Characteristic Genomic Imbalances in Pediatric Pheochromocytoma

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Pheochromocytoma (PCC) in children is rare, genetically not well described, and often related to a poor prognosis. We detected genomic imbalances in all 14 tumors from children analyzed by comparative genomic hybridization. A combinatorial loss of chromatin from 3p and 11p was a common feature in 10 of 14 (72%) patients, which was a result of either a loss of a total chromosome 3 and a total chromosome 11 in 6 of 10 patients, or confined deletions of their p arms in 4 of 10 patients. All patients exhibiting a loss of 3p and 11p carried *VHL* mutations. The *VHL* mutations were constitutive in 9 cases and somatic and restricted to tumor DNA in the remaining tumor. On the other hand, *VHL* mutations were absent in 4 patients, 2 who had other familial syndromes (NF1, SDHD) and 2 with unknown etiology. Our data show that the pattern of imbalances in the tumor DNA of PCC patients strongly correlated with an underlying familial *VHL* mutation. Furthermore, we show that true sporadic PCC is rare in childhood. Thus, children with PCC should be checked for a related predisposing gene. This would also identify familial syndrome patients requiring long-term monitoring for other syndrome-related malignancies.

INTRODUCTION

Pheochromocytoma (PCC) is a catecholamine-producing tumor that arises from either the chromaffin cells of the adrenal medulla (~90%) or the paraganglia outside the adrenals (~10%). In the latter instance, the tumor is also more specifically referred to as a paraganglioma. In general, about 90% of cases are sporadic, with the remaining 10% developing as part of a familial cancer syndrome such as multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease type 2 (VHL type 2), neurofibromatosis type 1 (NF1), or hereditary pheochromocytoma-paraganglioma (SDHD, SDHB, and SDHC) (Manger and Gifford, 2002; Neumann et al., 2002b; Gimm et al., 2004). Extra-adrenal pheochromocytoma has been found to be associated with constitutive *SDHD* mutations (Neumann et al., 2002a). Sporadically occurring pheochromocytoma and paraganglioma are associated with a higher risk of malignancy. At an early diagnostic stage, discriminating between malign and benign tumors usually is not possible, and malignancy is manifested only later with the appearance of metastases.

Although there are several genes known to be involved in hereditary and sporadic PCC, the molecular steps in tumorigenesis remain obscure. There is significant evidence for the 2-hit Knudsen model of tumor development of PCC (Knudson, 1986), which has been clearly established for patients with familial syndromes like MEN2 or VHL (Koch et al., 2002). In addition, there is also evidence based on comparative genomic hybridization (CGH) analyses of other additional and essential genetic alterations, such as loss of chromosome 11 in VHL-associated PCC (Lui et al., 2002). Unfortunately, available CGH data are largely restricted to adult tumors, with very limited data on childhood tumors (Dannenberg et al., 2000; Edstrom et al., 2000; Lui et al., 2002). This is partly because the annual incidence of PCC is only 0.4 per 100,000 inhabitants, only 10% of which develop in childhood. As such, PCC makes up about 4%–5% of all childhood neoplasms (Melicow, 1977;

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Chrousos, 1989). However, bear in mind that some tumor species have less complex chromosome aberrations in children than in adults and therefore may offer an easier way to discover the pathogenically relevant gene alterations responsible for PCC. In the present study, we performed CGH to analyze the genomic imbalances in 14 cases of childhood PCC. Our results revealed characteristic genomic alterations, suggesting that the concomitant deletion of 3p with 11p is a specific and pathogenically relevant feature of PCC.

MATERIAL AND METHODS

Clinical Samples

Tumor samples of 14 children (ratio of female to male 1:1.8) with PCC were obtained from various German hospitals. All patients had been registered in GPOH-MET 97, the interdisciplinary, prospective multicenter trial study of the German Society of Pediatric Oncology and Hematology—Malignant Endocrine Tumors (Parlowsky et al., 1996).

Comparative Genomic Hybridization

Genomic DNA was extracted from paraffin-embedded as well as from frozen tumor samples. Only specimens composed mostly of tumor cells were used for DNA analysis after histological examination and dissection. DNA preparation followed standard protocols using a Qiagen DNA Purification Mini-Kit (Qiagen, Hilden, Germany). DNA from paraffin-embedded samples was preamplified by DOP-PCR prior to labeling (Speicher et al., 1993). Probe detection was performed after conjugation of the test and reference DNA with Avidin-FITC and Digoxigenine-Cy3, respectively (Vector Laboratories, Burlingame, CA).

The target chromosomes where prepared according to standard protocols. Comparative genomic hybridization (CGH) was performed according to the protocol of Lichter et al. (1995) with modifications described by Heller et al. (2000). Each tumor sample was analyzed twice, in 2 hybridization experiments, each using a different reference DNA sample. Fluorescence imaging and analysis were performed with a Axioplan 2 microscope (Zeiss, Jena, Germany) and ISIS software (Metasystems, Altlussheim, Germany), respectively. Gain or loss of specific genomic parts was defined as a chromosomal region whose mean green-to-red fluorescence ratio exceeded or fell short of the accepted threshold, respectively. This threshold was set as 3 times the standard deviation of the mean green-to-red fluorescence ratio at a given chromosomal locus. Chromosomal regions that had previously been shown to frequently cause artifacts (Solinas-Toldo et al., 1996; Kirchhoff et al., 2001), such as 1p32–pter, 12q24 and all of chromosomes 19, 22, and Y, as well as regions containing a large amount of repetitive DNA, such as centromeres, 9q12–q13, 16q11–q12, and the p arms of the acrocentric chromosomes, were excluded from evaluation.

Gene Analyses

DNA sequence analyses for *VHL*, *SDHD*, *SDHB*, and *VHL* deletion analysis were performed as described elsewhere (Bender et al., 2001; Neumann et al., 2002a; Schouten et al., 2002; Neumann et al., 2004). Multiplex ligation-dependent probe amplification (MLPA) was performed as described by Schouten et al. (2002).

RESULTS

CGH revealed chromosomal imbalances in all 14 tumors examined. Among the 9 tumors from patients with familial VHL syndrome, we found a simple and uniform aberration pattern (Table 1). This pattern consisted of frequent loss of a whole chromosome 3 and a whole chromosome 11, in 6 of 9 (67%) cases, or deletion of 3p and 11p, in 3 of 9 (33%) cases (Fig. 1). For 1 patient (02-024) whose tumor tissue showed deletion of 3p and 11p, we could not detect a constitutive *VHL* point mutation or a deletion (Richards et al., 1993; Cybulski et al., 2002) by DNA sequencing and MLPA, respectively, in the DNA extracted from blood cells. Therefore, we sequenced the DNA from tumor cells and detected a *VHL* c605 A/G mutation that was absent in the peripheral-blood leucocytes. Microsatellite analysis confirmed that both samples were derived from the same patient. A comparison of our CGH patterns with data on adult patients reported in the literature showed an aberration pattern for the latter that was concordant with that of the VHL syndrome group in our study (Dannenberg et al., 2000; Edstrom et al., 2000; Lui et al., 2002).

Two patients, one with the mutation for familial SDHD (03-017) and the other with a mutation for NF1 (02-038), showed an aberration pattern distinct from that of the VHL syndrome patients. Both displayed loss of 3p and gain of 9p12 (Table 1). The tumor of one patient (01-003) showed deletion of 3p without deletion of 11p (see Table 1). For this patient, DNA sequencing revealed no constitutive VHL-causing point mutation and no mutation for *MEN2*. Another patient (01-038), who had no point mutation for *VHL* and no other syndrome detected

TABLE 1. Clinical, Morphological, and Genetic Data on the PCC Patients

	Patient data/genetic data		Tumor Characteristics		CGH imbalances		
Case	Sex/age (months)	Constitutive mutation	Size (cm^3)	Origin	Lost	Gained	Follow-up Actual state (months)
01-002	m/108	*VHL* (c406 T/G)	15		3, 11		CCR (74)
01-051	f/96	*VHL* (E2, c125)	21		3, 11	17	CCR (38)
01-011[a]	f/108	*VHL* (c505 T/C)	79		3, 11	7	CCR (54)
01-008[a]	m/85	*VHL* (c505 T/C)	nd[b]	paraganglioma	3, 11	15	metachrone (87)
02-011	m/147	*VHL* (c463 G/C)	48		3, 11		CCR (37)
01-016	m/170	*VHL* (c452 G/T)	52		3p, 11p		CCR (51)
01-005	f/151	*VHL* (c680 A/G)	10		3p, 11p	6, 12, 17	CCR/ifu[c] (14)
01-042	f/93	*VHL* (c680 A/G)	36		3p, 11p, 11q23–qter, 14q23–q31	2p14–p22	CCR/ifu[c] (19)
01-004	m/162	*VHL*[f]	nd		3, 11,17, 18 (left tumor) 3, 11 (right tumor)		nd[b]
02-024	m/103	*VHL*[e]/no *SDHB*/no *SDHD*/	nd[b]		3p, 11p	13	CCR (67)
03-017	m/159	*SDHD* (c331 del G)	35		11	9p12-p13, 17q	nd[b]
02-038	f/198	*NF1*	126		1p, 4, 13qter, 17pter–q12	3p21–pter, 5p14–p15, 9p12, 18	metastasis, tAML[d] (33)
01-038	m/72	no *VHL*	16	paraganglioma	11q22–qter, 20	11q12–q14 16p, 17	death from relapse (9)
01-003	m/169	no *VHL*	33	paraganglioma	3p14–pter, 11q23–qter, 8p21–pter, 8q24, 9q33–qter, 16, 15q22–qter, 17, 20		metachrone (29)

[a]Cousins.
[b]No data.
[c]Lost to follow-up.
[d]Therapy-induced acute myeloid leukemia (AML M5).
[e]No constitutive deletion as well, but a somatic c605 A/G mutation.
[f]*VHL* mutation positive but no sequence data available.

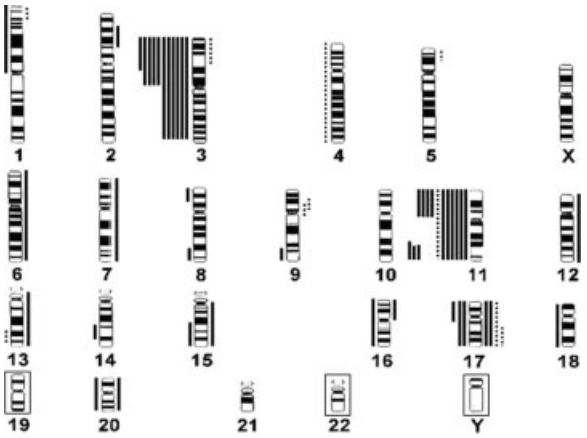

Figure 1. Copy number abnormalities in 14 PCC cases. Loss and gain of chromosomal material are depicted by vertical bars to the left and right of the chromosomes, respectively. Dotted bars indicate the imbalances of patients with familial mutations other than VHL. Boxes mark chromosomes excluded from evaluation.

so far, showed a loss in 11q22–11qter together with gains of other portions of the genome, but no deletion of 3p or 11p. This patient suffered from relapse (Table 1). Both tumors of patient 01-004, who had bilateral manifestation, displayed loss of chromosomes 3 and 11, with one of the tumors exhibiting an additional loss of chromosomes 17 and 18.

Gain of an extra chromosome 17 was the next most frequent aberration, found in 2 of the VHL patients, as well as in the SDHD (03-017) and NF1 (02-038) syndrome patients. We did not observe any correlation between genomic imbalance and levels of the typical hormones that are routinely monitored in PCC diagnostics.

DISCUSSION

Lui et al. (2002), in a study of adult patients with pheochromocytoma (PCC), reported a strong association between a *VHL* mutation and loss of chromosomes 3 and 11. In the present study, we extended this investigation to tumors of pediatric patients with PCC. We used comparative genomic hybridization (CGH) to analyze chromosomal imbalances in the PCC tumors of 14 children and proved these tumors had in common a high incidence [10 of 14 (72%)] of loss of either all or the p arms of both chromosomes. Nine of these patients carried a constitutive *VHL* mutation, whereas the other patient (02-024) carried a somatic c605 A/G *VHL* mutation confined to the tumor DNA. Such somatic *VHL* mutations are known to occur in sporadic PCC and in patients with germ-line deletions (Brauch et al., 1997; Mircescu et al., 2001; Vortmeyer et al., 2002). Our data suggest that mutations in VHL, which are either hereditary or somatic in origin, select for combinatorial deletions of 3p and 11p. An intriguing finding was that loss of 3p was always linked with loss of 11p ($n = 4$), whereas loss of a whole chromosome 3 was always linked with loss of a whole chromosome 11 ($n = 6$; Table 1). However, learning more about the mechanisms that drive these particular aberration patterns will have to await the availability of PCC karyotypes of patients with a VHL syndrome. Our data could not be matched with the little data available so far (Decker et al., 1994; Pfragner et al., 1998; Gunawan et al., 2004).

Lui et al. (2002) pointed out that loss of heterogeneity of chromosome 11 is a prerequisite cytogenetic aberration for the development of VHL-associated PCC (Lui et al., 2002), an assumption corroborated by our data. Because we identified a combinatorial deletion of only 3p and 11p in 4 (40%) of the 10 VHL-associated tumors, it is speculated that it is highly probable that genes presumed to be relevant in VHL-associated PCC are on the p arm of chromosome 11. This presumptive localization should aid faster identification of such genes. Potential candidate genes are numerous and include *WT1*, *CDKN1C*, *IGF2*, and *H19*. *WT1*, on 11p13, is known to play a role in renal and adrenocortical tumors. *CDKN1C* is a tumor suppressor on 11p15 that is affected in adrenocortical tumors and is known to inhibit cyclin-dependent kinase complexes (Henry et al., 1989; Bourcigaux et al., 2000). *IGF2* and *H19* are also on 11p15; the former is overexpressed in many tumors including PCC (Mircescu et al., 2001), and both have shown monoallelic expression. Mutter et al. (1993) demonstrated that a biparental genome may be required for the reciprocal *IGF2/H19* imprint to be expressed. Therefore, it cannot be ruled out that an additional contribution to the development of PCC comes from an imprinting effect as a result of deletion of 11p. There is compelling independent evidence for this: loss of the maternal 11p region seems to be essential for tumorigenesis of SDHD-linked paragangliomas and pheochromocytomas (Hensen et al., 2004; Riemann et al., 2004). Our patient 03-017, with a constitutive *SDHD* mutation and loss of one entire chromosome 11, might be a similar case, but we cannot determine decisively because we do not have clues on the parental origin of the residual chromosome 11. Loss of the maternal, rather than the paternal, chromosome 11 was also implicated in sporadic and familial cases of PCC in a previous study that performed a methylation analysis of 11p15 (Margetts et al., 2005). But,

again, our inability to determine if the retained 11p regions were of maternal or paternal origin leaves open the issue of how important parental origin is for tumor development in our cases.

Deletion of 1p has been found in 84% of sporadic and 18% of VHL-associated PCC tumors in adults (Edstrom et al., 2000). In addition, loss of 1p is more frequently found in MEN2-associated tumors than in VHL-associated tumors (Bender et al., 2000; Lui et al., 2002; Aarts et al., 2006). Interestingly, only the oldest patient (020-38) in our cohort, who lacked the characteristic *VHL* deletions of 3p and 11p, showed this chromosome aberration, raising the question of whether a deletion in 1p correlates with age rather than with a specific genotype. This patient had NF1 syndrome and a loss of 17pter–q12 in the tumor DNA, which is in accordance with the 2-hit Knudson model of tumor genesis.

Although the risk of malignant degeneration is relatively high in sporadic PCC, no histological or laboratory parameter currently exists that can determine the malignancy state. In one patient from this study (02-038), malignancy was confirmed because of metastasis, whereas malignancy was suspected in another patient but could not be confirmed. Neither patient belonged to the VHL syndrome group, and one of them (01-038) was supposed to have a sporadic tumor. CGH analysis of PCC tumors of children is a significant step toward determining the etiology of a tumor and will help to track patients with sporadic tumors who may face more adverse outcomes.

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