Gene Expression Profiling of Benign and Malignant Pheochromocytoma

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ABSTRACT: There are currently no reliable diagnostic and prognostic markers or effective treatments for malignant pheochromocytoma. This study used oligonucleotide microarrays to examine gene expression profiles in pheochromocytomas from 90 patients, including 20 with malignant tumors, the latter including metastases and primary tumors from which metastases developed. Other subgroups of tumors included those defined by tissue norepinephrine compared to epinephrine contents (i.e., noradrenergic versus adrenergic phenotypes), adrenal versus extra-adrenal locations, and presence of germline mutations of genes predisposing to the tumor. Correcting for the confounding influence of noradrenergic versus adrenergic catecholamine phenotype by the analysis of variance revealed a larger and more accurate number of genes that discriminated benign from malignant pheochromocytomas than when the confounding influence of catecholamine phenotype was not considered. Seventy percent of these genes were underexpressed in malignant compared to benign tumors. Similarly, 89% of genes were underexpressed in malignant primary tumors compared to benign tumors, suggesting that malignant potential is largely characterized by a less-differentiated pattern of gene expression. The present database of differentially expressed genes provides a unique resource for mapping the pathways leading to malignancy and for establishing new targets for treatment and diagnostic and prognostic markers of malignant disease. The database may also be useful for examining mechanisms of tumorigenesis and genotype–phenotype relationships. Further progress on the basis of this database can be made from follow-up confirmatory studies, application of bioinformatics approaches for data mining and pathway analyses, testing in pheochromocytoma cell culture and animal model systems, and retrospective and prospective studies of diagnostic markers.

KEYWORDS: pheochromocytoma; paraganglioma; gene expression; microarray; metastases; catecholamines

INTRODUCTION

Pheochromocytomas are rare catecholamine-producing neuroendocrine tumors with diverse clinical presentations and phenotypes depending on the interplay of numerous factors, including genetics, tumor location, and type of catecholamine produced.[1] Most are benign solitary tumors arising from adrenal medullary chromaffin tissue or more occasionally from extra-adrenal chromaffin tissue, where they are defined as paragangliomas. For these, treatment by surgical resection is relatively straightforward and usually curative. An important proportion of tumors, however, presents as or develops into metastatic disease. For these there is invariably no cure. There are also no pathological markers to reliably distinguish malignant from benign disease or to predict malignant tendency of a resected primary mass. Definitive diagnosis of malignant pheochromocytoma continues to rely on identification of metastases at non-chromaffin sites (e.g., liver, bones, and lungs) distant from that of the primary tumor.[1]

Although there remains a lack of pathological markers for malignant disease, several interdependent factors are now identified to be associated with increased risk for malignancy. Most malignant tumors produce predominantly norepinephrine (noradrenergic biochemical phenotype), in contrast to 50% of solitary adrenal tumors that produce a mixture of both norepinephrine and epinephrine (adrenergic biochemical phenotype).[2] Extra-adrenal tumors are almost always characterized by a noradrenergic phenotype and have a relatively high risk of malignancy.[3,4] More recently, germline mutations of the succinate dehydrogenase subunit B (SDHB) gene were identified to be associated with both high rates of extra-adrenal tumor locations and malignant disease.[5,6] This contrasts with other hereditary syndromes, such as multiple endocrine neoplasia type 2 (MEN 2) and von Hippel–Lindau (VHL) syndrome, both associated with low rates of malignancy, the former characterized by an adrenergic phenotype and predominantly adrenal tumors and the latter by a noradrenergic phenotype with both adrenal and extra-adrenal paragangliomas.[7]

In the present study we used oligonucleotide microarray analysis to examine gene expression profiles in a large series of benign and malignant pheochromocytomas with carefully characterized locations, hereditary or sporadic backgrounds, and catecholamine phenotypes. The study used sophisticated statistical approaches involving multiple comparisons—taking into account catecholamine biochemical phenotypes, hereditary factors, and tumor location—to establish differences in gene expression between benign and malignant pheochromocytoma. The ultimate goals of these comparisons are to establish the molecular pathways involved in malignant transformation, identify targets for new treatments, and develop new diagnostic markers for predicting malignant potential. This initial report, rather than addressing the aforementional goals, provides a description of the methods and design of the analyses, with reporting of results restricted to a general overview of global differences in gene expression for each of the numerous comparisons of the study.

MATERIALS AND METHODS

Tumor Procurement

Tumor specimens were collected from patients who underwent surgery at several centers: National Institutes of Health, Bethesda, MD; Yale University, New Haven, CT; University of Michigan, Ann Arbor, MI; and at several European university hospitals (Dresden, Germany; Florence, Italy; Nijmegen and Rotterdam, the Netherlands; and Bratislava, Slovak Republic). Specimens were procured under Institutional Review Board–approved protocols compliant with international guidelines with informed consent from patients.

Tumor samples were frozen and stored at –80°C shortly after surgical resection. Total RNA was extracted from tumors using a standard Trizol

preparation protocol (Life Technologies, Inc. Rockville, MD). Quality and quantity of RNA were sufficient for gene expression profiling in 103 tumors from 93 patients. Five tumors (seven slides, as two tumors were done in duplicate) were excluded on account of poor array spot quality ($n = 4$) or inability to classify a tumor as metastatic or recurrent ($n = 1$). Consequently, the final analysis was on the basis of 98 tumors collected from 90 patients (46 female, 42 male, and 2 unknown), including 20 patients with metastatic disease and 70 patients with benign disease. The majority of tumors came from patients with apparently sporadic pheochromocytoma, but the study set also includes tumors from 12 VHL, 12 MEN 2A (one with metastatic disease), 5 SDHB (three with metastatic disease), 4 SDHD patients, and 1 neurofibromato type 1 patient. Multiple tumors were included from three patients, including two with two separate extra-adrenal tumors each, and one patient with seven abdominal metastatic lesions.

Tumor Catecholamines

Tumor tissue concentrations of catecholamines (norepinephrine, epinephrine, and dopamine) were quantified by liquid chromatography with electrochemical detection.[8] Samples of tumor tissue were weighed frozen and homogenized in 5 to 10 volumes of 0.4 M perchloric acid containing 0.5 mM EDTA. Homogenates were centrifuged and supernatants collected for catecholamine determinations.

Oligonucleotide Microarrays

Microarray slides were generated from 34,580 longmer oligonucleotide probes obtained from the Human Genome Oligonucleotide Set Version 3.0 from Qiagen Inc. (Valencia, CA, USA). Each 70 basepair oligomer was sequence-optimized to represent a specific gene using BLAST (Basic Local Alignment Search Tool) for nucleotide sequence to minimize cross-hybridization with other genes. The set represented over 26,121 unique Refseq genes, over 24,048 unique Ensembl genes, and over 25,416 unique Unigene genes. The set also contained 12 unique positive controls (housekeeping genes) and 12 unique negative controls (random sequence) provided in 16 replicates. The gene description and annotation of these oligonucleotides were based on the Ensembl database dated from February 2005.

Dried oligonucleotides were resuspended in 3 $\times$ SSC solution and spotted on epoxy-coated slides. The slides were then cleaned by vigorous shaking in a 0.5% SDS solution for 2 min and incubated for 20 min in water at 50°C. The slides were finally air-dried by centrifugation and ready for hybridization.

Probe Labeling and Hybridization

The detailed protocols for probe labeling and hybridization are available through the website: http://research.nhgri.nih.gov/nhgri_cores/microarray.html. In brief: 5 μg of each total RNA sample or 150 ng of human normal adrenal medulla polyA reference (Cat. 637451, BD Biosciences Clontech, Palo Alto, CA, USA) was reverse-transcribed with random hexamers in the presence of 5-(3-aminoallyl)-dUTP. After purification, the cDNAs are coupled with either Cy3 or Cy5 for 1 h and then purified using the PCR cleaning kit from Qiagen. Labeled cDNAs were then hybridized to the slides in a 1 × final *in situ* hybridization buffer from Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA). The hybridization was performed overnight at 60°C in a rotisserie oven. The slides are then washed in a series of SSC/SDS buffers and dried by centrifugation.

Scanning and Image Analysis

A laser confocal scanner from Agilent Technologies was used to scan the hybridized Cy3 and Cy5 probes on the chips. The fluorescent intensities at the spot and nearby background locations on the array were measured using the DEARRAY software (http://www.scanalytics.com). For each spot, a quality indicator (was greater than 100 fluorescent units, spot size greater than 50 units) was recorded.

Clinical Characteristics for Data Analysis

Data were analyzed according to a series of comparisons on the basis of the hypothesis that gene expression profiles would differ according to multiple variables, including benign versus malignant potential, location of primary tumors and metastases, catecholamine biochemical phenotype, and presence of any underlying germline mutation.

Malignant disease was defined on the basis of the occurrence of metastases at sites where chromaffin cells are normally absent (e.g., liver, lungs, and bones). Tumor samples in patients with malignant disease included metastases and primary tumors from which metastases developed, the latter with both adrenal and extra-adrenal locations. Tumors were classified as benign if there was no evidence of metastases at resection, care being taken to distinguish multifocal paragangliomas from true metastatic lesions at nonchromaffin locations. Tumors were classified as having a noradrenergic versus an adrenergic catecholamine phenotype according to the relative tissue concentrations of norepinephrine and epinephrine, as described elsewhere.[9] The presence of a relevant disease-causing germline mutation was confirmed by analysis of genomic DNA for mutations of the SDHB, SDHD, VHL, and RET genes.

TABLE 1. Study comparisons

	Tumors (n)	Patients (n)
Series 1—Focus on malignant disease		
1. Metastatic versus benign tumors		
Metastatic	20	20
Benign	71	69
Total	91	89
2. Metastatic versus benign tumors with consideration of biochemical profile		
Metastatic	16	16
Noradrenergic	12	12
Adrenergic	4	4
Benign	61	60
Noradrenergic	33	32
Adrenergic	28	28
Total	77	76
3. Metastatic versus benign tumors with consideration of SDHB mutation status		
Metastatic	10	8
SDHB+	5	3
SDHB −	5	5
Benign	17	16
SDHB+	3	2
SDHB −	14	14
Total	27	24
4. Metastatic primary versus benign tumors		
Metastatic	8	8
Benign	24	23
Total	32	31
5. Metastases versus metastatic primary tumors		
Metastases	8	8
Primary	10	10
Total	18	18
6. Metastatic adrenal-derived versus metastatic PGG-derived tumors		
PGG	7	7
Adrenal	9	9
Total	16	16
Series 2—Focus on influences of hereditary factors and biochemical profiles		
A. MEN 2A versus VHL tumors		
MEN 2A	10	10
VHL	12	12
Total	22	22
B. Sporadic adrenergic versus noradrenergic tumors		
Adrenergic	16	16
Noradrenergic	17	17
Total	33	33
C. Relationships of gene expression with tumor epinephrine content	87	80
D. VHL versus SDHB tumors		
VHL	12	12
SDHB	6	5
Total	18	17

PGG-paraganglioma.

Study Comparisons

Study comparisons were divided into two series, with numbers of samples in each comparison dependent on the availability of clinical data required for defining the various groups of that comparison (TABLE 1). The first and major series of comparisons was directed at defining sets of genes involved in malignant transformation. This series comprised six comparisons: (1) metastatic versus benign tumors (all tumors included with no consideration of biochemical profile, underlying mutation, tumor location, or nature of malignancy as a primary malignant tumor or a metastatic lesion); (2) metastatic versus benign tumors with additional consideration of adrenergic versus noradrenergic biochemical profile by two-way analysis of variance (ANOVA) [but no consideration of underlying mutation, tumor location, or nature of malignancy as a metastatic lesion or a primary malignant tumor]; (3) metastatic versus benign tumors with additional consideration of SDHB mutation status by two-way ANOVA (confined to noradrenergic tumors from patients tested positive or negative for SDHB mutations and without VHL, RET, or SDHD mutations); (4) metastatic primary versus benign tumors (confined to malignant primary and benign noradrenergic tumors from patients without VHL, RET, or SDHD mutations); (5) metastatic primary tumors versus distant metastatic lesions (all involving different patients); and (6) metastatic paraganglioma-derived versus metastatic adrenal-derived tumors.

The second series of analyses was directed at defining sets of genes critical to the adrenergic versus noradrenergic phenotype or differentially expressed according to underlying mutations of VHL, RET, and SDHB genes. This series also served as a positive control, building on a similar series of comparisons carried out in a previous cDNA microarray study.[10] The series comprised three comparisons and one analysis by linear regression: (A) MEN 2A versus VHL tumors (excluding malignant tumors); (B) sporadic adrenergic versus sporadic noradrenergic tumors (excluding malignant tumors); (C) relationships of gene expression with tumor epinephrine content by linear regression analysis; and (D) VHL versus SDHB tumors.

Statistical Analysis

Image data for each chip were reduced to intensity values for the test and reference channels and quality indicators for each spot, as described above. Data were then normalized between chips. Only spots achieving the highest quality score (1.0) for all chips were included in the subsequent analysis. Quantile normalization was applied separately to intensities from each channel to adjust for chip-to-chip variation in responsivity. An adaptive, variance-stabilizing transform was then applied to each channel to establish a measurement scale in which the variance was uniform. This transformation (denoted as the "symmetric adaptive transform") has been extensively and successfully used with

single-channel oligonucleotide microarray data as an effective alternative to median-adjusted-log10-transform, to control for variation between chip responsiveness. We here extend it in a novel application to two-channel microarray data. Differences between transformed intensities for each channel were then computed for each spot and chip, yielding the relative intensity index used in subsequent analysis.

Quality Control, Printing Batch, and Lot Effects

Transformed intensity data from the two channels on each chip were found to be highly correlated ($R > 0.9$), as expected. Six chips with unusually low correlation values (R between 0.83 and 0.89), indicating possible problems with mRNA quality in one or the other channel, image registration, or scanning, were marked as outliers and dropped from the study. Transformed intensities were also subjected to a principal-components analysis (PCA), to further scan for potential outliers and other data artifacts. The six chips identified with low R values also appeared as outliers on the first principal component. Chips were printed in two separate batches, and reference RNA was obtained in six distinct lots. Plots of the first 10 principal components versus the sequence number of each chip revealed a clear batch and lot effect, necessitating the use of batch-corrected analysis subsequently.

ANOVA with Batch Correction

Data were analyzed in reference to each of the study comparisons using a one- or two-way ANOVA, with blocking. Two-way ANOVA is valuable when a confounding factor is known to influence the outcome variable, such as the greater incidence of noradrenergic tumors among malignancies. Both the confounding factor (noradrenergic status) and the factor of interest (malignant vs. benign) can be accounted for by entering both into a two-way ANOVA. Variation due to the six identified print batch or reagent lots (block effect) was removed before the study comparison was addressed using a blocked ANOVA. In some cases, a patient sample was hybridized to multiple chips. Rather than simply averaging the chip intensities, which would have confounded the batch correction, each replicate chip was given a fractional weight, inversely proportional to the number of replicate chips used. Thus, identical, unit statistical weight was applied to each unique sample. Significance values, direction, and magnitude of differential expression were computed for each gene (spot), for each comparison, and genes with $P < 0.001$ were reported in separate gene lists. False-discovery rate values (estimated number of false detection divided by total genes detected) were calculated for each comparison.

Gene Ontology Analysis

The functional distribution of the differentially expressed genes was established on the basis of the annotation provided by the gene ontology (GO) database (http://www.geneontology.org). Numbers of genes per GO term category were analyzed to detect disproportionate numbers of genes using the following formula for a hypergeometric distribution: $P = \Pr(X \geq x \mid N, K, n)$, where x is the number of genes with the GO term per comparison, N is the total number of genes in the database with any GO annotation, K is the total number of genes in database with the specific GO term, and n is the number of genes with any GO annotation per comparison. Statistical significance was defined by a P value less than 0.05 using Fisher's exact test.

RESULTS

Series 1: Malignant Pheochromocytoma

Comparison of the data for all benign and malignant pheochromocytomas (comparison 1) yielded a total of 636 genes that discriminated benign from malignant disease at a significance level of $P < 0.001$ (TABLE 2). Consideration of the influence of catecholamine biochemical phenotype in comparison 2 indicated a considerably larger number of 2,246 genes that discriminated adrenergic from noradrenergic tumors. Additional consideration of the confounding influence of catecholamine biochemical phenotype, by two-way ANOVA, yielded a 53% larger number of 976 genes that discriminated ($P < 0.001$) malignant from benign tumors in comparison 2 than in comparison 1. Among these 976 differentially expressed genes in comparison 2, 516 were also differentially expressed in comparison 1. Most (70%) differentially expressed genes showed lower expression in metastatic than benign tumors (TABLE 2).

Comparison of SDHB-positive with SDHB-negative tumors (comparison 3) yielded a total of 613 genes that differentiated ($P < 0.001$) these two groups of tumors, with 59% of differentially expressed genes more highly expressed in SDHB-positive than SDHB-negative tumors (TABLE 2). Additional consideration of the confounding influence of SDHB mutation status by two-way ANOVA, however, revealed only 19 genes that distinguished benign from malignant pheochromocytoma. This negligible yield did not exceed the number of genes expected to show a difference ($P < 0.001$) by chance alone (i.e., calculated from the expected false-discovery rate).

Excluding adrenergic tumors and metastases at sites distant from the primary source of the malignancy in comparison 4 indicated a total of 383 genes that differentiated ($P < 0.001$) benign from primary malignant tumors (TABLE 2). Eighty-nine percent of these differentially expressed genes were more highly expressed in benign than in malignant primary tumors.

TABLE 2. Numbers of differentially expressed genes at $P < 0.001$

Study comparison	Number (FDR)	Number of genes with higher expression in:	
	Series 1		
1. Metastatic versus benign tumors	636 (4.3%)	Metastatic tumors	134
		Benign tumors	502
2. Metastatic versus benign tumors with consideration of biochemical profile	2,246 (1.2%)	Noradrenergic tumors	1,354
		Adrenergic tumors	892
	976 (2.8%)	Metastatic tumors	293
		Benign tumors	683
3. Metastatic versus benign tumors with consideration of SDHB mutation status	613 (4.4%)	SDHB positive tumors	364
		SDHB negative tumors	249
	19 (100%)	Metastatic tumors	7
		Benign tumors	12
4. Metastatic primary versus benign tumors	383 (7.1%)	Metastatic primary tumors	44
		Benign tumors	339
5. Metastases versus metastatic primary tumors	2,836 (1.0%)	Metastases	1,372
		Metastatic primary tumors	1464
6. PGG-derived versus adrenal-derived metastatic tumors	619 (4.4%)	Metastatic PGG-derived	290
		Metastatic adrenal-derived	329
	Series 2		
A. MEN 2A versus VHL tumors	887 (3.0%)	MEN 2A tumors	259
		VHL tumors	628
B. Sporadic adrenergic versus noradrenergic tumors	179 (15.1%)	Adrenergic tumors	108
		Noradrenergic tumors	71
C. Relationships of gene expression with tumor epinephrine content	1,725 (1.6%)	Positive relationships	602
		Negative relationships	1,123
D. VHL versus SDHB tumors	1,165 (2.3%)	VHL tumors	355
		SDHB tumors	810

Abbreviation: FDR, false discovery rate (percent of genes expected to show differential expression at $P < 0.001$ by chance); PGG, paraganglioma.

Comparison of primary tumors from patients with malignant disease with metastatic lesions of other patients (comparison 5) yielded 2,836 genes that discriminated ($P < 0.001$) between these two groups of tumors (TABLE 2). Among these genes, there were near equal proportions showing higher (48%) and lower (52%) expression in metastatic lesions than in primary malignant tumors.

Among all malignant primary tumors and metastases, comparison of adrenal-derived with paraganglioma-derived malignancies yielded 619 differentially expressed genes that discriminated between ($P < 0.001$) tumors on the basis of the adrenal versus extra-adrenal location of the primary tumor (TABLE 2). Among these differentially expressed genes, 47% showed higher expression in paraganglioma-derived malignancies and 53% in adrenal-derived malignancies.

Series 2: Influences of Hereditary Factors and Biochemical Profiles

Comparison of benign adrenal pheochromocytomas from patients with MEN 2A and VHL syndrome (comparison A) yielded 887 differentially

($P < 0.001$) expressed genes that discriminated between these two groups of hereditary adrenergic and noradrenergic tumors (TABLE 2). Most (72%) of these genes showed higher expression in tumors from VHL than MEN 2A patients. Among benign sporadic pheochromocytomas there were 179 genes that discriminated ($P < 0.001$) adrenergic from noradrenergic tumors (comparison B). Sixty percent of these differentially expressed genes showed higher levels of expression in adrenergic than in noradrenergic tumors.

Linear regression analysis using all data from benign and malignant tumors (comparison C) indicated 1,725 genes showing significant ($P < 0.001$) positive or negative relationships with percentage of epinephrine contents of tumors (TABLE 2). Thirty-five percent of these genes showed positive relationships and 65% negative relationships with increasing epinephrine content. As might be expected, expression of the gene for phenylethanolamine-*N*-methyltransferase showed a positive relationship with tumor epinephrine content and, among all 1,725 genes, represented the single gene with the strongest relationship to epinephrine content.

Comparison of tumors from VHL patients with those from patients with SDHB mutations (comparison D) yielded 1,165 genes that discriminated ($P < 0.001$) between these two groups of tumors (TABLE 2). Seventy percent of these genes showed higher expression in SDHB than in VHL tumors.

GO Analysis

GO analysis of differentially expressed genes ($P < 0.001$ level of significance) for comparisons of benign with malignant primary tumors and metastases (comparisons 1 and 2) indicated disproportionate numbers of differentially expressed genes related to angiogenesis or translation (TABLE 3). The comparison of malignant primary with benign tumors (4) indicated a disproportionate number of differentially expressed genes associated with signal transduction, while the comparison of malignant primary tumors with metastases (5) indicated an importance of genes related to cell proliferation, cytoskeletal function, and regulation of transcription. The comparison of VHL with MEN 2A (A) tumors showed disproportionate numbers of differentially expressed genes associated with cell motility, cytoskeletal function, extracellular matrix, and nucleotide metabolism, while the comparison of VHL with SDHB tumors (D) showed an importance of genes related to ATP binding, nucleotide metabolism, and protein transport.

DISCUSSION

The global differences in gene expression among the multiple comparisons of this study illustrate the importance of a carefully collected comprehensive clinical dataset for analysis and interpretation of microarray-generated

TABLE 3. Selected gene ontology (GO) summaries

GO term (total of genes listed in database with this GO term)	Comparison							
	1	2	4	5	6	A	B	D
Genes $P < 0.001$ (n)	636	976	383	2,836	619	887	179	1,165
Genes with GO listings (%)	41	41	38	47	52	50	56	52
Angiogenesis (56)	4*	4*		1		3	2*	4
Apoptosis/antiapoptosis (556)	8	9	8	51	9	13	1	22
ATP binding (1,731)	23	33	9	131	30	37	11	75*
Cell cycle (575)	9	9		45	14	17	3	15
Cell motility (157)	1	2	1	9	3	9*	2	7
Cell proliferation (611)	8	12	7	59*	11	22	11*	21
Cytoskeleton (665)	11	11	8	64*	14	25*	5	24
Electron transport (583)	7	12	4	46	11	14	10*	19
Extracellular matrix (295)	2	5	5	23	7	22*	4	8
Glucose metabolism (32)				1	2	3		1
Ion transport (721)	12	22	3	54	10	20	3	32
Neurotransmitter (110)	4	4	2	10	1	4		5
Nucleotide (545)	7	17	3	43	15	21*	1	27*
Lipid metabolism (201)	4	6	3	16	2	5	1	3
Protein transport (258)	2	8	3	19	4	7	2	15*
Regulation of transcription (1,962)	24	34	16	174*	29	48	10	57
Signal transduction (1,003)	16	25	13*	77	16	22	6	27
Transcription factor (789)	12	16	4	70	8	18	3	28
Translation (151)	5	13*	2	7	2	1		7

*$P < 0.05$ indicates disproportionate number of genes with a particular GO annotation by Fisher's exact test.

Data for comparison 2 are restricted to genes differentially expressed between benign and malignant tumors. Data for comparison 3 are not shown. The total number of genes in the database with any GO annotation is 17,052.

gene expression profiles in human tumor samples. Consideration of germline mutations and variations in catecholamine biochemical phenotypes and tumor locations are several factors shown here to be associated with differences in gene expression that can assist in comparisons of benign with malignant pheochromocytoma.

The major strengths of our study are the large sample set and the detailed clinical information available for most tumors. The large sample set is extraordinary given the rarity of the tumor, and even more remarkable considering the inclusion of 20 malignant tumors, an even more infrequent entity than benign tumors, which, when diagnosed, are often not resected.

The detailed clinical information available to us allowed identification of confounding variables influencing comparisons of malignant with benign tumors. Use of ANOVA to correct for the influence of tumor catecholamine phenotype in comparisons of malignant versus benign tumors (comparison 2) exemplifies how such information can be applied to enrich a database of differentially expressed genes. This comparison yielded 50% more genes than

when the major influence of catecholamine biochemical phenotype was not considered (comparison 1). Moreover, the overlap of differentially expressed genes for both comparisons indicated that differential expression of almost one out of five genes in comparison 1 most likely reflected confounding influences (e.g., catecholamine phenotype) not directly related to malignancy. Thus, the overall yield of correctly identified differentially expressed genes in comparison 2 was nearly twice that of comparison 1. The foregoing differences in yields are explained by major differences in gene expression between adrenergic and noradrenergic tumors,[10] and the predominant noradrenergic phenotype of malignant tumors and adrenergic phenotype of many benign adrenal tumors.[2,9]

Separate identification and analysis of subgroups of malignant primary tumors and distant metastases arising from these primary tumors, SDHB mutation-positive and -negative malignancies, and malignancies arising from adrenal versus extra-adrenal locations, provide other information useful for identifying differences in gene expression crucial for determining malignant potential and pathways of malignant transformation. The distinction of malignant primary tumors from subsequent metastases is not often considered in other studies of malignant pheochromocytoma, but has several important implications. The genes distinguishing benign from malignant primary tumors (comparison 4) may be particularly important for identifying prognostic markers of malignancy. In contrast, the more than sevenfold-higher number of genes differentially expressed between malignant primary tumors and metastatic lesions (comparison 5) may reflect the malignant transformation process or adaptation to a new environment to facilitate survival.

A limitation accompanying the abundance of clinical information is that the necessary subgrouping requires a large dataset of tumors for the various comparisons. Although reasonably large, the sample size was not adequate enough to assess the contributions of all considered variables to the malignant process. The relatively small numbers of malignant and benign tumors identified as arising from SDHB mutations is one example where insufficient sample size limited interpretation. Although the comparison of SDHB-positive versus SDHB-negative benign and malignant tumors yielded a large number of differentially expressed genes, additional consideration of these two variables by ANOVA indicated little difference between SDHB-dependent benign and malignant tumors (comparison 3). Whether this reflects lack of difference between the two groups of SDHB-positive tumors or limited sample size and statistical power of the comparison is unclear.

Another limitation to this and any other study of malignant and benign pheochromocytoma is that there is currently no method other than lifelong follow-up to establish that a pheochromocytoma is not malignant (i.e., truly benign). Since malignant disease can occur 17 or more years after diagnosis of the initial tumor, with patients asymptomatic during this interval,[11,12] it is realistic to assume that some tumors classified as benign in this study may

later turn out to metastasize. Such misclassification may lead to a diminished yield of differentially expressed genes, but would not be expected to result in any increase in false-positive rates of gene detection.

What are the future directions for use of the database of differentially expressed genes? This database, available on request as a Filemaker Pro file (Santa Clara, CA), provides a valuable resource from which hypotheses may be generated and tested in further studies on pheochromocytoma. Identification of subsets of genes involved in the malignant process can benefit from application of data mining and pathway analysis tools, but will require validation studies—preferably in different tumor sample sets—before hypotheses about specific pathways of malignancy, targets for treatment, and diagnostic or prognostic markers of malignancy can be tested. Development of tissue arrays for detection of panels of differentially expressed proteins is one approach that might be used in retrospective studies involving archival paraffin-embedded tissues or prospective studies required to validate hypotheses about diagnostic and prognostic markers of malignancy.

Although primarily directed at benign versus malignant pheochromocytoma, the various comparisons in the database are also useful for generating other testable hypotheses about mechanisms of tumorigenesis and genotype–phenotype relationships, including insight into the key genes determining catecholamine biochemical phenotypes. Epinephrine-producing pheochromocytomas in MEN 2A and predominantly norepinephrine-producing tumors in VHL syndrome have highly distinct gene expression profiles, suggesting development from different populations of noradrenergic and adrenergic chromaffin cells.[10] Dahia and colleagues[13] have reported similar highly distinct expression profiles falling into two broad groups of pheochromocytomas, one group including tumors in MEN 2A patients, and the other tumors due to mutations of VHL, SDHD, and SDHB genes. These findings fit with others indicating that familial pheochromocytomas develop by a single pathway linking mutations in disease-causing genes to failure of apoptosis after withdrawal of growth factors during chromaffin cell development.[14] As advanced elsewhere in this volume by Huynh *et al*.,[15] epinephrine-producing tumors in MEN 2A are hypothesized to develop due to susceptibility of more fully developed PNMT-expressing chromaffin cells to the effects of activating RET mutations, whereas norepinephrine-producing pheochromocytomas in VHL syndrome develop from neural crest progenitors before their final development into differentiated epinephrine-producing adrenal chromaffin cells.

The findings presented here that close to 90% of differentially expressed genes in benign and malignant primary pheochromocytomas are underexpressed in malignant compared to benign tumors fits with other observations of a less differentiated biochemical phenotype in malignant tumors, as characterized by lack of production of epinephrine and relatively high production of dopamine compared to norepinephrine.[16,17] This may reflect predominant development of malignant pheochromocytomas from neural crest progenitors

arrested in an early stage of development with a malignancy-prone lineage able to populate both adrenal and extra-adrenal sites of chromaffin tissue. The matching of gene expression profiles in neural crest progenitors with those in different groups of pheochromocytomas and mechanistic studies linking these progenitors to effects of specific genes provides possible approaches to test the foregoing hypotheses.

In summary, the present gene expression profiling study in a large sample set, combined with use of advanced statistical approaches to analyze influences of multiple variables, provides a valuable database of differentially expressed genes to help address outstanding questions about genotype–phenotype relationships, tumorigenesis, and malignant potential and transformation pathways in pheochromocytoma.

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