Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel–Lindau syndrome

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Abstract

Pheochromocytomas in von Hippel–Lindau (VHL) syndrome produce exclusively norepinephrine, whereas those in multiple endocrine neoplasia type 2 (MEN 2) produce epinephrine. This study examined the pathways activated in VHL-associated pheochromocytomas by comparing gene expression profiles in VHL and MEN 2 tumors in relationship to profiles in sporadic norepinephrine- and epinephrine-producing tumors. Larger and more distinct differences in gene expression among hereditary than sporadic tumors indicated the importance of the underlying mutation to gene expression profiles. Many of the genes over-expressed in VHL compared with MEN 2 tumors were clearly linked to the hypoxia-driven angiogenic pathways that are activated in VHL-associated tumorigenesis. Such genes included those for the glucose transporter, vascular endothelial growth factor (VEGF), placental growth factor, angiopoietin 2, tie-1, VEGF receptor 2 and its coreceptor, neuropilin-1. Other up-regulated genes, such as connective tissue growth factor, cysteine-rich 61, matrix metalloproteinase 1, vascular endothelial cadherin, tenascin C, stanniocalcin 1, and cyclooxygenases 1 and 2 are known to be involved in VEGF-regulated angiogenesis. Shared differences in expression of subsets of genes in norepinephrine- versus epinephrine-producing hereditary and sporadic pheochromocytomas indicated other differences in gene expression that may underlie the biochemical phenotype. Over-expression of the hypoxia-inducible transcription factor, HIF-2 α , in norepinephrine-predominant sporadic and VHL tumors compared with epinephrine-producing tumors indicates that expression of this gene depends on the noradrenergic biochemical phenotype. The findings fit with the known expression of HIF-2 α in norepinephrine-producing cells of the sympathetic nervous system and might explain both the development and noradrenergic biochemical phenotype of pheochromocytomas in VHL syndrome.

Endocrine-Related Cancer (2004) 11 897–911

Introduction

Pheochromocytomas are rare chromaffin cell tumors characterized by synthesis, storage, metabolism, and release of the catecholamines, norepinephrine and epinephrine. Clinical manifestations of these tumors are highly variable depending, in part, on the type of catecholamine produced. Location of tumors also contributes to variation in presentation. Most form in the adrenal glands, but some arise from extra-adrenal chromaffin tissue. The latter usually produce exclusively norepinephrine and tend to be more aggressive and likely to metastasize than tumors of the adrenal glands (Kimura *et al.* 1984, John *et al.* 1999, van der Harst *et al.* 2002).

Advances in molecular diagnostics are leading to increasing recognition of underlying hereditary conditions as causes of pheochromocytoma (Dluhy 2002, Neumann *et al.* 2002, Bryant *et al.* 2003). Such conditions include multiple endocrine neoplasia type 2 (MEN 2) due to mutations of the *ret* gene, von Hippel–Lindau (VHL) syndrome due to mutations of the *VHL* gene, von Recklinghausen's disease due to mutations of the *neurofibromatosis type 1* gene, and familial paragangliomas due to mutations of genes encoding subunits for succinate dehydrogenase (SDHB and SDHD).

At least some of the variation in clinical presentation of hereditary pheochromocytomas appears to reflect the underlying mutation. Pheochromocytomas in MEN 2 express the enzyme phenylethanolamine-N-methyltransferase (PNMT), that converts norepinephrine to epinephrine, whereas those in VHL syndrome do not express PNMT and consequently do not produce epinephrine (Eisenhofer *et al.* 2001). The above differences in expression of PNMT and synthesis of epinephrine in VHL- and MEN 2-associated pheochromocytomas were suggested to reflect development of tumors from different populations of epinephrine- and norepinephrine-producing chromaffin cells within the adrenal medulla.

The molecular mechanisms by which the various mutations predispose to development of pheochromocytoma and the bases for the variable clinical presentation of the tumor are largely unknown. Presumably, however, these molecular mechanisms involve differences in expression of genes influenced by the particular mutation responsible for the hereditary syndrome. We therefore hypothesized that pheochromocytomas from VHL and MEN 2 patients would exhibit differences in gene expression that might be explained by relationships to known functions of the genes responsible for these syndromes, and that such differences might also account for variations in phenotypic features of the tumors.

There has been one previous pheochromocytoma gene expression profiling study involving a single patient with a sporadic tumor (Yang *et al.* 2003). The present study

involved tumor samples from 39 patients, utilizing cDNA microarrays to compare gene expression in pheochromocytomas from patients with VHL syndrome and MEN 2. Since these hereditary tumors are characterized biochemically by differences in synthesis of epinephrine, we also compared gene expression in sporadic pheochromocytomas that produced predominantly norepinephrine (non-adrenergic phenotype) with those that also produced epinephrine (adrenergic phenotype). The analysis presented here focuses mainly on the genes and pathways activated in VHL-associated pheochromocytomas in relationship to those activated in sporadic norepinephrine-producing tumors.

Materials and methods

Patients

Patients included 12 with VHL syndrome, seven with MEN 2A and 20 without evidence of an underlying hereditary condition for pheochromocytoma (i.e. patients with sporadic pheochromocytoma). VHL patients were 11–60 years old (mean 31 years) at the time of surgical resection of pheochromocytomas and included five females and seven males. MEN 2 patients were 27–43 years old (mean 33 years) and included five females and two males. Patients with sporadic pheochromocytoma were 30–71 years old (mean 51 years) and included eight females and 12 males.

The diagnosis of VHL syndrome or MEN 2A was indicated by a clinical and family history of multiple tumors characteristic of the particular disorder and confirmed by identification of germ-line mutations of the *VHL* tumor suppressor gene or the *ret* gene. Patients with sporadic pheochromocytoma were not routinely tested for the presence of underlying germ-line mutations of relevant disease-causing genes. None of these patients, however, had multiple tumors or a family history of pheochromocytoma or other tumors associated with these mutations. Nevertheless, on the basis of a recent report (Neumann *et al.* 2002) it might be expected that up to five of the 20 patients with apparent sporadic pheochromocytoma might have had underlying germ-line mutations, including up to two with mutations of the *VHL* gene and one with a mutation of the *ret* gene.

Tissue procurement

Tissue samples were procured from patients operated on between 1997 and 2001 under studies that were approved by the appropriate Institutional Review Boards with informed consent obtained from all patients. Approved protocols did not allow for germ-line mutation testing in patients with apparently sporadic pheochromocytoma.

All patients had pheochromocytomas arising from the adrenal glands. Samples of tumor tissue were obtained within 90 min of surgical removal of pheochromocytomas. Dimensions of tumors were recorded, and small samples (50–400 mg) were dissected away from surrounding tissue, placed on dry ice and then stored at -80°C .

Determination of catecholamine biochemical phenotype

The biochemical phenotype of tumors was determined by analysis of tissue catecholamine contents. Samples of tumor tissue were weighed frozen and homogenized in 5–10 volumes of 0.4 M perchloric acid containing 0.5 mM EDTA. Homogenates were centrifuged and supernatants collected and stored at -80°C . Supernatants were diluted 1000-fold in 0.2 M acetic acid, after which catecholamines in 100 μl samples were extracted onto alumina and measured using high performance liquid chromatography as described elsewhere (Eisenhofer *et al.* 1986).

Expression profiling

cDNA microarrays were constructed as described elsewhere (DeRisi *et al.* 1996), and contained 13 826 cDNA clones. Excluding housekeeping genes and duplicate clones, there were a total of 12 210 spots corresponding to 11 754 unique cDNA clones. Over 85% of these cDNA clones represented functionally known genes. Gene names are listed according to the Unigene database (<http://www.ncbi.nlm.nih.gov/entrez>). RNA was extracted from the frozen tumors after homogenization in TRIzol reagent (Invitrogen) followed by RNeasy Maxi (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. All tumor samples were referenced to the same universal human RNA source (Stratagene, La Jolla, CA, USA). Microarrays were hybridized, scanned, and analyzed as described previously (DeRisi *et al.* 1996, Chen *et al.* 2002). Fluorescence intensities of the genes expressed in tumor samples were compared with a common universal human RNA reference, from which calibrated ratios were generated.

Data analysis

Data were subject to two sets of filtering criteria and statistical analyses: (1) a primary analysis involving selection of differentially expressed genes based on a false-discovery rate of 10% and (2) a secondary analysis involving more stringent filtering criteria, a different statistical test, and selection of genes based on a difference in *P* value of less than 0.001. The raw data used in these analyses can be made available upon request to the corresponding authors.

For the primary analysis, the data were required to pass three quality filters. First, the average spot quality score (which ranges from 0, being worst, to 1, best) over all samples in the study was required to be at least 0.5 (Chen *et al.* 2002). Of the original 12 210 spots, 11 578 spots passed this first filter. Second, the normalized ratio to reference RNA was required to be above 2 or below 0.5 in three or more experiments. This led to a filtering of a further 4288 spots, leaving 7290 spots that passed both requirements. Third, data were required to show a standard deviation of the decimal log ratio of greater than 0.10, indicating that an important variation in ratio took place at this spot. This resulted in a final 6854 spots that passed all three requirements. Data were next inspected using principal components analysis, and a scatterplot of samples on the first and second principal components was reviewed. Consistent differences between MEN 2 and VHL tumors could be seen, as could important but less consistent differences between sporadic norepinephrine- and sporadic epinephrine-producing tumors. Genes which differed significantly between MEN 2 and VHL tumors, and between sporadic norepinephrine- and sporadic epinephrine-producing tumors were selected using a two sample Student's *t*-test on the median normalized log-ratios. Genes were then selected so that the false-discovery rate was 10% (estimated number of false detections divided by total genes detected) as described previously (Benjamini & Hochberg 1995). Fold-change scores were computed for each comparison by averaging the log-ratios for each group, computing the difference, and applying the anti-log transform. Principal components analysis, gene selection and false-discovery rate calculations were made in the JMP δ statistical package (SAS, Inc, Cary, NC, USA) using the analyst's toolbox scripts developed by one of us, available at <http://affylims.cit.nih.gov>.

Genes that differed significantly between MEN 2 and VHL tumors, and between sporadic norepinephrine- and sporadic epinephrine-producing tumors were also selected using a second series of filtering criteria and a different method of statistical analysis. Except for the first filter, the same filters were used as described above. For the first filter, the average spot quality score was required to be at least 0.5 in each of the two individual comparisons among hereditary and sporadic tumors. This more rigorous initial filtering step led to a loss of a further 1764 spots, leaving 5090 spots that passed all three requirements. These data were analyzed by generation of a weighted list of genes followed by random permutation analysis, as described elsewhere (Golub *et al.* 1999, Allander *et al.* 2001, Kees *et al.* 2003). The tools and the statistical methods used for this particular analysis are available at <http://arrayanalysis.nih.gov/>. Genes that differed with *P* values less than 0.001

after this test were selected, and only genes that passed both statistical tests are presented in any detail.

Hierarchical clustering, gene selection, and multi-dimensional scaling analysis were performed as described elsewhere (Khan *et al.* 1998, Allander *et al.* 2001). The functional distribution of the differentially expressed genes was established based on the annotation provided by the Gene Ontology database (<http://www.geneontology.org>).

Results

Catecholamine biochemical phenotypes

In the 20 sporadic adrenal pheochromocytomas where there was no evidence of an underlying hereditary condition, tissue contents of epinephrine varied widely from 0.02% to 90% of the total catecholamine content (i.e. sum of norepinephrine, epinephrine, and dopamine). All sporadic and hereditary tumors had tissue contents of dopamine of less than 2.8% (mean 0.4%).

Eight of the 20 sporadic tumors had tissue contents of epinephrine of less than 5% and norepinephrine contents greater than 95%. These tumors were designated with a noradrenergic biochemical phenotype. The other 12 tumors had epinephrine contents above 12% and were designated with an adrenergic biochemical phenotype.

Tissue contents of epinephrine in tumors from the VHL patients averaged 1.2% (range 0.1–4.0%) of the total catecholamine content, similar to the sporadic tumors with a noradrenergic phenotype where the epinephrine content averaged 1.3% (range 0.02–4.3%). In contrast, the tissue content of epinephrine in tumors from MEN 2 patients averaged 48% (range 9–71%) of the total catecholamine content and did not differ from sporadic tumors with an adrenergic phenotype where the epinephrine content was 49% (range 13–90%) of the total.

Differential gene expression based on a 10% false-discovery rate

Of the 7290 cDNA clones that passed the three filtering steps in the primary analysis, a total of 2187 showed differential expression between tumors from MEN 2 and VHL patients based on an expected 10% false-discovery rate. In contrast, based on this same criterion, only 321 of the 7290 clones showed differential expression between sporadic noradrenergic and adrenergic tumors. Among the 321 differentially expressed genes in sporadic tumors, 203 (63%) were also differentially expressed in hereditary tumors.

Among the genes showing differential expression between tumors from VHL and MEN 2 patients, 1185 had higher expression in VHL than MEN 2 tumors and 1002 higher expression in MEN 2 than VHL tumors.

Among the genes showing differential expression between sporadic noradrenergic and adrenergic tumors, 249 had higher expression in noradrenergic than adrenergic tumors, and 72 higher expression in adrenergic than noradrenergic tumors.

A scatterplot of differentially expressed genes in hereditary versus sporadic tumors revealed highly significant positive relationships for genes that were differentially expressed only in hereditary tumors ($r = 0.63$, $P < 0.001$), for genes that were differentially expressed only in sporadic tumors ($r = 0.71$, $P < 0.001$), and for genes that were differentially expressed in both hereditary and sporadic tumors ($r = 0.91$, $P < 0.001$) (Fig. 1). Thus, genes that were more highly expressed in MEN 2 than VHL tumors also tended to be more highly expressed in sporadic adrenergic than noradrenergic tumors. Similarly, genes that were more highly expressed in VHL than in MEN 2 tumors tended to be more highly expressed in sporadic noradrenergic than adrenergic tumors.

Final selection of differentially expressed genes

Supervised hierarchical clustering of the 5090 genes that passed the three filtering steps of the second analysis yielded a total of 518 genes that showed differential expression ($P < 0.001$) between tumors from VHL and MEN 2 patients (Fig. 2) and 82 genes that showed differential expression between sporadic noradrenergic and adrenergic tumors (Fig. 3). Among hereditary tumors, the selection of genes generated by this second analysis included a total of 497 out of 518 (96%) genes that were also differentially expressed according to the first analysis, which utilized less stringent filtering criteria and selection based on a 10% false-discovery rate. Similarly, 77 out of the 83 (93%) differentially expressed genes in sporadic tumors derived from the second analysis were also differentially expressed according to the first analysis.

Further analysis of the data was restricted to the 497 genes in hereditary tumors and the 77 genes in sporadic tumors that were differentially expressed according to both analyses. Complete listings of the differentially expressed genes for hereditary tumors are available at <http://www.pressor.org/VHLvsMEN2.html> and for sporadic tumors at <http://www.pressor.org/SNAvsSA.html>.

Examination of the 497 differentially expressed genes in hereditary tumors revealed that most (74%) of the differences involved higher expression of genes in VHL than MEN 2 tumors. Similarly, among these 77 differentially expressed genes, most (87%) were more highly expressed in the sporadic noradrenergic than the sporadic adrenergic tumors.

Figure 1 Relationships between the differential expression of genes in hereditary noradrenergic (VHL) and adrenergic (MEN 2) tumors compared with sporadic adrenergic and noradrenergic tumors. Separate relationships are shown for the 1984 genes that were differentially expressed only in hereditary tumors (black), for the 118 genes differentially expressed only in sporadic tumors (red), and for the 203 genes differentially expressed in both hereditary and sporadic tumors (blue). The positive relationships establish that genes more highly expressed in MEN 2 than VHL tumors (i.e. high MEN 2/VHL ratio) also tend to be more highly expressed in sporadic adrenergic than noradrenergic tumors (i.e. high sporadic adrenergic/noradrenergic ratio), and vice versa for genes more highly expressed in VHL than MEN 2 tumors.

Comparison of the expression profiles of differentially expressed genes in the hereditary pheochromocytomas with those of the same genes in sporadic tumors revealed a

similar but less profound pattern of expression in sporadic adrenergic and noradrenergic tumors compared with MEN 2 and VHL tumors (Fig. 2). Also, comparison of

Figure 2 Gene expression profiles after supervised hierarchical cluster analysis of tumors from patients with MEN 2 (M) and VHL syndrome (V). The gene expression profiles in sporadic adrenergic (SA) and sporadic noradrenergic (SN) tumors are also shown for comparison. Genes with a relatively higher level of expression are shown in red and those with a lower level of expression are shown in green. 3σ , standard deviation cut off.

the expression profiles of differentially expressed genes in the sporadic pheochromocytomas with expression of the same genes in hereditary tumors revealed a similar pattern of expression in MEN 2 and VHL tumors compared with sporadic adrenergic and noradrenergic tumors (Fig. 3). More specifically and, as also indicated by the data in Fig. 1, the genes more highly expressed in MEN 2 than VHL tumors tended to be also more highly expressed in

Figure 3 Gene expression profiles after supervised hierarchical cluster analysis of tumors from patients with sporadic adrenergic (SA) and sporadic noradrenergic (SN) tumors. The gene expression profiles in tumors from MEN 2 (M) and VHL (V) patients are shown for comparison. Genes with a relatively higher level of expression are shown in red and those with a lower level of expression are shown in green. 3σ , standard deviation cut off.

sporadic adrenergic than sporadic noradrenergic tumors. Similarly, the genes more highly expressed in VHL than MEN 2 tumors tended to be also more highly expressed in sporadic noradrenergic than sporadic adrenergic tumors.

Shared differential expression of genes in hereditary and sporadic tumors

Comparison of the list of 77 differentially expressed genes in sporadic tumors with the list of 497 differentially expressed genes in hereditary pheochromocytoma revealed that 47 out of the 77 genes (61%) differentially

Table 1 Genes with shared differential expression in both hereditary and sporadic tumors

Gene name	Clone ID	Unigene ID
Sporadic adrenergic > noradrenergic and MEN 2 > VHL		
Mitogen-activated protein kinase-activated protein kinase 3	2406134	Hs.234521
N-acylaminoacyl-peptide hydrolase	813279	Hs.221589
Phenylethanolamine N-methyltransferase	1957136	Hs.1892
Retinoblastoma binding protein 8	51737	Hs.437224
Sporadic noradrenergic > adrenergic and VHL > MEN 2		
A disintegrin-like and metalloprotease with thrombospondin type 1 motif 2 (ADAMTS1)	2229965	Hs.120330
Adenosine A2a receptor	279970	Hs.197029
Aquaporin 1 (channel-forming integral protein, 28 kDa)	51950	Hs.76152
Cadherin 12, type 2 (N-cadherin 2)	2703024	Hs.333997
Cadherin 13, H-cadherin (heart)	31093	Hs.63984
Calcitonin receptor-like	1326920	Hs.152175
Carbonic anhydrase II	2569769	Hs.155097
Collagen, type IV, α 2	769959	Hs.407912
Collagen, type V, α 3	154173	Hs.235368
Cytochrome b-5	196189	Hs.83834
Death-associated protein kinase 2	343871	Hs.129208
Downregulated in ovarian cancer 1	344189	Hs.15432
Endothelial PAS domain protein 1 (HIF-2 α)	869187	Hs.8136
Ephrin-B2	796198	Hs.30942
ERO1-like (<i>S. cerevisiae</i>)	810448	Hs.10949
FAT tumor suppressor homolog 1 (<i>Drosophila</i>)	591266	Hs.166994
G protein-coupled receptor 124	486493	Hs.17270
Heparan sulfate proteoglycan 2 (perlecan)	770059	Hs.470489
Histone 1, H2am	1713730	Hs.134999
Hypothetical protein FLJ10159	376844	Hs.346203
Hypothetical protein FLJ10439	773324	Hs.415220
Integrin, α 7	377671	Hs.74369
Jagged 1 (Alagille syndrome)	1474023	Hs.409202
NADH dehydrogenase (ubiquinone) 1, α/β subcomplex, 1, 8 kDa	782635	Hs.5556
Neurocalcin δ	838478	Hs.90063
Notch homolog 3 (<i>Drosophila</i>)	81475	Hs.8546
Phospholipase A2, group IVB (cytosolic)	2910181	Hs.198161
Plexin D1	811048	Hs.301685
Plexin domain containing 1	47781	Hs.125036
Podocalyxin-like	290378	Hs.16426
Polymerase (RNA) II (DNA directed) polypeptide G	740672	Hs.14839
Protein tyrosine phosphatase type IVA, member 3	375827	Hs.43666
Protocadherin 17	1579610	Hs.106511
PTPL1-associated RhoGAP 1	884783	Hs.430919
Rap1 guanine-nucleotide - exchange factor directly activated by cAMP	795382	Hs.8578
Receptor (calcitonin) activity modifying protein 2 (RAMP-2)	379604	Hs.155106
Regulator of G-protein signalling 4	429349	Hs.386726
Sema domain, immunoglobulin domain (Ig), short basic domain (semaphorin 3F)	809526	Hs.32981
Transducin (β)-like 1X-linked	840766	Hs.76536
Transforming growth factor β -stimulated protein, TSC-22	868630	Hs.114360
Translocase of outer mitochondrial membrane 7 homolog (yeast)	529307	Hs.112318
v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (Ets1)	489729	Hs.18063
Zinc finger protein 219	772904	Hs.250493

expressed in sporadic adrenergic and noradrenergic pheochromocytomas were also differentially expressed in VHL and MEN 2 tumors (Table 1). All genes with higher expression in adrenergic than noradrenergic tumors had higher expression in MEN 2 than VHL tumors and all

genes with higher expression in noradrenergic than adrenergic tumors also had higher expression in VHL than MEN 2 tumors.

As expected, PNMT was one of the four genes that showed significantly ($P < 0.001$) higher expression both in

sporadic adrenergic than noradrenergic tumors and in MEN 2 than VHL tumors (Table 1). The gene for neurocalcin- δ , which has previously been shown to be expressed most abundantly in norepinephrine- rather than epinephrine-containing populations of adrenal chromaffin cells (Iino *et al.* 1997), also showed the expected higher ($P < 0.001$) expression in sporadic noradrenergic than adrenergic tumors and in VHL than MEN 2 tumors. The gene for endothelial PAS domain protein 1 (EPAS-1), also known as hypoxia-inducible transcription factor (HIF)-2 α , was another gene with both higher expression in sporadic noradrenergic than adrenergic tumors and in VHL than MEN 2 tumors. The cDNA arrays included two separate clones (no. 869187 and no. 1855332) for this gene, both of which showed significantly ($P < 0.0003$) greater expression in sporadic noradrenergic than adrenergic tumors and in VHL than MEN 2 tumors.

Functional analysis of differentially expressed genes

Using the Gene Ontology database (<http://www.geneontology.org>) we were able to assign functions to most of the differentially expressed genes as detailed elsewhere for lists of genes for hereditary (<http://www.pressor.org/VHLvsMEN2.html>) and sporadic pheochromocytomas (<http://www.pressor.org/SNAvsSA.html>). Comparisons of the assigned functions for the 369 genes more highly expressed in VHL than MEN 2 tumors with functions for the 128 genes more highly expressed in MEN 2 than VHL tumors suggested that the relative expression of different functional groups of genes depended on the particular mutation (Fig. 4). In particular, larger proportions (30% vs 6%) of genes encoding extracellular matrix constituents or regulatory proteins were more highly expressed in VHL than in MEN 2 tumors. There were also larger numbers of genes more highly expressed in VHL than MEN 2 tumors that were associated with angiogenesis (eight versus none) or cellular proliferation (six versus none).

Due the limited number (ten) of genes that were more highly expressed in sporadic adrenergic than noradrenergic tumors it was not possible to accurately compare functions of genes differentially expressed in these two groups of tumors. Nevertheless, the distribution of relative functions of the 67 genes that were more highly expressed in sporadic noradrenergic than adrenergic tumors closely mirrored the distribution of functions for the 369 genes more highly expressed in VHL than MEN 2 tumors.

Among the genes with assigned functions that were more highly expressed in VHL than MEN 2 tumors, over 80 were identified that could be linked to hypoxia-driven processes or functions associated with angiogenesis or the extracellular matrix (Fig. 5). Close to 20% of these

particular genes were also more highly expressed ($P < 0.001$) in sporadic noradrenergic than adrenergic tumors. Many other genes (about 30%) also showed shared over-expression in hereditary and sporadic noradrenergic tumors, but at a lower level of significance ($P < 0.05$ to $P < 0.001$) that did not reach the required cut-off limits for inclusion in the list of 77 differentially expressed genes in sporadic tumors (e.g. vascular endothelial growth factor (VEGF) at $P = 0.015$ and VEGF receptor 2 (VEGFR-2) at $P = 0.0014$). Genes that were over-expressed in VHL tumors, apparently independently of the noradrenergic phenotype, included those involved in glucose utilization (e.g. glucose transporter 1 (GLUT1) and hexokinase 2) and others associated with the extracellular matrix (Fig. 5).

Discussion

This study has established that pheochromocytomas from patients with hereditary syndromes are characterized by distinct gene expression profiles that depend on the mutation and also correlate with the catecholamine biochemical phenotype. As indicated by the relationships in gene expression among hereditary and sporadic pheochromocytomas (Fig. 1), many of the differences in gene expression between VHL and MEN 2 tumors were shared by similar, though overall weaker, differences between sporadic noradrenergic and adrenergic tumors. As discussed later, the nature of the germ-line mutation may predispose to development of tumors with a specific biochemical phenotype and exaggerate some aspects of gene expression profiles associated with these phenotypes. This and the more variable nature of the underlying somatic mutations in sporadic pheochromocytoma could account for the stronger and more distinctive gene expression profiles among hereditary noradrenergic (VHL) and adrenergic (MEN 2) tumors than among sporadic noradrenergic and adrenergic tumors.

The multiple filtering and statistical steps left subsets of 77 differentially expressed genes in the sporadic groups of tumors and 497 differentially expressed genes in hereditary groups. Among the latter, more genes were over-expressed in VHL than in MEN 2 tumors, and many of these were clearly linked to the hypoxia-regulated angiogenic pathways known to be involved in VHL-associated tumorigenesis (Fig. 5). In this syndrome, mutations of the *VHL* tumor suppressor gene lead to a VHL protein that is defective in targeting HIF- α subunits (HIF-1 α and HIF-2 α) for ubiquitin-mediated proteolysis. The resulting accumulation of these transcription factors leads to increased utilization of glucose, facilitated by increased expression of the glucose transporter, GLUT1, and hexokinase 2 (Iliopoulos *et al.* 1996, Mathupala *et al.* 2001), and to

VHL

(369 genes, 261 with known function)

MEN 2

(129 genes, 77 with known function)

Sporadic - noradrenergic

(67 genes, 61 with known function)

Sporadic - adrenergic

(10 genes, 7 with known function)

Figure 4 Pie charts showing the functional analysis of differentially expressed genes in VHL versus MEN 2 tumors (top) and sporadic noradrenergic versus adrenergic tumors (bottom). Functions of genes for VHL tumors represent those with higher expression in VHL than MEN 2 tumors, whereas functions of genes in MEN 2 tumors are those with higher expression in MEN 2 than VHL tumors. Similarly, functions of genes in sporadic noradrenergic tumors represent those with higher expression in noradrenergic than adrenergic tumors, whereas functions of genes in sporadic adrenergic tumors are for those showing higher expression in adrenergic than noradrenergic tumors. Functions were established based on the annotation provided by the Gene Ontology database (<http://www.geneontology.org>) and are shown only for genes with known functions.

Figure 5 Diagram showing a selection of 82 genes more highly expressed in VHL than MEN 2 tumors. The selection represents genes known to be involved in hypoxia or VEGF signaling pathways or with established functions in angiogenesis and extracellular matrix reorganization. In these processes, endothelial cells are activated and then secrete proteases to dissolve basement membranes, thereby allowing detachment of endothelial cells from blood vessel walls. The detached endothelial cells send out cytoplasmic projections, migrate, proliferate, undergo cytoskeletal rearrangement, and interact with other cells eventually differentiating and forming new vessels. This neovascularization process is associated with a reorganization of the extracellular matrix that requires resynthesis of an array of extracellular matrix constituents. A deregulated process of angiogenesis appears critical to development of many tumors, particularly those associated with VHL syndrome. Unrestrained production of angiogenic factors may also contribute to abnormal cell–cell and cell–matrix interactions involved in solid tumor growth. The black arrows indicate connections between genes known to be induced by an up-stream signaling protein (e.g. VEGF, CYR61) or transcription factor (e.g. EPAS-1/HIF-2 α , Ets-1). The grey arrows show pathways regulating amounts of HIF-1 α or HIF-2 α protein. The asterisks indicate a gene identified to be also more highly ($P < 0.001$) expressed in sporadic noradrenergic than adrenergic tumors. The daggers indicate a gene showing no difference in expression ($P > 0.05$) among sporadic noradrenergic and adrenergic tumors.

angiogenesis, stimulated by increased expression of VEGF and other mitogens (Forsythe *et al.* 1996). Consistent with the above, the genes for GLUT1, hexokinase 2, and VEGF were all over-expressed in VHL compared with MEN 2 tumors.

Cell surface receptors to VEGF that were over-expressed in VHL tumors included VEGFR-2 (or Flk-1) and neuropilin-1, an isoform-specific co-receptor for VEGF and receptor for class 3 semaphorins (Neufeld *et al.* 1999). VEGFR-2 is directly induced by the co-operative

actions of HIF-2 α and Ets-1 (Elvert *et al.* 2003), which were both over-expressed in VHL tumors. Ets-1 is an oncogenic transcription factor that can be induced by HIF-1 α and VEGF (Oikawa *et al.* 2001), and which promotes expression of several hypoxia-driven angiogenic genes, including neuropilin-1 (Teruyama *et al.* 2001). VEGFR-2, together with neuropilin-1, form a receptor complex specific for a VEGF165 isoform particularly important in facilitating endothelial cell migratory responses (Wang *et al.* 2003). Associated over-expression in VHL tumors of semaphorin

3F and plexin-D1 suggests an additional level of influence on angiogenic migratory processes via semaphorin–neuropilin/plexin signaling pathways (van der Zwaag *et al.* 2002, Lantuejoul *et al.* 2003).

Other hypoxia-inducible genes for mitogens involved in angiogenic signaling that were over-expressed in VHL tumors included placental growth factor, platelet-derived growth factor- α (PDGF- α) and angiopoietin 2 (Schweda *et al.* 2000, Koga *et al.* 2001, Yamakawa *et al.* 2003). Associated over-expressed hypoxia- or VEGF-inducible genes for cell-surface receptors included the PDGF- β receptor (Lafuente *et al.* 1999), the endothelial receptor tyrosine kinase, Tie-1 (McCarthy *et al.* 1998), and the calcitonin receptor-like receptor which, together with the receptor activity-modifying proteins, form integral components of the adrenomedullin-signaling system (Nikitenko *et al.* 2003).

Genes for connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61), two members of a family of mitogens with established functions in hypoxia-driven angiogenic processes (Brigstock 2002), were also over-expressed in VHL tumors. Expression of CTGF and CYR61 can be induced by VEGF (Suzuma *et al.* 2000), and both regulate the production and function of numerous extracellular proteins involved in angiogenic extracellular matrix remodeling, including other mitogens, such as VEGF (Brigstock 2002, Hashimoto *et al.* 2002).

Up-regulation in VHL tumors of matrix metalloproteinase 2, a proteinase active in dissolving basement membranes during extracellular matrix reorganization, is consistent with the known induction of this gene by both VEGF and Ets-1 (Lamoreaux *et al.* 1998, Reisdorff *et al.* 2002). Similarly, increased expression in VHL tumors of the gene for the tissue inhibitor of the metalloproteinase 1 (TIMP1) is consistent with the associated role of this factor in extracellular matrix remodeling and induction of the gene by CYR61 (Chen *et al.* 2001). TIMP3, which is known to block binding of VEGF to VEGFR-2 and antagonize VEGF-mediated angiogenesis (Qi *et al.* 2003), was also over-expressed in VHL tumors. α -2-Macroglobulin is another matrix metalloproteinase inhibitor up-regulated in VHL tumors and known to contribute to tumorigenic extracellular matrix remodeling and to be induced by VEGF (Abe & Sato 2001). α -2-Macroglobulin binds to numerous cytokines and growth factors, including VEGF and PDGF, thereby modulating the clearance and receptor binding of angiogenic growth factors (Soker *et al.* 1993). Increased expression of the gene for the disintegrin-like metalloproteinase, ADAMTS1, which binds to the VEGF₁₆₅ isoform and blocks VEGFR-2 phosphorylation (Luque *et al.* 2003), is consistent with the role of this extracellular protease as a modulator of angiogenesis.

Other VHL up-regulated genes known to be induced by HIF-1 α , HIF-2 α , Ets-1, or VEGF and involved in angiogenic extracellular matrix reorganization and cell–cell interactions included VE-cadherin (Lelievre *et al.* 2000, Wary *et al.* 2003), stanniocalcin 1 (Liu *et al.* 2003), platelet endothelial cell adhesion molecule-1 (Bautch *et al.* 2000, Cao *et al.* 2002), vascular cell adhesion molecule-1 (Kim *et al.* 2001), phosphatidic acid phosphatase 2b (Humtsoe *et al.* 2003), cyclo-oxygenases 1 and 2 (Murphy & Fitzgerald 2001, Liu *et al.* 2003), thrombomodulin (Calnek & Grinnell 1998, Abe & Sato 2001), and tenascin C (hexabrachion) (Lal *et al.* 2001), the latter being an extracellular matrix protein that promotes endothelial cell sprouting, proliferation, and migration (Grant *et al.* 1999, Cha *et al.* 2003). Ephrin-B2 and ephrin-B3 were other VHL up-regulated genes, with established functions in the patterning of arterial and venous endothelial cell migratory responses in angiogenesis (Adams *et al.* 1999). Similarly, the genes for Notch-3 and its ligand, Jagged 1, which are involved in arterial vascular development, remodeling, and cell survival (Lindner *et al.* 2001), also showed increased expression in both VHL and sporadic norepinephrine-producing pheochromocytomas. Interestingly, the Notch-3 signaling pathway has been linked to development of chromaffin cell phenotypes characterized by expression of specific catecholamine-synthesizing enzymes (Van Limpt *et al.* 2003), suggesting a possible role in the noradrenergic phenotype of pheochromocytoma tumor cells.

Members of the integrin family (integrin subunits α 1, α 5, and α 7) were other VHL up-regulated genes known to be involved in angiogenic signaling, endothelial cell sprouting, migration, adhesion, and extracellular matrix reorganization (Ponce *et al.* 2001, Senger *et al.* 2002). VEGF has been established to induce expression of α 1-integrin (Senger *et al.* 1997), whereas CYR61 and the Ets-1 transcription factor induce expression of the integrin α 5 subunit of the fibronectin receptor, thereby facilitating fibronectin-stimulated cell migration and adhesion (Chen *et al.* 2001, Kita *et al.* 2001). These pathways may also be involved in VHL tumorigenesis, where up-regulation of integrin levels and changes in cell–extracellular matrix signaling have been suggested to induce improper assembly of the extracellular fibronectin matrix and proliferative and disorganized growth (Davidowitz *et al.* 2001, Esteban-Barragan *et al.* 2002).

The integrins interact or serve as receptors for numerous angiogenic signaling ligands and extracellular matrix constituents, including members of the laminin and collagen families (Ignatius *et al.* 1990, Ponce *et al.* 2001), many of which showed up-regulation in VHL tumors consistent with the extensive nature of extracellular matrix reorganization and resynthesis in these

tumors. The gene for perlecan, a heparin sulfate proteoglycan and a major component of basement membranes, was also up-regulated in VHL tumors, consistent with established functions of this extracellular matrix protein in integrin-mediated adhesion, laminin matrix assembly processes, and in inducing tumor growth and angiogenesis (Sharma *et al.* 1998, Henry *et al.* 2001, Jiang & Couchman 2003).

Up-regulated expression in VHL tumors of the gene for HIF prolyl-4-hydroxylase 3 presumably reflects a negative feedback response to activation of hypoxia-inducible pathways. The three prolyl-4-hydroxylase enzymes identified to date regulate hypoxia-driven processes by catalyzing the hydroxylation of key proline residues on the HIF- α subunits, thereby facilitating recognition of these transcription factors by the VHL tumor suppressor protein for proteasome-mediated degradation (Bruick & McKnight 2001). The enzymes are only active under normoxic conditions, so that under hypoxic conditions the proline residues are not hydroxylated and the HIF- α subunits accumulate. Interestingly, whereas enzyme activity is reduced under hypoxic conditions, gene expression is increased and, importantly, this appears to be specific for HIF prolyl-4-hydroxylase 3, consistent with the presence of several hypoxia response elements on the upstream regulatory region of the gene for this particular enzyme (Cioffi *et al.* 2003).

Since regulation of HIF- α occurs primarily through changes in protein stability, the increased HIF-2 α mRNA levels in VHL compared with MEN 2 tumors were unexpected findings. However, the HIF-2 α gene was also more highly expressed in sporadic pheochromocytomas with a noradrenergic than an adrenergic phenotype. This indicates that increased expression of HIF-2 α mRNA in VHL tumors is not dependent on the VHL mutation, but rather reflects the noradrenergic phenotype that characterizes pheochromocytomas from patients with VHL syndrome. This and the additional influence of VHL mutations to increase HIF-2 α protein stability explain some of the exaggerated differences in gene expression profiles among hereditary compared with sporadic noradrenergic and adrenergic tumors.

Of particular relevance to the above findings, HIF-1 α and HIF-2 α have different patterns of baseline and hypoxia-influenced mRNA and protein expression and variable functions in the hypoxia-driven process among different tissues and cell types (Sowter *et al.* 2003, Wiesener *et al.* 2003). Apart from endothelial cells, HIF-2 α is also expressed in developing sympathetic nerves, where it regulates the expression of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis (Tian *et al.* 1998, Favier *et al.* 1999). Presumably, as in noradrenergic nerves of the sympathetic nervous system,

HIF-2 α might also be expressed in noradrenergic chromaffin cells of the adrenal medulla. If so, then this might confer onto such cells susceptibility to VHL-associated tumorigenesis, thereby explaining both the development of such tumors and their associated noradrenergic phenotype. The above possibility is in line with previous observations on the relative contributions of HIF-1 α and HIF-2 α to VHL-associated renal cell carcinomas, where it was suggested that the pattern of HIF-2 α abundance might be responsible for the observed organ specificity of the VHL syndrome (Maranchie *et al.* 2002).

The importance of angiogenic processes in VHL pheochromocytomas, as indicated by the present gene expression profiling study, is consistent with histopathologic studies where VHL tumors had a prominent vasculature closely intermixed with tumor cells, whereas MEN 2 tumors had a less prominent vasculature largely confined to the stromal septae between nests of tumor cells (Koch *et al.* 2002). The presence of a myxoid and hyalinized stroma was another distinct morphological feature of VHL tumors consistent with the present findings of pronounced over-expression in VHL tumors of numerous genes for extracellular matrix constituents.

In conclusion, the present analysis supports an important role of HIF-2 α -mediated angiogenic processes and associated extracellular matrix reorganization in the development of VHL-associated pheochromocytomas. Nevertheless, despite the consistency of the present findings with known hypoxia-driven angiogenic pathways, any suggestion of a central role of HIF-2 α -mediated processes in the development of VHL-associated pheochromocytomas must be tempered by findings from two other groups of rare mutations in the VHL gene that are not associated with impaired targeting of HIF- α for proteasome-mediated degradation, but which still cause pheochromocytomas (Clifford *et al.* 2001, Hoffman *et al.* 2001). Additional analyses of such tumors appears important to establish the critical pathways involved in development of VHL-associated pheochromocytomas.

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