Frequent loss of 17p, but no *p53* mutations or protein overexpression in benign and malignant pheochromocytomas

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Genetic changes in the tumorigenesis of sporadic pheochromocytomas are poorly understood, and there are no good markers to discriminate benign from malignant pheochromocytomas. *p53* is a tumor suppressor gene and aberrations in this gene are frequently found in many tumor types. The role of *p53* in pheochromocytoma tumorigenesis is unclear, with some studies suggesting that *p53* mutations can be used to discriminate benign from malignant pheochromocytomas while other studies do not find such an association. Because most of these investigations were hampered by small series of tumors and the use of varying methods, we have performed a comprehensive analysis of *p53* aberrations in a large series of pheochromocytomas. Comparative genomic hybridization analysis of 31 benign and 20 malignant tumors showed loss of the *p53* locus at chromosome 17p13.1 in 23/51 (45%) cases, and most of these results were confirmed by fluorescence *in situ* hybridization. Forty-three tumors, including the malignant tumors and the tumors with loss of the *p53* locus, were analyzed for *p53* mutations in exons 5–8, but none were found. Furthermore, p53 immunohistochemistry on 35 cases revealed strong nuclear p53 expression in only two pheochromocytoma metastases, all other tumors being negative. We conclude that, although there is frequent loss of the *p53* locus on 17p, the *p53* gene does not appear to play a major role in pheochromocytoma tumorigenesis.
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Pheochromocytomas are neuroendocrine tumors derived from chromaffin cells of the adrenal medulla. Extra-adrenal tumors of chromaffin tissue have recently been renamed sympathetic paragangliomas. Pheochromocytomas are usually benign (90%), but there are no markers that can distinguish the remaining 10% malignant pheochromocytomas. Histologically confirmed metastases in places where chromaffin tissue does not normally occur, such as in bone, lung, liver, or lymph nodes, are the only accepted sign of malignancy.[1]

Whereas most pheochromocytomas occur sporadically, up to 24% of these tumors appear in familial cancer syndromes, such as multiple endocrine neoplasia 2, Von Hippel–Lindau disease, neurofibromatosis type 1, and pheochromocytoma-paraganglioma syndrome.[2] In some sporadic pheochromocytomas, genes associated with the inherited tumor syndromes underlie tumorigenesis, but in the majority of sporadic pheochromocytomas molecular tumorigenesis is poorly understood. Loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) studies performed by us and others have reported allelic losses at 1p, 3pq, 17p, and 22q, but corresponding genes have not been identified.[3–6] Particularly 17p, the chromosome arm where the *p53* gene is located, is interesting to investigate knowing that aberrations in this gene are implicated in many inherited and sporadic forms of malignancy, such as colon, lung, brain, and breast tumors.[7]

p53 is functionally involved in guarding the stability of the genome. In case of DNA damage, it will downregulate the cell cycle and inhibit cell division.[8] Mutations in and inactivation of one or both *p53* alleles can promote tumorigenesis, whereas restoration of *p53* function results in tumor regression in *in vivo* animal models.[9] Over the past decade, several studies have addressed the possible role of *p53* in pheochromocytoma tumorigenesis. However, in many reports, only few pheochromocytomas were investigated.[10–12] Five studies, including one from our own group, exclusively described the results of immunohistochemical staining.[13–17] Other studies, examining *p53* mutations with or without concomitant protein expression, included only benign or very few malignant pheochromocytomas, and generally did not show *p53* gene mutations.[18–20] In contrast, Yoshimoto *et al*[21] described a relatively high frequency of single-strand conformation polymorphism (SSCP) analysis abnormalities and *p53* gene mutations in multiple and malignant pheochromocytomas from an Asian population.

To examine the involvement of *p53* in pheochromocytoma development and malignant progression, we set out for CGH analysis of a uniquely large series of well-characterized benign and malignant pheochromocytomas. We identified chromosome alterations at chromosome 17 by using conventional CGH analysis and confirmed these data by using double-target fluorescence *in situ* hybridization (FISH) for the *p53* gene and centromere loci. Subsequently, we used polymerase chain reaction (PCR) and SSCP analysis to detect *p53* gene mutations in exons 5–8 and immunohistochemistry to visualize nuclear p53 overexpression.

Materials and methods

Tissue Samples

Tissue samples of pheochromocytomas ($n = 63$ from 48 patients) were collected from the pathology archive of the Departments of Pathology, Erasmus MC, Rotterdam ($n = 16$), Radboud University Nijmegen ($n = 43$), and University Hospital Zurich ($n = 4$). Clinical and tumor data are listed in Table 1. All tumors were collected between 1978 and 2003. All malignant cases had histologically confirmed metastases, but only 12 of the latter are included in this study, because not all were available. There is no overlap between the tissue samples in this study and any of our previous studies, except for six samples that had been used for prior p53 immunostaining.[5,15]

DNA Extraction

DNA extraction was performed as described before.[5,22] Genomic DNA from 50 frozen samples was isolated by homogenizing approximately $5 \, mm^3$ of each tissue sample prior to proteinase K treatment and DNA purification using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Genomic DNA from 13 paraffin-embedded tumors was isolated from $5–10$-μm-thick tissue sections after deparaffinization. DNA quality was checked with agarose gel electrophoresis and quantified by spectrophotometry.

CGH

CGH was used to analyze genome-wide DNA copy number imbalances in 51 pheochromocytoma samples as described previously.[23] This approach uses differentially labelled tumor and 'reference' DNA, which are competitively hybridized to normal metaphase chromosomes. The ratio of the fluorescence intensities detected is indicative of the relative DNA copy number in tumor *vs* reference DNA.

FISH

Touch preparations of $n = 29$ frozen pheochromocytomas and 4-μm-thick tissue sections of $n = 4$ routinely fixed, paraffin-embedded tumors were subjected to FISH ($n = 32$) as described previously.[22,23] A combination of centromere 17 (p17H8) and a PAC probe containing the *p53* gene on chromosome 17p13.1, labelled with digoxigenin and biotin, respectively, was used for hybridization. Digoxigenin was detected by sheep anti-digoxigenin fluorescein (Roche) and biotin by two layers of avidin-rhodamine connected by a biotinylated goat anti-avidin antibody (Vector). Probe visualization and nuclear counterstaining were carried out as described for CGH, and slide evaluation and signal scoring to detect DNA copy number gain and losses were performed as described previously.[22,24]

PCR–SSCP

Exons 5–8 of the *p53* gene, including the exon–intron boundaries, were investigated by PCR–SSCP in $n = 43$ pheochromocytoma samples. As controls, DNA samples from normal individuals were used. In addition, DNA from the prostate carcinoma cell lines PC-3 and Du-145, and from the colorectal carcinoma cell lines Colo-320 and HT-29, with known *p53* mutations in exons 5, 6, 7, and 8, respectively, served as positive controls, shown by van Nederveen *et al*.[25] The DNA isolated from routine formalin-fixed, paraffin-embedded tissues is highly degraded; therefore, we used small amplicons (<200 bp) with overlapping primers for each exon to maximize chances of detecting mutations whenever they would be present. The primers are listed in Table 2. PCR was performed with $1–3 \, \mu l$ of isolated DNA in a final reaction volume of $15 \, \mu l$
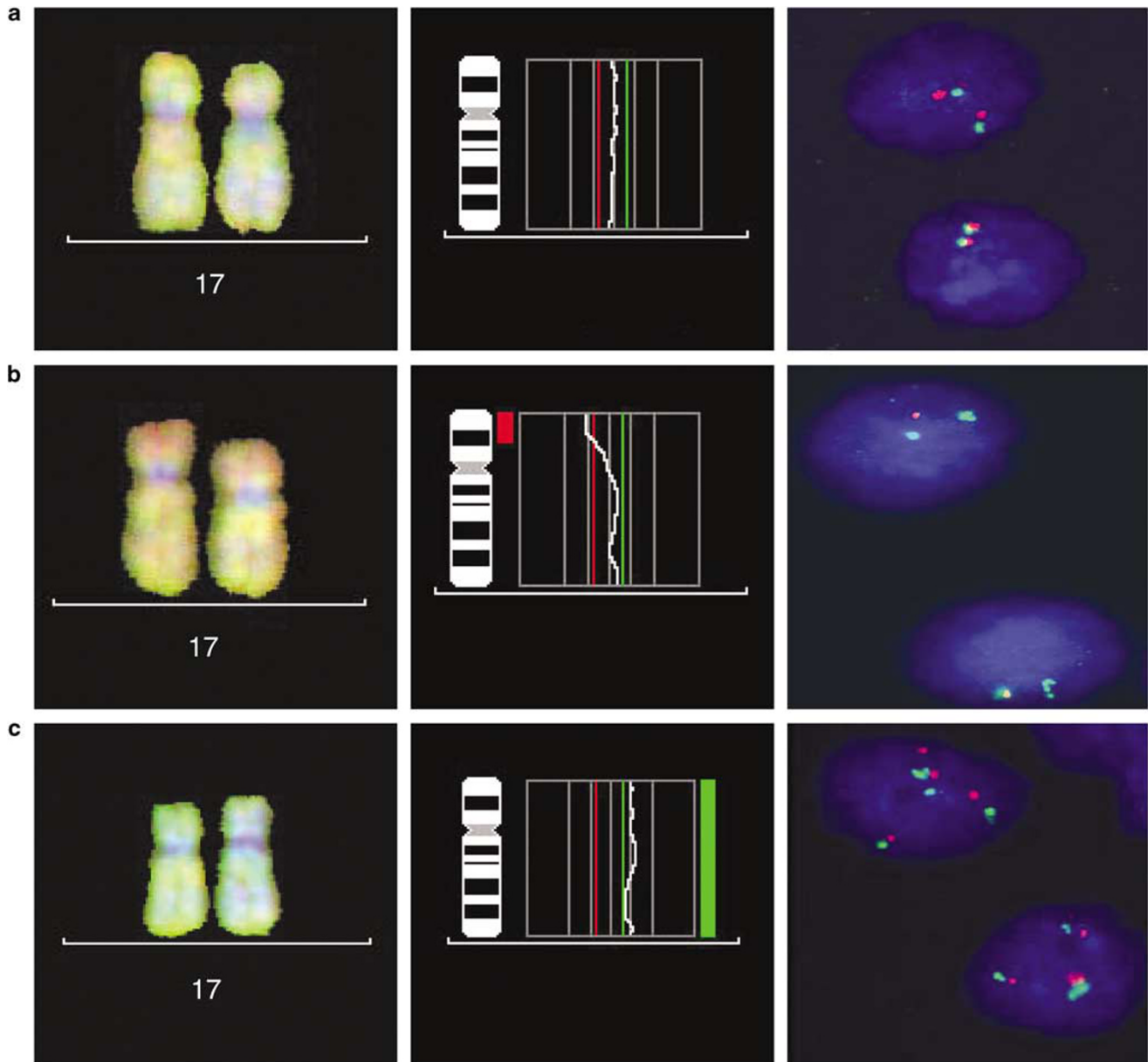
Figure 1 Representative results of chromosome 17 CGH analysis confirmed by FISH of the p53 locus in three cases of malignant PCC. (**a**) Malignant PCC (case 15), not showing loss or gain in CGH and FISH analysis. (**b**) Malignant PCC (case 8) with loss of 17p in the CGH analysis, including the *p53* gene locus, also showing loss in FISH. (**c**) Malignant PCC (case 13) showing gain of chromosome 17 in the CGH analysis and multiple *p53* gene loci by FISH.

containing, in mmol/l, MgCl$_2$ 1.5, dATP 0.02, dGTP 0.2, dTTP 0.2, and dCTP 0.2, [α-^{32}P]dATP 0.8 μCi (Amersham, Buckinghamshire, UK), 20 pmol of each primer, and 0.2 U of *Taq* polymerase (Promega, Madison, WI, USA). PCR was performed for 35 cycles (denaturing at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min) in a Biometra thermocycler (Biometra, Göttingen, Germany). A final extension was carried out at 72°C for 10 min. PCR products were diluted with loading buffer (95% formamide, 10 mmol/l EDTA (pH 8.0), 0.025% bromophenol blue, and 0.025% xylene cyanol), and denatured at 95°C for 10 min.

The solution was chilled on ice and 4 μl was loaded on an 8% polyacrylamide gel (acrylamide to bisacrylamide, 49:1) containing 10% glycerol. Electrophoresis was performed at 8 W for 16 h at room temperature. Gels were vacuum dried at 80°C and exposed to X-ray films.

Immunohistochemistry

p53 immunohistochemistry was performed using a monoclonal mouse antibody directed against normal and mutated p53 (Do-7, Dako, Glostrup, Denmark).
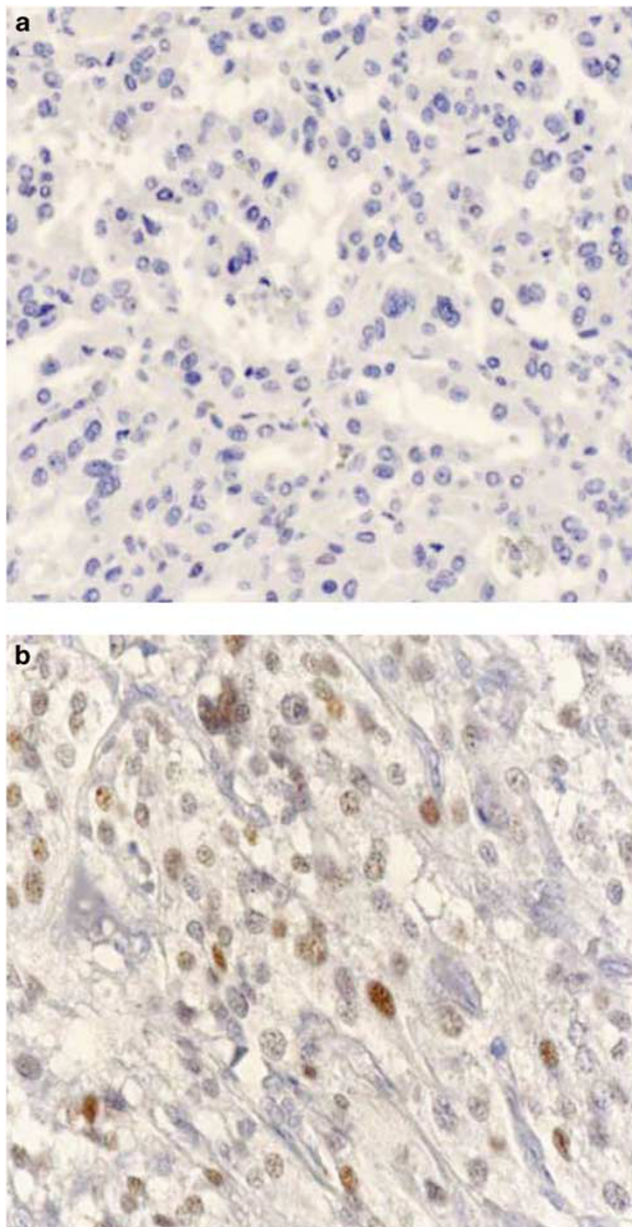
Figure 2 Representative results of p53 immunohistochemistry in malignant PCC. (**a**) Primary tumor of a malignant PCC (case 6) not showing nuclear overexpression of p53 protein (score 0). (**b**) Malignant PCC (case 7) showing the corresponding metastasis of the primary tumor shown in (**a**) with positive nuclear immunostaining for p53 (score 6).

Tissue samples of 36 pheochromocytomas were formalin-fixed and embedded in paraffin. Sections of 4 μm thickness were cut and mounted on Superfrost Plus slides. The sections were deparaffinized, dehydrated, exposed to microwave heating (in Tris/EDTA pH 9.0) at 100°C for 15 min, rinsed with tap water followed by incubation in H_2O_2 30%/PBS 1:10 for 15 min. The p53 antibody was diluted 1:100 with normal antibody diluents (Klinipath, Duiven, The Netherlands), and slides were incubated with 150 μl per slide for 30 min, followed by rinsing with Tris/

Table 1 Clinical and tumor data of 63 PCC from 48 patients

Sex (M/F)	19/29
Average age (years)	45.7 (range 23–72)
Malignant/benign	18/30
Metastases	12
Sporadic cases	39
Familial cases	8
*MEN2a	6
*NF1	2
Adrenal cases	41
Extra-adrenal cases	7 (malignant/benign 4/3)
Unilateral cases	43
Bilateral cases	5 (all MEN 2a)

*Indicates that of the 8 familial cases, there are 6 MEN2 cases and 2 NF1 cases.

Table 2 p53 primer sets exons 5–8 for SSCP

	Forward 5′→3′	Reverse 5′→3′	Size
Exon 5-I	cctgactttcaactcttgctc	actgcttgtagatggccatg	158 bp
Exon 5-II	cagctgtgggttgattccac	ctggggaccctgggcaac	176 bp
Exon 6-I	agcctctgattcctcactg	gaccaccacactatgtcga	127 bp
Exon 6-II	ccctcagcatcttatccga	ccactgacaacccaccctt	159 bp
Exon 7-I	aggcgcactggcctcatctt	tccagtgtgatgatggtgagg	141 bp
Exon 7-II	catgtgtaacagttcctgcatg	gcggcaagcagaggctgg	135 bp
Exon 8-I	ccttactgcctcttgcttctc	cttgcggagattctcttcctc	130 bp
Exon 8-II	tggtgcctgtcctgggagag	ctccaccgcttcttgtcct	127 bp

Tween 0.5%, pH 8.0. Dako ChemMate Envision HRP was applied for 30 min (150 μl, Dako envision kit), followed by rinsing with Tris/Tween 0.5%, pH 8.0. Diaminobenzidine tetrahydrochloride (150 μl, Dako envision kit) was applied twice, without rinsing, and rinsed with tap water. Slides were counterstained with Harris haematoxylin for 1 min and rinsed with tap water. Slides were rehydrated and covered. In the negative control reactions, the primary antibodies were omitted from the dilution buffer. A p53-positive breast carcinoma was used as positive control. Scoring of p53 staining was based on a method described by Sinicrope et al.[26] Two independent observers scored the tumors based on intensity and the percentage of nuclei showing immunostaining. Intensity is scored in three groups: weak = 1, moderate = 2, and strong = 3. Percentages are divided in five groups: ≤5% = 0, 5–25% = 1, 25–50% = 2, 50–75% = 3, and ≥75% = 4. A multiplied score of ≥6 was considered positive staining and a score <6 was considered negative.

Results

CGH Findings

Table 3 shows the losses and gains on chromosome 17 of the 51 tumor (31 benign and 20 malignant) samples that were examined by CGH. We found CGH abnormalities on chromosome 17 in 29/51 (57%) tumors, including 23/51 (45%) at the *p53* gene locus at 17p13.1. In 18 of these 23 cases (78%)

Table 3 Results of CGH analysis, FISH, SSCP analysis, and immunohistochemistry in benign PCC

Samples	Patients	Characteristics	CGH 17p	FISH p53	SSCP	IH
1	1	F/62 sp/adr/u	●	●	○	○
2	2	M/30 sp/adr/u	●	●	○	—
3	3	F/41 sp/adr/u	●	●	○	—
4	4	F/24 n/adr/u	●	●	○	—
5	5	M/66 sp/adr/u	●	—	○	—
6	6	M/49 m/adr/bi	●	—	○	○
7	7	M/42 sp/adr/u	●	—	○	○
8	8	M/47 sp/ea/u	●	—	○	—
9	9	F/69 sp/adr/u	●	—	○	—
10	10	F/37 sp/adr/u	●	—	○	○
11	11	M/41 sp/adr/u	●	—	—	—
12	12	F/30 sp/adr/u	●	—	○	○
13	13	F/56 sp/ea/u	○	●	—	—
14	14	M/41 sp/adr/u	○	●	○	—
15	15	F/33 sp/adr/u	○	●	○	○
16	16	M/72 m/adr/bi I	○○	○	—	○
17		II	○	●	○	○
18	17	M/40 m/adr/u	○○	○	○	—
19	18	M/53 m/adr/bi I	○○	○	○	—
20		II	○	—	—	○
21	19	F/33 sp/adr/u	○○	○	—	○
22	20	F/44 sp/adr/u	○	○	—	○
23	21	M/24 m/adr/u	○	○	—	○
24	22	M/58 sp/adr/u	○	○	—	○
25	23	F/53 n/adr/u	○	○	—	○
26	24	M/53 sp/adr/u	○	—	○	—
27	25	F/59 sp/adr/u	○	○	—	○
28	26	F/49 sp/adr/u	○	○	—	○
29	27	F/48 sp/adr/u	○	○	—	—
30	28	F/24 m/adr/bi I	○	○	—	—
31		/27 II	○	—	—	○
32	29	F/59 m/adr/bi	—	—	—	○
33	30	M/43 sp/adr/u	—	—	—	○

In CGH analysis, ○ represents 'no gain or loss,' ● is 'loss,' and ○ ○ means 'gain' of chromosome 17. In FISH analysis, ○ is 'retention of heterozygosity,' ● means 'loss of heterozygosity,' and ○ ○ represents 'gain' of the *p53* locus on chromosome 17. In SSCP, ○ means no mutation. In immunohistochemistry, ○ represents 'no overexpression' and ● means p53 protein overexpression. In all four techniques, — means that these samples are not examined. M = male; F = female; sp = sporadic; m = men; n = neurofibromatosis; adr = adrenal; ea = extra-adrenal; u = unilateral; bi = bilateral; LN = lymph node.

Table 4 Results of CGH analysis, FISH, SSCP analysis, and immunohistochemistry in malignant PCC

Samples	Patients	Characteristics	CGH 17p	FISH p53	SSCP	IH
1	1	F/47 sp/adr/u	●	●	○	○
2	2	F/65 sp/adr/u	●	●	○	○
3	3	F/70 sp/adr/u	●	●	○	○
4	4	F/56 sp/adr/u	●	—	○	—
5		meta	—	—	○	○
6	5	F/30 sp/ea/u	○	●	—	○
7		Meta intercost I	○	—	○	●
8		Meta intercost II	●	●	○	—
9	6	M/70 sp/adr/u	●	—	—	○
10		Meta LN	●	●	○	—
11	7	F/53 sp/ea/u	—	—	—	○
12		Meta	○	—	○	○
13	8	F/27 sp/adr/u	○○	○○	○	○
14	9	F/32 sp/adr/u	○	—	○	—
15		Meta LN I	○	○	○	○
16		Meta LN II	○	—	○	○
17	10	M/32 sp/ea/u	○	○	○	○
18	11	M/36 sp/adr/u	○	○	○	○
19		Meta LN	—	—	○	○
20	12	F/23 sp/adr/u	○	○	○	—
21		Meta bone	—	—	○	●
22	13	F/62 sp/adr/u	○	○	○	○
23	14	M/42 sp/adr/u	○	—	○	—
24	15	M/42 sp/adr/u	—	—	○	—
25	16	F/39 sp/adr/u	—	—	○	○
26		/49 meta LN	○	—	○	—
27	17	F/45 sp/adr/u	○	—	○	—
28		Meta LN	—	○	—	—
29	18	F/32 sp/adr/u	—	—	○	—
30		Meta liver	○	—	○	—

In CGH analysis, ○ represents 'no gain or loss,' ● is 'loss,' and ○ ○ means 'gain' of chromosome 17. In FISH analysis, ○ is 'retention of heterozygosity,' ● means 'loss of heterozygosity,' and ○ ○ represents 'gain' of the *p53* locus on chromosome 17. In SSCP, ○ means no mutation. In immunohistochemistry, ○ represents 'no overexpression' and ● means p53 protein overexpression. In all four techniques, — means that these samples are not examined. M = male; F = female; sp = sporadic; m = men; n = neurofibromatosis; adr = adrenal; ea = extra-adrenal; u = unilateral; bi = bilateral; Meta = metastasis; LN = lymph node.

there was loss, and in 5 of 23 (22%) we found gain of chromosomal material.

Confirmation of CGH Data by FISH

FISH analysis on 29 touch preparations and 4 paraffin-embedded tissue sections confirmed the CGH results at the *p53* locus. We found abnormalities in the FISH analysis in 16/33 (48%) cases, including 14/33 (42%) cases with loss of the *p53* locus and 2/33 (6%) with gain of the *p53* locus. There was almost complete concordance between the FISH and CGH data for those samples that were analyzed with both techniques, except for the benign cases 13, 14, 15, and 17 in which there was loss in the FISH analysis but not in the CGH and benign cases 16, 18, 19, and 21 in which we observed gain in the CGH but not in the FISH

analysis. The results are shown in Tables 3 and 4 and a representative result of CGH and FISH analysis is shown in Figure 1.

PCR–SSCP analysis

PCR–SSCP analysis was performed on 43 samples to examine sequence abnormalities in exons 5–8 of the *p53* gene. No band shifts were found at SSCP, indicating that there were no mutations in $n = 17$ benign and $n = 26$ malignant pheochromocytomas, as our positive control yielded the expected results. The results are shown in Tables 3 and 4.

Immunohistochemistry

Immunohistochemical staining was performed on 36 samples to analyze whether there was overexpression of the p53 protein. Two of 36 samples

(6%) showed overexpression (score ≥ 6), whereas all other samples, including the corresponding primary tumor of one of these two metastases, were negative (score ≤ 5) (Figure 2). One of these two positive samples was available for SSCP analysis, but did not show a band shift, nor did we find loss in the CGH analysis of the other primary tumor. The results are summarized in Tables 3 and 4.

Discussion

In this study, we have performed a comprehensive analysis of a large series of benign and malignant pheochromocytomas to detect abnormalities in *p53* at the DNA and protein level using a variety of techniques. Although we found a loss rate of 45% in CGH analysis at the *p53* locus, which was confirmed by FISH analysis, we could not detect gene mutations in the *p53* exons 5–8, nor did we find a high frequency of p53 protein overexpression by immunohistochemistry, with only two metastases showing nuclear immunostaining. This leads us to suggest that *p53* abnormalities apparently do not appear to play a major role in the tumorigenesis of benign and malignant pheochromocytomas, except for a possible minor role in metastatic progression. *p53* abnormalities thus cannot be used for the distinction between benign and malignant pheochromocytomas.

Until now, the molecular mechanisms underlying sporadic pheochromocytoma tumorigenesis have largely remained elusive. Because of its frequent involvement in carcinogenesis, *p53* has been the topic of several investigations, employing a range of techniques. However, most of these studies were limited to a single technique or considered only small numbers of tumors. Our study is the first to specifically address loss of 17p, including the *p53* locus, by conventional CGH analysis. This analysis, which we confirmed by FISH, was performed in the context of a genome-wide CGH study, which is the topic of a separate paper. In four cases, we observed a discrepancy between the CGH and the FISH analysis, identifying loss by FISH but no alterations by CGH. This may be due to the fact that not all deletions are picked up in CGH analysis due to its limitations in resolution.[23] The observed loss rate of 45% is in accordance with our previous work, where we found 31% loss in a series of 29 pheochromocytomas, and with LOH analyses performed by others, which showed loss rates between 18 and 24%.[5,18,27] In five pheochromocytomas (10%), gain of 17p was found in CGH, although copy number gain could only be confirmed in two instances by FISH analysis. This may most probably be due to heterogeneity in the tumors or may be partly due to the pheochromocytomas being rather hypodiploid.

In the majority of cases in which we found loss of 17p at the CGH analysis and in almost all malignant

pheochromocytomas, we performed mutation analysis of exons 5–8 using SSCP analysis, which allows the detection of over 90% of mutations. However, we did not find a single mutation in our analysis, which is in accordance with several previous studies.[18,20] However, two Asian studies reported 83% (5/6) and 40% (4/10) *p53* mutations, respectively.[10,21] The reason for the discrepancy between these two groups of studies is not known, but may be related to geographical factors. Alternatively, we cannot entirely exclude the fact that mutations are present in other parts of the *p53* gene, but it is known that more than 95% of mutations occur in exons 5–8.[7]

In normal cells, nuclear p53 expression is usually below the detection level of immunohistochemistry. Mutant *p53*, because of its longer half-life, and cellular stress can induce overexpression, resulting in positive nuclear immunostaining. In the present study, we did not find a single positive case in the entire group of primary pheochromocytomas. It must be noted that we employed very strict criteria before a tumor was scored as positive (see Materials and methods for scoring system). This is based on the notion that for *p53* to play a major role in tumorigenesis, whether in pheochromocytomas or in other tumors, a large proportion of cells should show moderate or strong immunostaining to be considered physiologically relevant. In addition, in our experience, no *p53* mutations, for example in intestinal or breast tumors, are found if only weak or focal staining is present (Dinjens, personal communication). In many of the studies on p53 immunohistochemistry in pheochromocytomas that have been published so far, a variety of scoring systems have been used, usually yielding a low percentage of cases that were considered positive. Whenever cases were considered positive, these were frequently malignant pheochromocytomas or metastases thereof, which is in line with our finding of two metastases showing p53 overexpression. The corresponding primary tumors, however, were negative.

Taken together, we have found a relatively high frequency of loss at the *p53* gene locus on chromosome 17p, without concomitant *p53* mutations or signs of protein overexpression by immunohistochemistry. These results were similar between pheochromocytomas and sympathetic paragangliomas. This suggests that *p53* does not appear to play a major role either in tumorigenesis or in the clinical behavior of human pheochromocytomas. Because of the relatively high frequency of 17p loss, one may hypothesize the existence of other tumor suppressor genes on this chromosomal arm, which may be involved in pheochromocytomas. An example of these is described in patients with breast cancer and endocrine pancreatic tumors.[28,29] However, it cannot be excluded that loss of 17p is the result of stochastic accumulation of genetic abnormalities in pheochromocytomas.

Acknowledgement

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