Sporadic and Familial Pheochromocytomas Are Associated with Loss of at Least Two Discrete Intervals on Chromosome 1p

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ABSTRACT

Pheochromocytomas are tumors of the adrenal medulla originating in the chromaffin cells derived from the neural crest. Ten % of these tumors are associated with the familial cancer syndromes multiple endocrine neoplasia type 2, von Hippel-Lindau disease (VHL), and rarely, neurofibromatosis type 1, in which germ-line mutations have been identified in RET, VHL, and NF1, respectively. In both the sporadic and familial forms of pheochromocytoma, allelic loss at 1p, 3p, 17p, and 22q has been reported, yet the molecular pathogenesis of these tumors is largely unknown. Allochic loss at chromosome 1p has also been reported in other endocrine tumors, such as medullary thyroid cancer and tumors of the parathyroid gland, as well as in tumors of neural crest origin including neuroblastoma and malignant melanoma. In this study, we performed fine structure mapping of deletions at chromosome 1p in familial and sporadic pheochromocytomas to identify discrete regions likely housing tumor suppressor genes involved in the development of these tumors. Ten microsatellite markers spanning a region of ~70 cm (1pter to 1p34.3) were used to screen 20 pheochromocytomas from 19 unrelated patients for loss of heterozygosity (LOH). LOH was detected at five or more loci in 8 of 13 (61%) sporadic samples and at five or more loci in four of five (80%) tumor samples from patients with multiple endocrine neoplasia type 2. No LOH at 1p was detected in pheochromocytomas from two VHL patients. Analysis of the combined sporadic and familial tumor data suggested three possible regions of common somatic loss, designated as PC1 (D1S243 to D1S244), PC2 (D1S228 to D1S507), and PC3 (D1S507 toward the centromere). We propose that chromosome 1p may be the site of at least three putative tumor suppressor loci involved in the tumorigenesis of pheochromocytomas. At least one of these loci, PC2 spanning an interval of <3.8 cm, is likely to have a broader role in the development of endocrine malignancies.

INTRODUCTION

Pheochromocytomas are tumors of the adrenal medulla, originating in the chromaffin cells derived from the neural crest. Pheochromocytomas most often occur sporadically (90%) but may also present as a component of three autosomal dominantly inherited cancer syndromes, commonly in MEN 2, in 10–14% of VHL patients and rarely in NF1 (1). These familial forms of pheochromocytoma have been associated with germ-line mutations in RET, VHL, and NF1, respectively, and a small subset of sporadic tumors have been shown to have somatic RET or VHL mutations (2, 3). Allelic loss at chromosomes 1p, 3p, 17p, and 22q has also been reported in both sporadic and familial forms of pheochromocytomas (4). The region of allelic loss at chromosome 1p includes 1p34–p36, with the frequency of reported loss being between 29 and 45% in sporadic pheochromocytomas (4–6) and 100% in pheochromocytomas from MEN 2 patients at this locus (5).

There is also evidence from LOH studies for the involvement of a putative tumor suppressor locus at chromosome 1p in the development of other endocrine tumors such as medullary thyroid carcinoma, the primary component tumor of MEN 2 (4, 5, 7, 8). In parathyroid adenosomas, a SRO has been defined by a 4-cM region at 1p35–p36 flanked by the markers D1S228 and D1S507 (9). Furthermore, a common region of loss from D1S214 to D1S228 was defined in neuroblastomas (10), and the combination of this result with those of three previous studies (11–13) determined a 7-cM consensus region of loss from D1S214 to D1S244 (10). Another SRO has been identified in neuroblastomas and named the NB3 locus flanked by D1J22 and p73 and including the p73 gene itself (14).

To generate a fine map of the regions of allelic loss present in both familial and sporadic pheochromocytomas, we have performed LOH studies using 10 microsatellite markers spanning a region of ~70 cm at 1pter to 1p34.3. Furthermore, we have shown overlap of these regions with regions of LOH identified previously in other endocrine- and neural crest-derived tumors.

PATIENTS AND METHODS

Patients. The specimens used in this analysis were from a patient cohort of 13 sporadic pheochromocytomas (Table 1) including 5 males (mean age at surgery, 57.2 ± 12.2 years) and 8 females (mean age, 47.1 ± 16.1 years), as well as 6 familial cases (Table 2), including a 40-year-old male and 5 females (mean age, 39.6 ± 16.5 years). Of the five female patients presenting with familial disease (Table 2), three were diagnosed with MEN 2A (P14, P15, and P16) and one with MEN 2B (P17). Two patients, one male and one female, were diagnosed with VHL (P18 and P19).

Tissue and Blood Samples. Twenty fresh pheochromocytoma samples from 19 patients were obtained and immediately frozen in liquid nitrogen before being transferred to −70°C for storage. Corresponding peripheral blood samples were also collected into EDTA tubes and frozen at −70°C. Pheochromocytoma from both left and right adrenal glands (removed at the same surgery) of one of the MEN 2A patients (P16) were collected. Tumor and constitutional DNA were extracted using a standard protocol (15).

RET Mutation Analysis. For the MEN 2 patients, RET mutation analysis of genomic DNA had been performed prior to this study for exons 10, 11, 13, 14, 15, and 16 according to protocols described previously (16–19). All MEN 2 patients had been shown to harbor a germ-line RET mutation prior to this study (Table 2).

LOH Analysis. LOH analysis was undertaken using DNA from 20 pheochromocytoma/blood paired samples and 10 microsatellite markers linked to chromosome 1p, from 1pter to 1p34.3. The markers are ordered as follows from the most telomeric to the most centromeric, D1S243-D1S468-D1S244-D1S2667-D1S228-D1S2728-D1S507-D1S478-D1S513-MYCL1 (20–24). Genomic and tumor DNA (50 ng) were amplified by PCR in a total volume of 10 μl containing 50–200 μM each deoxynucleotide triphosphate (Boehringer Mannheim); 0.5 μM each primer, the forward primer end labeled with 1.5 μCi/reaction [γ-33P]dATP (DuPont NEN, Boston, MA); 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1–1.5 mM MgCl2, and 0.5 unit of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). The following PCR conditions were
The ratio of the normalized tumor band density to the normalized band of tumor DNA was normalized against the corresponding retained band. The residual density of the deleted band in the tumor DNA was normalized against the density of the corresponding allelic band in the genomic DNA. A clear reduction in the intensity of one of the allelic bands in the tumor sample, and this was confirmed by scanning densitometry (4). The residual density of the deleted band was normalized against the density of the retained band, and the band in the genomic DNA corresponding to the deleted band was normalized against the density of the corresponding allelic band in the genomic DNA. A clear reduction in the intensity of one of the allelic bands in the tumor sample, and this was confirmed by scanning densitometry (4).

LOH at 1p in Pheochromocytomas

RESULTS

LOH Analysis. Using microsatellite markers located at 1pter to 1p34.3, analysis of DNA from 20 blood/pheochromocytoma paired samples showed LOH in 8 of 13 (61%) sporadic samples (Fig. 1A). Of the 8 sporadic tumors exhibiting LOH, 4 (P3, P5, P6, and P9) showed LOH at each informative marker, and in the remaining 4 sporadic tumors, LOH was detected at each informative marker with the exception of retention of heterozygosity. For the five sporadic tumors with retention of heterozygosity, whereas the right adrenal tumor showed loss at five of eight informative loci with retention of heterozygosity observed at the markers D1S243, D1S244, and D1S228.

Thus, in familial pheochromocytoma, two distinct regions of LOH were identified: SRO1-f corresponding to a <22.9 cM interval flanked by D1S243 and D1S244, and SRO2-f, a >39 cM interval flanked by D1S228 and extending toward the centromere (Fig. 1B).

Analysis of the combined sporadic and familial results suggested three regions of common loss: PC1 identical to SRO1-f (D1S243 to D1S244); PC2 identical to SRO2-s (D1S228 to D1S507); and PC3 identical to SRO3-s, a >35.3 cM interval flanked by D1S507 and extending toward the centromere.

Table 1. Patient information on sporadic pheochromocytomas indicating the sex of the patient and patient age at the time of surgery

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>F</td>
<td>62</td>
<td>MEN 2A</td>
</tr>
<tr>
<td>P2</td>
<td>F</td>
<td>27</td>
<td>MEN 2A</td>
</tr>
<tr>
<td>P3</td>
<td>F</td>
<td>63</td>
<td>MEN 2A</td>
</tr>
<tr>
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<td>F</td>
<td>40</td>
<td>MEN 2A</td>
</tr>
<tr>
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<td>F</td>
<td>23</td>
<td>MEN 2A</td>
</tr>
<tr>
<td>P6</td>
<td>F</td>
<td>44</td>
<td>MEN 2A</td>
</tr>
<tr>
<td>P7</td>
<td>M</td>
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<tr>
<td>P13</td>
<td>M</td>
<td>76</td>
<td>VHL</td>
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Table 2. Patient information on familial pheochromocytomas indicating the sex, age at time of surgery, and diagnosis for each patient including the results of RET testing on genomic samples for the MEN 2 patients

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
<th>RET mutation analysis</th>
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<td>63</td>
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<td>C634Y</td>
</tr>
<tr>
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<td>F</td>
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<td>MEN 2A</td>
<td>C634S</td>
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<td>P16-bilateral</td>
<td>F</td>
<td>21</td>
<td>MEN 2A</td>
<td>C634R</td>
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<td>P17</td>
<td>F</td>
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<td>M918T</td>
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<tr>
<td>P18</td>
<td>F</td>
<td>27</td>
<td>VHL</td>
<td></td>
</tr>
<tr>
<td>P19</td>
<td>M</td>
<td>40</td>
<td>VHL</td>
<td></td>
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</tbody>
</table>

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used: a denaturation step of 94°C for 4 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 15 s (for all markers except D1S507 and D1S513, where a 64°C annealing temperature was used), and extension at 72°C for 30 s. Electrophoresis was used to resolve the denatured samples (2 μl of each) for 3–4 h, followed by drying and overnight exposure to a Molecular Dynamics PhosphorImager screen (PhosphorImager SP; Molecular Dynamics, Sunnyvale, CA). LOH was indicated if there was loss or clear reduction in the intensity of one of the allelic bands in the tumor sample, and this was confirmed by scanning densitometry (4). The residual density of the deleted band in the tumor DNA was normalized against the density of the retained band, and the band in the genomic DNA corresponding to the deleted band of tumor DNA was normalized against the corresponding retained band. The ratio of the normalized tumor band density to the normalized corresponding genomic band was calculated, and a ratio of <0.5 was defined as representing LOH (4).

LoH AT 1p IN PHEOCHROMOCYTOMAS

LOH at chromosome 1p34.3, analysis of DNA from 20 blood/pheochromocytoma paired samples showed LOH in 8 of 13 (61%) sporadic samples (Fig. 1A). Of the 8 sporadic tumors exhibiting LOH, 4 (P3, P5, P6, and P9) showed LOH at each informative marker, and in the remaining 4 sporadic tumors, LOH was detected at each informative marker with the exception of retention of heterozygosity. For the five sporadic tumors with retention of heterozygosity, whereas the right adrenal tumor showed loss at five of eight informative loci with retention of heterozygosity observed at the markers D1S243, D1S244, and D1S228.

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Fig. 1. LOH analysis of 10 microsatellite markers at chromosome 1pter to 1p34.3 in pheochromocytomas. A, loss of heterozygosity; O, noninformative (homozygosity); ○, retention of heterozygosity; △, indeterminate case. A, analysis of 13 sporadic pheochromocytomas at this interval. B, analysis of seven familial pheochromocytomas consisting of three cases of MEN 2A (P16 with both left and right adrenals analyzed), one case of MEN 2B, and two cases of VHL. * PC1, PC2, and PC3 indicate the SROs from combining data from SROs of sporadic (SRO-s) and familial (SRO-f) pheochromocytomas.

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DISCUSSION

In this study of LOH in pheochromocytoma at 1p34–36, we have identified three putative tumor suppressor loci likely to be involved in the pathogenesis of this tumor. Specifically, these regions are PC1 (D1S243 to D1S244), PC2 (D1S228 to D1S507), and PC3 (D1S507 toward the centromere). This fine structure mapping of 1p involvement in pheochromocytoma refines previously reported gross loss (4, 6–8) with a SRO defined previously as bounded proximally by D1S15 (1pter–p22) and distally by D1Z2 (1p36.3; Ref. 5).

In our study, allelic loss at distal 1p was found in 8 of 13 (61%) sporadic pheochromocytomas compared with previous reports of loss at that region in the range of 28–54% (4, 5, 6, 8). Possible reasons for the differences in frequency of loss observed between these reports include the size of the cohorts studied and the range of markers used. Allelic loss at distal 1p was also observed in four of seven familial pheochromocytomas, including MEN 2 and VHL patients. Of considerable interest, two of the familial tumors that did not exhibit LOH at this locus were pheochromocytomas from VHL patients. This is in contrast to another report that identified 1p loss in one of two VHL pheochromocytomas studied (5).

An additional familial tumor exhibiting retention of heterozygosity at 1p34–36 for all informative markers was the left adrenal gland of a MEN 2A patient with the corresponding right adrenal gland showing LOH at D1S468 and loss flanked by D1S228 toward the centromere. This patient had undergone bilateral adrenalectomy, and pheochromocytoma was present in both glands. Although anecdotal, this raises the interesting possibility that somatic mutation of different genes, in combination with a germ-line mutation in the RET proto-oncogene, may lead to the formation of bilateral pheochromocytomas in MEN 2A patients along different genetic pathways.

We did not find any significant differences in the frequency of 1p LOH in sporadic versus familial (MEN and VHL) pheochromocytomas (61 and 57%, respectively), an observation that is consistent with previous reports (4, 8). By excluding the VHL samples from the familial analysis, the frequency of 1p LOH for MEN 2 pheochromocytomas was 80%. Interestingly, a previous study had reported 1p LOH in 100% of MEN 2 pheochromocytomas (nine of nine), not dissimilar to the findings reported in the current study (5). However, this same study reported only 29% (two of seven) 1p loss in sporadic pheochromocytomas. Whether this discrepancy of the frequency of 1p loss in sporadic pheochromocytomas is a true finding or rather reflects the limited sample size of each study remains to be clarified by analysis of a larger number of samples. However, our data do support the possibility of chromosomal loci in addition to RET and 1p having a role in the pathogenesis of both familial and sporadic pheochromocytomas.

It is interesting to compare common regions of loss in 1p in neuroblastoma, another tumor of neural crest-derived tissue, with the regions identified in our study on pheochromocytomas (Fig. 3). In neuroblastomas, a compilation of results from four studies (10–13) defined a common 7-cM region of loss from D1S214 to D1S244. This region falls within our defined PC1 region, which contains several potential candidate genes likely to function as tumor suppressors and be active in endocrine or neural crest cell-derived tissues. Such candidate genes include TFNFRSF12 (DR3; Ref. 25), PIK3CD (26), and p73 (27). In addition, our PC3 region partly overlaps a region of LOH described previously (12) in neuroblastoma and harbors potential suppressor genes such as CDC42 (28), E2F2 (29), HEIR 1 (30), and RAPIA (31).

In the current study, we have identified the c.3,8-cM region flanked by D1S228 and D1S507 and designated it PC2. This region has been reported previously as a SRO in parathyroid adenomas and additionally as part of a gross region of loss in pituitary adenomas (9) and neuroblastomas (12). Thus, PC2 has been shown to be deleted in a range of endocrine tumors, including familial and sporadic pheochromocytoma, and parathyroid tumors, as well as tumors of neural crest-derived tissue including neuroblastoma. Possible candidate genes in the PC2 region are caspase 9 (32), ZNF151 (33), and CD30 (34).

Therefore, it is likely that a putative tumor suppressor(s) at PC2 has a broad role in the pathogenesis of both endocrine and neural crest cell-derived tumors. It is also possible that the regions PC1 and PC3 may be additional sites of possible tumor suppressor genes involved in the pathogenesis of tumors of neural crest origin.

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