial and cerebral infarctions, leading to morbidity and mortality. In up to 25% of PCC, the tumors occur in the context of four hereditary tumor syndromes, including multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau disease (VHL), the PCC–PGL syndrome, and neurofibromatosis type 1 (NF1; Neumann *et al.* 2002). Patients with MEN2 have mutations of the *RET* oncogene located at 10q11.21, the *VHL* tumor suppressor gene is located on 3p25.3, the *SDHD* gene in PGL–PCC patients is located on 11q23.1, and the related *SDHB* gene, also involved in the PCC–PGL syndrome is located on 1p36.13. Finally, the *NF1* gene is located on 17q11.2. In the remaining 75% of sporadic PCC somatic mutations of these five genes play a minor role, and the pathogenesis of these tumors is largely unknown.

Both syndrome-related PCC and sporadic PCC have been analyzed by comparative genomic hybridization (CGH). Interestingly, PCC from MEN2 patients, NF1

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patients, and the majority of sporadic PCC show similar genomic aberrations with a characteristic loss of 1p and 3q (Dannenberg *et al*. 2000, Edstrom *et al*. 2000, Cascon *et al*. 2005). VHL-related PCC, however, show distinct genetic aberrations consisting of loss of chromosomes 3 and 11 (Lui *et al*. 2002, Hering *et al*. 2006).

With the introduction of high-resolution array-CGH, it has become technically feasible to study small (submegabase) chromosomal deletions and gains that escaped detection by conventional CGH due to the low resolution. This technique has facilitated the analysis of chromosomes 21 and 22 that were difficult to analyze in conventional CGH. Recently, copy number imbalances affecting chromosome 22 were confirmed by submegabase array-CGH in 44% (29/66) of PCC analyzed, a percentage that had not been described in conventional CGH (Jarbo *et al*. 2006). In addition, the tiling order of bacterial artificial chromosome (BAC) clones also has the advantage to rule out mismapped clones, and gives precise breakpoint information. A similar array-CGH analysis has been performed on chromosome arm 1p, with 24 samples from hereditary and sporadic PCC, in which breakpoints of chromosome 1p could be identified precisely. These studies illustrate important differences between conventional and array-CGH (Aarts *et al*. 2006). To further clarify the pathogenesis of sporadic PCC, we analyzed 36 sporadic benign PCC using a tiling array consisting of 32 433 BAC clones.

Materials and methods

Patients and tumor samples

A series of 40 benign PCC of 40 patients was obtained from the archives of the Departments of Pathology of the Erasmus MC-University Medical Center Rotterdam, Maastricht University, Academic Medical Center Amsterdam, University Medical Center St Radboud Nijmegen, and University Medical Center Utrecht, the Netherlands and stored at the Erasmus MC tissue bank. Patients with each of the following characteristics were excluded from this study: positive family history of an endocrine hereditary cancer syndrome, evidence of NF1, multiple PCC, and/or PGL or the presence of germline mutations. In addition, none of the patients had other tumors related to MEN2, VHL or the PCC–PGL syndrome. After this selection, the study was performed with 36 benign truly sporadic tumors of 36 patients, of which two patients with sympathetic PGL. The cohort consisted of 17 females and 19 males. The mean age was 49 years (range 9–76), with an average follow-up of 4.4 years ($n=25$ patients, 11 patients were lost to follow-up). None of the patients had evidence of metastatic or recurrent disease during follow-up, and therefore are considered benign. The mean diameter of the tumors was 5.6 cm (range 2.5–18). Most tumors produced mainly norepinephrine, six patients had mainly or only epinephrine production, and two patients showed increase in ACTH. The clinical data are detailed in Table 1. Histology of all tumors was reviewed to confirm the diagnosis of PCC. None of the tumors had adverse histopathological characteristics as published by Thompson (2002), supporting the diagnosis of a benign PCC in all cases. Tumor DNA was isolated from fresh-frozen tumor tissue, except for four tumors in which no frozen tissue was available and DNA was isolated from paraffin-embedded archival material. DNA from both fresh frozen and paraffin-embedded material was isolated using the D-5000 Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturers' recommendations.

Array CGH labeling and hybridization

The submegabase tiling arrays (SMRT) previously described by Ishkanian *et al*. (2004) were used, consisting of 32 433 overlapping BAC clones. Test DNA and pooled reference male DNA (Novagen, Mississauga, ON, Canada; 300 ng each) were labeled with cyanine-3 and cyanine-5 (PerkinElmer, Woodbridge, ON, Canada) respectively, according to a random-priming protocol. After 18 h of random priming, the reference and test DNA were combined and 100 µl of Cot-1 DNA (Invitrogen) was added. The mixture was purified using Microcon YM-30 columns (Millipore, Mississaga, ON, Canada). The purified mixture was washed with 200 µl H_2O, and resuspended in 45 µl DIG easy hybridization solution (Roche), containing 20 mg/ml sheared herring sperm (Sigma-Aldrich) and 10 mg/ml yeast tRNA (Calbiochem, Mississauga, ON, Canada). The probe was denatured at 85 °C for 10 min, followed by 60 min at 45 °C to block repetitive sequences, and subsequently applied in a volume of 43 µl to the slide surface after which coverslips were applied. The slides were incubated at 45 °C for 36 h, washed four times for 5 min in 0.1× SSC, 0.1% SDS at room temperature, and finally rinsed by 0.1× SSC for five times and dried by centrifugation.

Array imaging and analysis

Hybridized slides were scanned using a charge-coupled device camera system (Applied Precision,

Table 1 Clinical data

Patient number	m/f	Age	Location	Diameter (cm)	Weight (g)	Follow-up (months)	Hormone
1	m	43	A	–	42	72	***NE***, E, D
2	m	65	A	18	458	60	***NE***, E, D
3	f	70	A	2.5	–	60	E
4	m	59	A	13	240	48	***E***, NE
5	m	63	A	5	67	3	E
6	f	40	A	7	180	9[a]	NE
7	f	38	A	8	123	60	NE
8	m	29	A	12	710	37	–
10	f	25	EA	5	–	264	NE
11	m	24	A	2.5	–	72	***NE***, E
12	m	67	A	5.5	70	84	***NE***, E
13	m	24	A	6	66	4	E
14	m	46	A	7	246	4	NE
15	f	32	A	6	32	108	E
16	m	46	A	11.5	340	–	–
17	f	65	A	6	49	–	–
18	m	50	A	9	260	24	NE
19	m	56	A	16	–	12	–
20	m	43	A	7	79	–	–
21	f	63	A	4	–	–	–
22	m	53	EA	9	193	24	NE
23	f	52	A	7	93	84	NE, E, D
24	f	24	A	3.5	–	36	***NE***, E, D
25	f	70	A	8.5	234	–	E
26	f	70	A	6.5	137	–	–
27	m	78	A	4.2	50	–	–
28	m	41	A	7	–	2	NE, E, D
29	f	74	A	4	20	12	NE
30	m	29	A	7	–	60	ACTH
31	m	40	A	4.5	–	–	–
32	f	64	A	10	450	24	NE, E
33	m	9	A	4.8	–	18	–
34	f	48	A	8	–	–	–
36	f	26	A	7	–	–	ACTH
37	f	60	A	5	–	132	–
38	f	76	A	4	–	–	NE

A, adrenal; EA, extra-adrenal; NE, norepinephrine; E, epinephrine; D, dopamine; ACTH, adrenocorticotropic hormone. Bold italic hormones are dominantly produced.
[a]Died, not related to PCC.

Issaquah, WA, USA), and analyzed by SoftWoRx Tracker Spot Analysis software (Applied Precision). Resultant data were normalized using a stepwise normalization process (Khojasteh *et al*. 2005). Copy number alterations were identified via data visualization using custom software called 'SeeGH' (freely available at http://www.flintbox.ca/technology.asp?tech=FB312FB) and loss, normal, and gain probabilities for each clone as determined by a modified hidden Markov model (Chi *et al*. 2004, Shah *et al*. 2006). Data were filtered based on both replicate S.D. (data points with greater than 0.1 S.D. removed) and signal-to-noise ratio (data points with a signal-to-noise ratio <10 removed).

Mutation analysis

Mutation analysis was performed on a CGH profile basis. Tumors showing loss of 1p were screened for *SDHB* mutations ($n=26$), those with loss of 3p for *VHL* mutations ($n=11$), and those with loss of 11q for *SDHD* mutations ($n=10$). Because no specific profile is indicative of involvement of the *RET* proto-oncogene, all 35 tumors were tested for *RET* mutations. All exons including the intron–exon boundaries were screened, with the exception of *RET* for which only exons 10, 11, 13, and 16 were investigated. PCR and sequencing conditions have been previously described by Korpershoek *et al*. (2007). Corresponding normal DNA was tested when an alteration was found in the tumor DNA.

Statistical analysis

Fisher's exact test was applied, using SPSS version 11.5. P values <0.05 were considered to indicate statistical significance.

Results

Array CGH

All but two tumors included in this study yielded interpretable array results. A frequency plot, adding up percentages of loss and/or gain of each individual BAC clone of all 34 analyzable tumors is shown in Fig. 1. A representative karyogram of one tumor with highlighted losses and polymorphisms is shown in Fig. 2. Regions of previously reported natural copy number variation were not included in the analysis of these samples (Shah *et al*. 2006). The commonly observed aberrations in each individual tumor sample are summarized in Table 2. Interestingly, there were an overwhelming number of copy number losses compared with copy number gains. In addition, most alterations encompassed whole chromosomes or chromosome arms.

In general, loss of 1p was found in 76% (26/34) of cases, where 88% (23/26) of these showed loss of the entire p-arm. Three tumors showed regional loss, consisting of 1p12–1p13.3, 1p31.3–1p36.33, and 1p12–1p35.1. Loss of 3q was observed in 59% ($n=20$) of the 34 tumors. No regional losses were observed. Loss of 1p and additional 3q loss were shown to be significantly associated ($P<0.05$).

Chromosome 3p loss was seen in 32% ($n=11$) of the tumors. In addition, eight of these tumors concordantly showed loss of chromosome 11p. Chromosome 3p loss was significantly associated with chromosome 11p loss ($P<0.05$).

Loss of 11q was found in 29% ($n=10$), with loss of the whole arm in 80% of these ($n=8$). The two tumors that had a regional loss showed an overlap from 11q14.3 until the telomeric end of the q-arm. Loss of chromosome 17p was found in 35% ($n=12$), but no regional losses were observed. Loss of 21q was observed in 21% ($n=7$), with one tumor showing a regional loss of 21q22.11 until the telomere. Loss of 21q was shown to be significantly associated with loss of 17p ($P<0.05$).

Finally, loss of chromosome 22 was found in 35% ($n=12$) with no regional losses. Interestingly, one tumor displayed a high negative Log_2Ratio, suggesting more than just a single copy loss of that region of the chromosome. However, with additional loss of heterozygosity (LOH) analysis of several polymorphic markers in that region, no homozygous deletion could be identified (data not shown).

Because of the association between 1p and 3q loss on one hand, and the association between 3p and 11p loss on the other hand, there appear to be two distinct groups of PCC. The first group ($n=20$) encompassed tumors showing 1p and/or 3q loss,

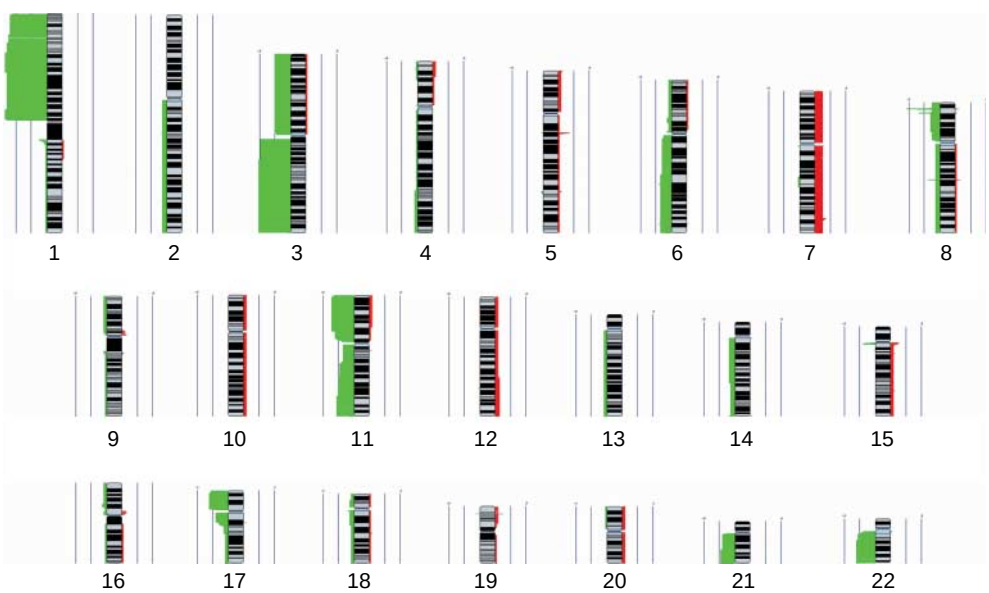

Figure 1 Frequency plot of all 38 PCC. Green lines on left side indicate loss of BAC clones situated in that area; red lines on right side indicate gain. Blue bars on either side represent 25 and 50%.

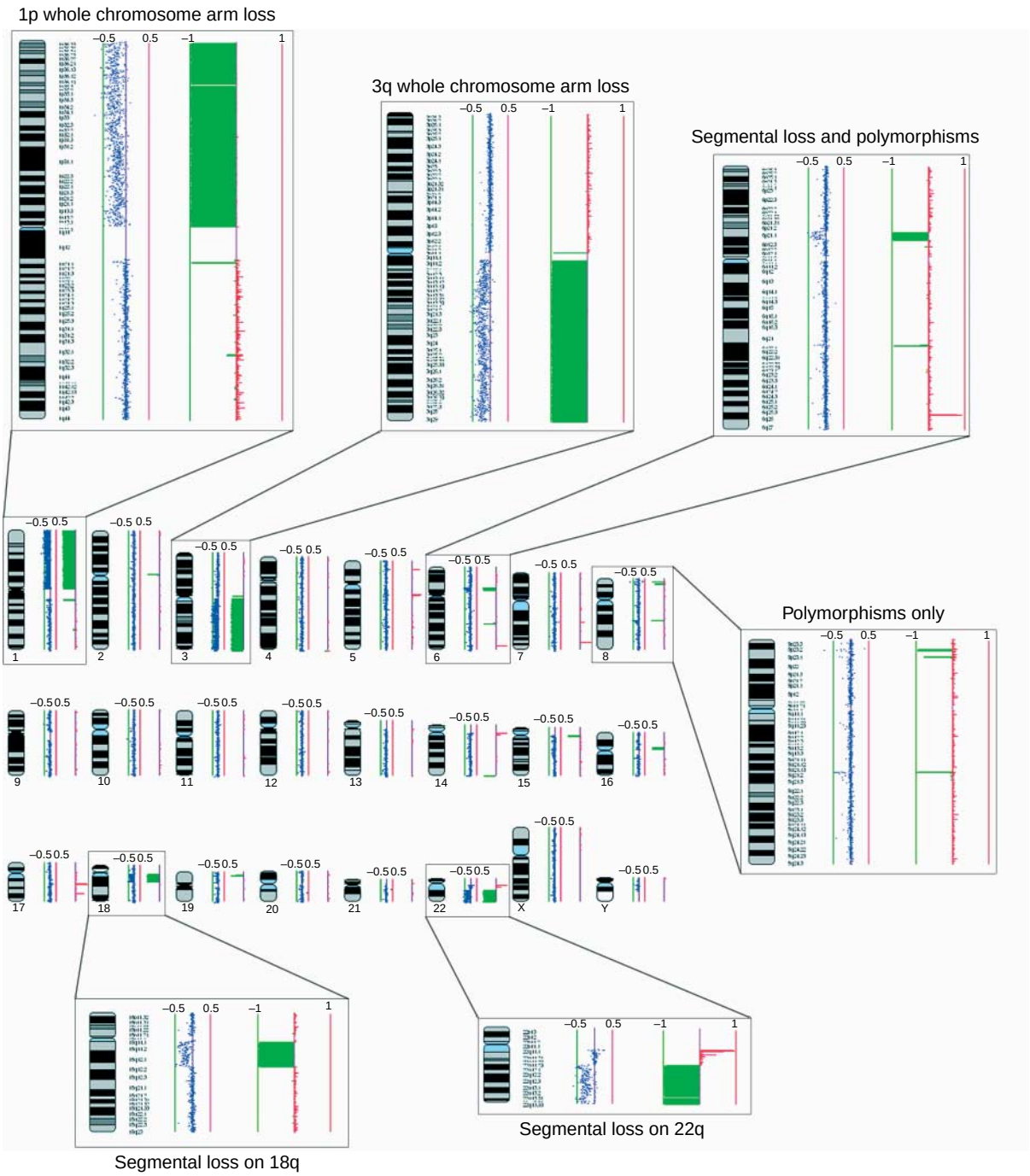

Figure 2 Karyogram of no. 28 showing the entire genome with magnified views of chromosomes 1, 3, 6, 8, 18, and 22. Each BAC clone on the array is displayed as a point representing the segment of the genome covered. The shift of each data point to the left of 0 represents a loss of copy numbers, while a shift to the right represents a gain in copy numbers. The bars to the left and right of CGH data represent Log_2 ratios of -0.5 and $+0.5$ respectively. To the right of the CGH data, hidden Markov model probabilities are displayed. Probabilities range from -1 (100% probability of copy number loss, green) to $+1$ (100% probability of copy number gain, red). Well-known polymorphisms are evident throughout the genome and are apparent in the magnified views of chromosomes 6 and 8.

Table 2 Loss of common regions

Patient number	1p	3p	3q	11p	11q	17p	21q	22q
1	x		x		reg	x	x	x
2	reg							x
3	x		x					x
4	reg	x	x			x	x	x
6	reg			x		x	reg	
7	x							x
8	x		x	x	x			
10	x							
12	x		x				x	
13	x	x	x	x				
14	x			x	reg			
16	x			x	x			
17	x	x	x	x				
19	x					x	x	x
20	x	x	x	x				
22	x	x	x			x		x
23	x		x			x	x	
25	x	x	x					x
26	x					x		x
27	x		x			x		
28	x		x					x
29	x		x					
30	x			x				
31	x		x			x		x
32	x		x			x		
15		x	x	x	x			
18		x		x				
24		x	x	x	x			
33		x	x	x	x			
34		x	x	x	x			
21						x		
37				x	x	x	x	

x, loss; reg, regional loss (see text for locations).

without having concurrent 3p loss. The second smaller group ($n=11$) showed loss of 3p with or without concomitant loss of 11p. In addition, there was a limited number of PCC ($n=3$) that revealed no losses of the previously mentioned chromosomal regions (1p, 3p). One of these tumors showed gain of chromosomes 15 and 20. The second tumor had loss of the chromosomes 17 and 19, and gain of chromosome 7. The third tumor showed loss of chromosomes 11, 17, and 21.

Table 3 Mutation analysis results

Patient number	Gene	Mutation→cDNA	Mutation→protein	Hormone
28	*RET*	c.1894_1899delGAGCTG	**p.E632_L633del**	NE, E, D
6	*RET*	c.2332G > A	**p.V778I**	NE
12[a]	*RET*	c.2753T > C	**p.M918T**	NE, E
10	*SDHB*	c.299C > T	**p.S100F**	NE
5	*SDHB*	c.487C > T	*p.S163P*	E
29	*SDHB*	c.487C > T	*p.S163P*	NE
23	*SDHD*	c.149A > G	**p.H50R**	NE, E, D
20	*VHL*	c.169_212delGGG_GCC	**p.G57LfsX60**	–
15	*VHL*	[c.364G > A; c.365C > T]	**p.A122I**	E
13	*VHL*	c.482G > A	**p.R161Q**	E
34	*VHL*	c.500G > A	**p.R167Q**	–

Mutations are in bold and polymorphism in italic. NE, norepinephrine; E, epinephrine; D, dopamine.
[a]It is not known whether this mutation was germline or somatic. All other mutations were somatic.

Mutation analysis

Sequence analysis of the four PCC susceptibility genes revealed mutations in seven tumors, of which three occurred in *RET*, one in *SDHB*, and three in *VHL* (Table 3). Analysis of corresponding germline DNA confirmed that six of the mutations were somatic. Corresponding germline DNA was not available from patient 12 with the *RET* p.M918T mutation. With the exception of the p.H50R polymorphism, no alterations were found in *SDHD*. Furthermore, two additional polymorphisms were found in *SDHB* which were both p.S163P (Table 3).

The patients wit the *RET* mutations all had mainly norepinephrine overproduction, of the four patients with somatic *VHL* mutations only two had available information on hormone production, both these tumors showed only epinephrine overproduction.

Discussion

This study represents the first comprehensive analysis of a large series of sporadic benign PCC using a genome-wide submegabase-resolution tiling array (SMRT-array). On the basis of DNA aberrations, we could distinguish two distinct subgroups of PCC, one with loss of 1p and/or 3q, representing more than 56% of all PCC investigated, and a second, smaller, group with loss of 3p with or without concurrent 11p loss, representing 32% of these PCC. These findings may relate to the different pathways of tumorigenesis in PCC.

The majority (76%) of PCC in this analysis of 36 benign sporadic tumors showed loss of 1p, which is comparable with the frequency of loss that has been reported in previous studies (Dannenberg *et al*. 2000, Edstrom *et al*. 2000, Cascon *et al*. 2005). Moreover, most PCC in our study (22/36) had loss of the entire short arm of chromosome 1, by contrast to our previous study, where we found regional 1p loss in half of the cases (Aarts *et al*. 2006). We speculate that the observed difference with our own previous studies and with series from others is related to the composition of the study group, which in the only present study comprised sporadic cases. In the few cases with partial loss, no minimal region of common loss could be determined, preventing us to speculate on the presence of one region harboring tumor suppressor genes on 1p, which have been postulated by various authors (Geli *et al*. 2005, Aarts *et al*. 2006). Still, based on the high frequency of 1p loss in PCC, we support the idea of one or more tumor suppressor genes on this chromosome arm. In most cases, 1p loss was accompanied by loss of 3q, which occurred in 62% of all cases with 1p loss, a figure that is comparable with that reported in the literature (Dannenberg *et al*. 2000, Edstrom *et al*. 2000, Cascon *et al*. 2005). Four PCC were found with loss of 3q without chromosome 1p loss. These four tumors displayed loss of the entire chromosome three in combination with loss of the entire chromosome 11 (see below).

Apart from the large group of PCC displaying a $1p^{-}/3q^{-}$ genotype, a smaller group of PCC was identified with loss of 3p, which was frequently accompanied by the loss of 11p. This pattern of loss has been previously mentioned in PCC from VHL patients, but has so far not been related to a subgroup of sporadic PCC (Lui *et al*. 2002, Hering *et al*. 2006). In order to exclude that this subgroup represented occult VHL disease, we performed mutation analysis of the entire VHL coding region, in which we could not detect germline mutations. However, we found three cases showing somatic VHL abnormalities. The p.R161Q and p.A122I VHL mutations have been described previously in an apparently sporadic PCC (Neumann *et al*. 2002). The p.G57LfsX59 has never been described before. Although, epigenetic silencing of the VHL gene by hypermethylation is not inconceivable, as seen in familial and non-familial renal cell carcinoma, no methylation has been described in PCC (Prowse *et al*. 1997). The fact that a subgroup of sporadic PCC, without VHL germline mutations, shows an identical genotype as VHL-related PCC, leads to the suggestion that this group of PCC follows similar pathways of tumorigenesis. Indeed, this might also be the case for MEN2-related PCC and the above-mentioned subgroup of sporadic PCC, which have been shown to have similar frequencies of 1p and 3q loss in previous studies (Dannenberg *et al*. 2000, Edstrom *et al*. 2000, Cascon *et al*. 2005).

This is also supported by the hormone production in these tumors, where the RET-related PCC showed dominant norepinephrine production, and VHL-related PCC showed only epinephrine production.

In addition to losses affecting chromosomes 1, 3, and 11, we observed the highest frequency of loss in chromosomes 21 and 22, concerning 21 and 35% of all PCC respectively. Loss of chromosome 21 has so far not been described at this relatively high frequency in benign sporadic PCC. All tumors with 22q loss also displayed 1p loss, and all but one tumor with chromosome 21q loss also revealed 1p loss. Therefore, these regions could be involved in the spectrum of the sporadic and/or MEN2-related PCC. However, as there was only one tumor with regional loss of 21q, we cannot draw conclusions with respect to the presence of potential tumor suppressor genes on this

chromosomal arm. Previous reports on the loss of chromosome 22 have been based on LOH analysis and showed loss of chromosomal bands 11.21–13.31, or 11.21 alone (Khosla *et al*. 1991, Tanaka *et al*. 1992, Shin *et al*. 1993). Furthermore, in a recent array-CGH study on 66 PCC, copy number alterations of 22q were found in 44% (Khosla *et al*. 1991, Tanaka *et al*. 1992, Shin *et al*. 1993). In eight of these cases (8/29), there was regional loss with a minimal region of common overlap from 22q11.23 until the telomeric end of chromosome 22. One additional interstitial deletion was found from 22q11.23 to 22q12.3. In our analysis, we did not find a regional loss concerning chromosome 22q. These findings might indicate the presence of tumor suppressor genes on 22q that could be involved in the pathogenesis of sporadic PCC, however, due to the large regions involved, combined with a gene-rich chromosome it is not possible at this time to pinpoint candidate genes.

Apart from the 31 PCC that could be fitted in either of the two groups already mentioned, there were three tumors that did not have losses in 1p, 3p or 3q. No common pattern could be derived from these three tumors, although it is interesting to note that two of these presented with chromosomal gains. It is of relevance to note that none of these were from an extra-adrenal location, as these two PGL presented with a CGH pattern that fitted well with that of the PCC.

Taken together, the predominant chromosomal abnormalities found in this genome-wide array-CGH study of 36 benign sporadic PCC concern losses of various chromosomal arms, most notably 1p, 3p, 3q, 11p, 11q, 17p, 21q, and 22q. By contrast, we observed no consistent gain of any chromosomal region. Furthermore, we could not confirm abnormalities of other chromosomes that have been suggested in the literature, such as aberrations of chromosomes 2 and 16 (Dahia *et al*. 2005). In addition, there appear to exist two different groups of benign sporadic PCC, each of them characterized by a specific genotypic pattern of chromosomal loss: a predominant form showing a 1p/3q-genotype, which can also be found in MEN2-related PCC; and a minor form showing a 3p-/11p-genotype, which can also be found in VHL-related PCC. Apart from this, the high frequency of loss of 21q and 22q indicates that these chromosomal arms might also be important in the pathogenesis of benign sporadic PCC.

Declaration of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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