# Identification of Potential Gene Markers and Insights into the Pathophysiology of Pheochromocytoma Malignancy

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**Context:** Pheochromocytomas are catecholamine-producing tumors that are generally benign but that can also present as or develop into malignancy. Occurrence of malignant pheochromocytomas can only be asserted by imaging of metastatic lesions.

**Objectives:** We conducted a gene expression profiling of benign and malignant tumors to identify a gene signature that would allow us to discriminate benign from malignant pheochromocytomas and to gain a better understanding of tumorigenic pathways associated with malignancy.

**Design:** A total of 36 patients with pheochromocytoma was studied retrospectively. There were 18 (nine benign and nine malignant) tumors used for gene expression profiling on pangenomic oligonucleotide microarrays.

**Results:** We identified and validated a set of predictor genes that could accurately distinguish the two tumor subtypes through unsupervised clustering. Most of the differentially expressed genes were down-regulated in malignant tumors, and several of these genes encoded neuroendocrine factors involved in prominent characteristics of chromaffin cell biology. In particular, the expression of two key processing enzymes of trophic peptides, peptidylglycine $\alpha$-amidating monooxygenase and glutaminyl-peptide cyclotransferase, was reduced in malignant pheochromocytomas.

**Conclusion:** The gene expression profiling of benign and malignant pheochromocytomas clearly identified a set of genes that could be used as a prognostic multi-marker and revealed that the expression of several genes encoding neuroendocrine proteins was reduced in malignant compared with benign tumors.

PHEOCHROMOCYTOMAS ARE CATECHOLAMINE-producing tumors that occur from chromaffin cells of adrenal medulla or extra-adrenal locations, leading to paroxysmal or persistent hypertension in most patients. They are mainly sporadic tumors but familial forms resulting from mutations of the oncogene RET or the oncosuppressors von Hippel-Lindau (VHL), neurofibromatosis 1 (NF1), and succinate dehydrogenase subunits are increasingly recognized (1–4). Familial pheochromocytomas represent approximately 25% of cases, and are observed as part of multiple endocrine neoplasia type 2, VHL and NF1 syndromes, and as paraganglioma tumors (5). Measurement of the concentrations of plasma free metanephrines or urinary fractionated metanephrines represents the test of choice for the diagnosis of pheochromocytomas (6).

Pheochromocytoma generally occurs as a benign tumor, but 10–25% of cases are malignant at the first surgery or at recurrence, with metastasis development at lymph node, bone, liver, or lung (7). Unlike benign tumors that can be diagnosed and surgically treated, there is currently no method to identify, predict, or cure malignant pheochromocytomas. Malignancy of pheochromocytoma can be diagnosed only after metastasis appearance. Therefore, malignancy development and the underlying molecular pathways remain poorly understood.

Here, we used pangenomic human oligonucleotide arrays to profile the gene expression of benign and malignant pheochromocytomas, to identify a set of genes that could distinguish the two types of tumors on the one hand, and to gain insights into the mechanisms underlying the occurrence of malignancy on the other hand.

## Materials and Methods

### *Tumor samples*

Tumor specimens were collected during surgery (≤15 min after the operation) from patients with pheochromocytoma and immediately frozen in liquid nitrogen. Nine benign and nine malignant tumors, classified based on the absence or presence of metastatic lesions, were used for microarray analysis. The average duration of the follow-up of the patients with benign tumors was 41 ± 28 months (range 1–84). Tumors used for microarray analysis were collected in two Hospital Centers of the COritco and MEdullo-surrénales Tumeurs Endocrines (COMETE) network (COMETE-2 network, PHRC AOM 02068), according to standardized guidelines of tumor collection established by contributing teams of the network. Of these tumors, 14 (nine benign and five malignant) were without evidence of a hereditary disease, *i.e.* sporadic tumors, whereas three malignant tumors had an succinate dehydrogenase B mutation, and one malignant tumor had a VHL mutation. Pheochromocytoma genetic testing has been performed as previously described (1). Among the benign tumors, seven were located in the adrenal, and two were at an extra-adrenal site. Among the malignant tumors, five were located in the adrenal, three at an extra-adrenal site, and one was located at both the adrenal and an extra-adrenal site. Malignant tumors used in microarray analysis were from the primary tumoral site. An additional five malignant (three familial, one sporadic, and one not genotyped) and 13 benign (three multiple endocrine neoplasia type 2, two NF1, one sporadic, and seven not genotyped) tumors were used for quantitative PCR analysis. These tumor samples were provided by the Rouen (Hôpital C. Nicolle, Rouen, France), the Nancy (Hôpital de Brabois, Nancy, France), and the Lausanne (Hôpital Vaudois, Lausanne, Switzerland) University Hospital Centers.

### *RNA extraction*

Total RNA was extracted using the Tri-Reagent (Sigma-Aldrich, Saint Quentin Fallavier, France), further purified on RNeasy mini Spin Columns (QIAGEN, Courtaboeuf, France), and analyzed on a denaturing agarose gel.

### *Microarray processing and data analysis*

The oligo microarray chips were generated from 34,580 longmer probes set obtained from the QIAGEN Human Genome Oligo Set version 3.0 (QIAGEN, Valencia, CA). The set corresponds to approximately 25,400 Unigene nonredundant human genes and covers 85% of the human genome. The gene description and annotation of these oligonucleotides were based on the Ensembl database (Cambridge, UK) dated from December 2006. The details of the whole protocol can be accessed through the web site: http://research.nhgri.nih.gov/nhgri_cores/microarray.html. Briefly, fluorescence-labeled cDNA from each tumoral sample was hybridized to a microarray slide concomitantly with fluorescence-labeled reference cDNA prepared from normal human adrenal medulla (BD Biosciences Clontech, Palo Alto, CA). Each tumoral RNA was used in two independent labeling and hybridization experiments. Microarrays were subsequently scanned with a laser confocal scanner (Agilent Technologies, Palo Alto, CA), and the fluorescence intensities were measured in the spots and their surrounding areas. The values were filtered through quality control variables, analyzed, and quantified using the DEARRAY IPLab image processing package (Scanalytics, Fairfax, VA). A dye bias analysis was performed on the reference and six tumoral samples when sufficient RNA was available. All the protocols are Minimum Information About a Microarray Experiment compliant.

The data were subjected to a set of filtering criteria, statistical analysis, and gene selection based on a difference in $P$ value as described elsewhere (3). First, the average spot quality score (which ranges from zero, being worst, to one, best) over all samples in the study was required to be at least 0.5 (8). Second, the normalized ratio to reference RNA was required to be above two or less than 0.5 in three or more experiments. The averaged duplicate ratios were log transformed, and a T-statistic/F-statistic algorithm was applied. The resulting data were analyzed by generation of a weighted list of genes followed by 10,000 random permutation analysis, as described elsewhere (9). The tools and statistical methods used for this particular analysis are available at http://arrayanalysis.nih.gov/.

### *Real-time PCR*

PCR amplification was performed using the SYBR Green I Master Mix Buffer (Applied Biosystems, Courtaboeuf, France) in an ABI PRISM 7000 Sequence Detector (Applied Biosystems). PCR results were analyzed using the qBase program (10).

### *Statistical analysis*

Statistical analyses were performed using the nonparametric Mann-Whitney $U$ test. Data were analyzed using the Prism program (GraphPad Software, Inc., San Diego, CA).

## Results

### *Gene expression profiling of benign and malignant pheochromocytomas*

An unsupervised hierarchical clustering of hybridization data yielded a four-branch dendrogram: two branches for malignant tumors and two branches for the benign tumors. There was only one benign tumor (sample no. 6) that clustered with malignant ones (Fig. 1A). Interestingly, the four hereditary cases included in the present analysis exhibited a significant aggregation among the malignant tumors. We performed a T-statistic/F-statistic discriminative gene selection followed by a supervised clustering method to identify the genes that best differentiate between the malignant and benign tumors. Analysis of hybridization data revealed about 100 genes (Fig. 1B) showing a statistically significant differential expression between benign and malignant pheochromocytomas ($P < 0.001$, Student's $t$ test). The differentially expressed genes were functionally categorized on the basis of known or inferred biological function of their protein product using gene ontology. Table 1 summarizes the functional clustering of the identified genes. Among these, about a third had unknown function, and, more importantly, only 16 were up-regulated in malignant pheochromocytomas.

### *Validation of microarray analysis*

To confirm the results obtained by microarray analysis, we used real-time PCR to determine the mRNA levels of glutaminyl-peptide cyclotransferase (QPCT), peptidylglycine $\alpha$-amidating monooxygenase (PAM), neuropeptide Y (NPY), and calcium/calmodulin-dependent protein kinase II (CAMKIIN$\alpha$) in a collection of tumors comprising the samples used for microarray analysis, and an additional three malignant and 11 benign pheochromocytomas with different characteristics. Differential expression between malignant and benign pheochromocytomas was confirmed for the four genes: $P = 0.0078$ for QPCT; $P = 0.0245$ for PAM; $P = 0.0292$ for NPY; and $P = 0.0302$ for CAMKIIN$\alpha$ (Fig. 2).

## Discussion

Among the main clinical challenges presented by pheochromocytomas, malignancy remains the most problematic

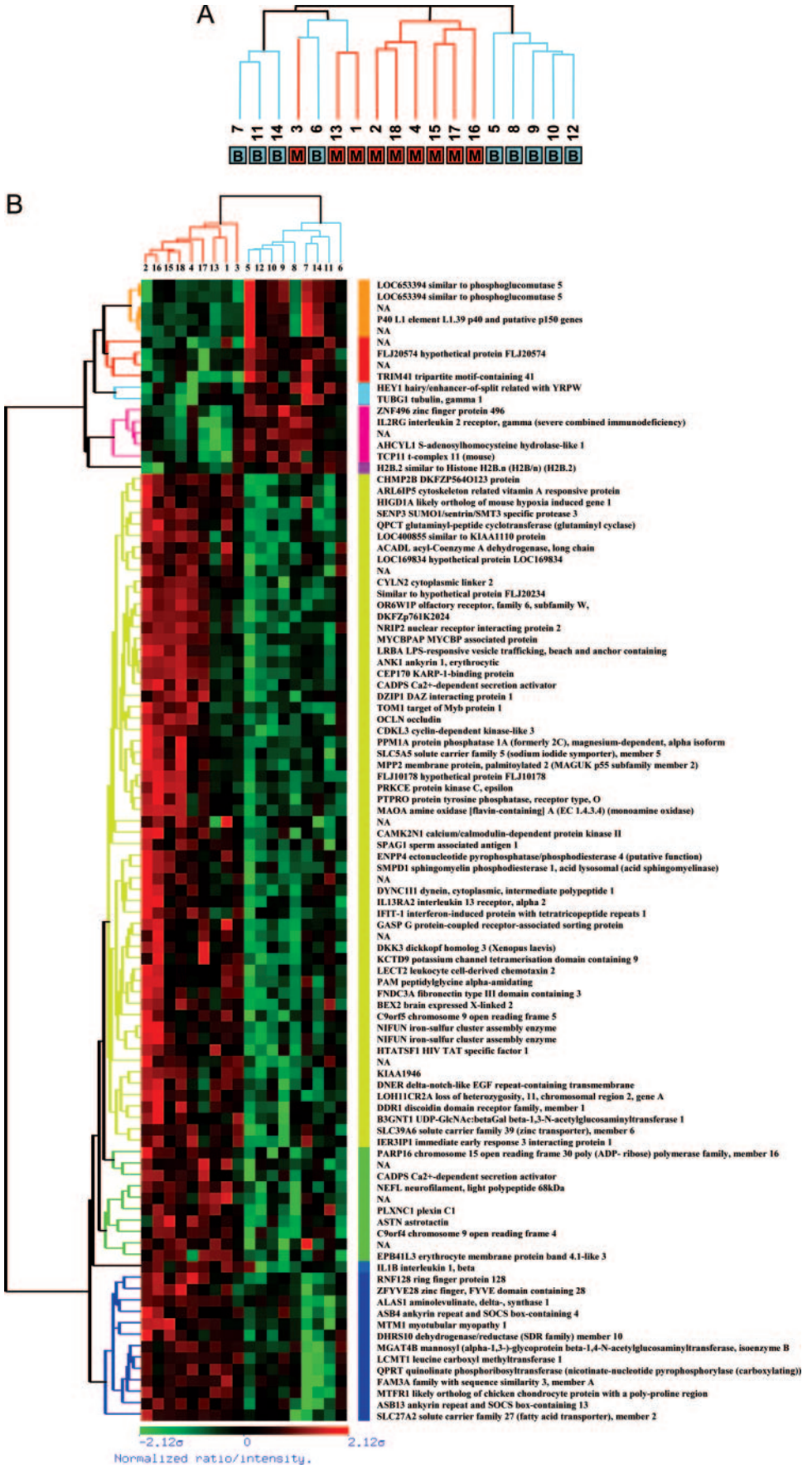


FIG. 1. A, Unsupervised hierarchical clustering of benign (B) and malignant (M) pheochromocytoma samples based on gene expression profiling. B, Supervised hierarchical clustering of pheochromocytomas (*columns*) and genes identified by microarray analysis (*rows*) on the basis of their expression profile. Dendrograms of tumoral samples (*above the matrix*) and genes (*at the left of the matrix*) represent similarities in gene expression profiles. Genes with a relatively higher level of expression in benign compared with malignant tumors are shown in *green*, and those with a lower level are shown in *red* according to the color scale *at the bottom*. Only genes exhibiting a differential expression with $P < 0.001$ were included in this analysis. The name of each gene is indicated *at the right of the matrix* [not applicable (NA) indicates expressed sequence tags (ESTs) with no functional annotation]. The number of each tumor is indicated *above the matrix*. Malignant tumors correspond to the *first nine columns*, and benign ones correspond to the *last nine columns* as grouped by the clustering method described in *Materials and Methods*.

**TABLE 1.** Ratios of gene expression in benign *vs.* malignant pheochromocytomas for differentially expressed genes

| Gene name | Symbol | Ratio | Accession no. | Unigene |
|---|---|---|---|---|
| Cell adhesion | | | | |
| Occludin | OCLN | 2.13 | NM_002538 | Hs.519367 |
| Discoidin domain receptor family, member 1 | DDR1 | 1.75 | NM_013994 | Hs.423573 |
| Astrotactin | ASTN | 1.72 | NM_004319 | Hs.6788 |
| Plexin C1 | PLXNC1 | 1.63 | NM_005761 | Hs.286229 |
| Cytoskeleton | | | | |
| KARP-1-binding protein | CEP170 | 2.44 | NM_014812 | Hs.25132 |
| Cytoplasmic linker 2 | CYLN2 | 2.38 | NM_032421 | Hs.104717 |
| Cytoskeleton related vitamin A responsive protein | ARL6IP5 | 2.17 | NM_006407 | Hs.92384 |
| Ankyrin 1, erythrocytic | ANK1 | 1.96 | NM_020477 | Hs.443711 |
| Neurofilament, light polypeptide 68 kDa | NEFL | 1.89 | NM_006158 | Hs.107600 |
| Dynein, cytoplasmic, intermediate polypeptide 1 | DYNC1I1 | 1.75 | NM_004411 | Hs.65248 |
| Erythrocyte membrane protein band 4.1-like 3 | EPB41L3 | 1.67 | NM_012307 | Hs.103839 |
| **Tubulin, $\gamma$ 1** | **TUBG1** | **0.58** | **NM_001070** | **Hs.21635** |
| **Centromere protein J** | **CENPJ** | **0.44** | **NM_018451** | **Hs.513379** |
| Metabolism | | | | |
| Tyrosine hydroxylase | TH | 4.44 | NM_000360 | Hs.435609 |
| Iron-sulfur cluster assembly enzyme | NIFUN | 1.96 | NM_014301 | Hs.350702 |
| Aminolevulinate, $\delta$-, synthase 1 | ALAS1 | 1.92 | NM_000688 | Hs.511918 |
| Solute carrier family 27 (fatty acid transporter), member 2 | SLC27A2 | 1.72 | NM_003645 | Hs.11729 |
| Solute carrier family 5 (sodium iodide symporter), member 5 | SLC5A5 | 1.72 | NM_000453 | NA |
| Amine oxidase (flavin-containing) A (EC 1.4.3.4) (monoamine oxydase) | MAOA | 1.72 | NM_000240 | NA |
| UDP-GlcNAc:betaGal $\beta$-1,3-N-acetylglucosaminyltransferase 1 | B3GNT1 | 1.69 | NM_006577 | Hs.173203 |
| Acyl-coenzyme A dehydrogenase, long chain | ACADL | 1.69 | NM_001608 | Hs.430108 |
| Quinolinate phosphoribosyltransferase (nicotinate-nucleotide pyrophosphorylase (carboxylating) | QPRT | 1.69 | NM_014298 | Hs.335116 |
| Fumarylacetoacetate hydrolase (fumarylacetoacetase) | FAH | 1.67 | NM_000137 | Hs.73875 |
| Chromosome 9 open reading frame 4 | C9orf4 | 1.67 | XM_378078 | Hs.347537 |
| Dehydrogenase/reductase (SDR family) member 10 | DHRS10 | 1.64 | NM_016246 | Hs.18788 |
| Solute carrier family 39 (zinc transporter), member 6 | SLC39A6 | 1.64 | NM_012319 | Hs.79136 |
| Major vault protein | MVP | 1.51 | NM_005115 | Hs.80680 |
| Solute carrier family 26 (sulfate transporter), member 2 | SLC26A2 | 1.51 | NM_000112 | NA |
| Chromosome condensation-related SMC-associated protein 1 | CNAP1 | 1.43 | NM_014865 | Hs.5719 |
| Mannosyl ($\alpha$-1,3-)-glycoprotein $\beta$-1,4-N-acetylglucosaminyltransferase, isoenzyme B | MGAT4B | 1.39 | NM_054013 | Hs.437277 |
| Glutathione synthetase | GSS | 1.35 | NM_000178 | Hs.82327 |
| **Aldehyde dehydrogenase 6A1** | **ALDH6A1** | **0.60** | **NA** | **Hs.293970** |
| **Similar to phosphoglucomutase 5** | **LOC653394** | **0.52** | **XM_372112** | **NA** |
| Protein processing | | | | |
| Glutaminyl-peptide cyclotransferase (glutaminyl cyclase) | QPCT | 2.22 | NM_012413 | Hs.79033 |
| SUMO1/sentrin/SMT3 specific protease 3 | SENP3 | 2.17 | NM_015670 | Hs.255022 |
| Peptidylglycine $\alpha$-amidating monooxygenase | PAM | 2.08 | NM_000919 | Hs.352733 |
| Ring finger protein 128 | RNF128 | 1.96 | NM_194463 | Hs.9238 |
| Serine (or cysteine) proteinase inhibitor, clade I (neuroserpin), member 1 | SERPINI1 | 1.79 | NM_005025 | Hs.78589 |
| Ring finger protein ($C^3H2C3$ type) 6 | RNF6 | 1.79 | NM_183044 | Hs.136885 |
| Heat shock protein (hsp110 family) | HSPA4liter | 1.69 | NM_014278 | Hs.135554 |
| Ceroid-lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease) | CLN3 | 1.49 | NM_000086 | Hs.446747 |
| Leucine carboxyl methyltransferase 1 | LCMT1 | 1.37 | NM_016309 | Hs.411135 |
| **Tripartite motif-containing 41** | **TRIM41** | **0.68** | **NM_201627** | **Hs.519822** |
| Secretion | | | | |
| Secretogranin II | SCG2 | 3.13 | NM_003469 | Hs.516726 |
| LPS-responsive vesicle trafficking, beach and anchor containing | LRBA | 2.77 | NM_006726 | Hs.209846 |
| $Ca^{2+}$-dependent secretion activator | CADPS | 1.92 | NM_003716 | Hs.441050 |

**TABLE 1.** *Continued*

| Gene name | Symbol | Ratio | Accession no. | Unigene |
|---|---|---|---|---|
| G protein-coupled receptor-associated sorting protein | GASP | 1.92 | XM_377032 | Hs.113082 |
| Synaptotagmin-like 3 | SYTL3 | 1.75 | XM_087804 | Hs.436977 |
| Synaptosomal-associated protein 25 | SNAP25 | 1.60 | NM_130811 | Hs.167317 |
| Pleckstrin and Sec7 domain containing 3 | PSD3 | 1.42 | NM_015310 | Hs.521426 |
| Mal, T-cell differentiation protein 2 | MAL2 | 1.35 | NM_052886 | Hs.76550 |
| Signaling | | | | |
| Protein tyrosine phosphatase, receptor type, O | PTPRO | 2.77 | NM_030671 | Hs.160871 |
| Dickkopf homolog 3 (Xenopus laevis) | DKK3 | 2.77 | NM_013253 | Hs.130865 |
| IL13 receptor, $\alpha$ 2 | IL13RA2 | 2.63 | NM_000640 | Hs.336046 |
| Protein phosphatase 1A (formerly 2C), magnesium-dependent, $\alpha$ isoform | PPM1A | 2.56 | NM_177951 | Hs.130036 |
| Neuropeptide Y | NPY | 2.38 | NM_000905 | Hs.1832 |
| IL1, $\beta$ | IL1B | 2.08 | NM_000576 | Hs.126256 |
| Leukocyte cell-derived chemotaxin 2 | LECT2 | 2.04 | NM_002302 | Hs.512580 |
| Membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2) | MPP2 | 2.27 | NM_005374 | Hs.436326 |
| Calcium/calmodulin-dependent protein kinase II | CAMK2N1 | 1.92 | NM_018584 | Hs.197922 |
| Sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase) | SMPD1 | 1.75 | NM_000543 | Hs.77813 |
| Protein kinase C, $\epsilon$ | PRKCE | 1.75 | NM_005400 | Hs.155281 |
| Target of Myb protein 1 | TOM1 | 1.72 | O60784 | Hs.474705 |
| DAZ interacting protein 1 | DZIP1 | 1.69 | NA | Hs.60177 |
| Ankyrin repeat and SOCS box-containing 4 (ASB4), transcript variant 2 | ASB4 | 1.67 | NM_145872 | Hs.413226 |
| Period homolog 2 (Drosophila) | PER2 | 1.59 | NM_022817 | Hs.410692 |
| HIV TAT specific factor 1 | HTATSF1 | 1.52 | NM_014500 | Hs.204475 |
| Low-density lipoprotein receptor (familial hypercholesterolemia) | LDLR | 1.49 | NM_000527 | Hs.213289 |
| Ankyrin repeat and SOCS box-containing 13 | ASB13 | 1.47 | NM_024701 | Hs.300063 |
| **IL2 receptor, $\gamma$ (severe combined immunodeficiency)** | **IL2RG** | **0.63** | **NM_000206** | **Hs.84** |
| **Hairy/enhancer-of-split related with YRPW motif 1** | **HEY1** | **0.59** | **NM_012258** | **Hs.234434** |
| **Immediate early response 3** | **IER3** | **0.59** | **NA** | **Hs.591785** |
| **Colony stimulating factor 2 (granulocyte-macrophage)** | **CSF2** | **0.56** | **NM_000758** | **Hs.1349** |
| **Zinc finger protein 496** | **ZNF496** | **0.51** | **NA** | **Hs.22051** |
| Unknown | | | | |
| MYCBP associated protein | MYCBPAP | 3.33 | NM_032133 | Hs.398196 |
| Hypothetical protein FLJ10178 | FLJ10178 | 2.94 | NA | Hs.274267 |
| $\delta$-Notch-like EGF repeat-containing transmembrane | DNER | 2.44 | NM_139072 | Hs.234074 |
| KIAA1946 | KIAA1946 | 2.17 | NP_803237 | Hs.172792 |
| Similar to hypothetical protein FLJ20234 | NA | 2.13 | BC008091 | NA |
| Chromosome 14 open reading frame 1 | C14orf1 | 2.13 | NM_007176 | Hs.15106 |
| TPR domain containing STI2 | STI2 | 2.08 | NA | Hs.376300 |
| Similar to KIAA1110 protein | LOC400855 | 2.04 | XM_375928 | NA |
| DKFZP564O123 protein | CHMP2B | 1.92 | NM_014043 | Hs.11449 |
| Fibronectin type III domain containing 3 | FNDC3A | 1.92 | NM_014923 | Hs.103329 |
| DKFZp761K2024 protein | NA | 1.89 | AL161983 | Hs.21415 |
| Ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative function) | ENPP4 | 1.89 | XM_376503 | Hs.54037 |
| Brain expressed X-linked 2 | BEX2 | 1.85 | NM_032621 | Hs.398989 |
| Interferon-induced protein with tetratricopeptide repeats 1 | IFIT-1 | 1.85 | NA | Hs.20315 |
| DKFZP434F2021 protein | C3orf17 | 1.85 | NM_015412 | Hs.377974 |
| Nuclear receptor interacting protein 2 | NRIP2 | 1.82 | NM_031474 | NA |
| Loss of heterozygosity, 11, chromosomal region 2, gene A | LOH11CR2A | 1.82 | NM_014622 | Hs.152944 |
| Cyclin-dependent kinase-like 3 | CDKL3 | 1.79 | NM_016508 | Hs.105818 |
| Likely ortholog of mouse hypoxia induced gene 1 | HIGD1A | 1.75 | NM_014056 | Hs.7917 |
| Olfactory receptor, family 6, subfamily W, member 1 pseudogene | OR6W1P | 1.75 | NR_002140 | NA |
| Zinc finger, FYVE domain containing 28 | ZFYVE28 | 1.72 | NA | Hs.292056 |
| Potassium channel tetramerization domain containing 9 | KCTD9 | 1.72 | AA_H68518 | Hs.72071 |
| Myotubular myopathy 1 | MTM1 | 1.69 | NM_000252 | Hs.386205 |
| RNA polymerase II associated protein 1 | RPAP1 | 1.66 | NA | Hs.4849 |

**TABLE 1.** *Continued*

| Gene name | Symbol | Ratio | Accession no. | Unigene |
|---|---|---|---|---|
| Chromosome 9 open reading frame 5 | C9orf5 | 1.64 | NM_032012 | Hs.418097 |
| Hypothetical protein LOC169834 | LOC169834 | 1.64 | XM_095965 | NA |
| Immediate early response 3 interacting protein 1 | IER3IP1 | 1.64 | NM_016097 | Hs.406542 |
| Chromosome 15 open reading frame 30 poly (ADP-ribose) polymerase family, member 16 | PARP16 | 1.61 | NM_017851 | Hs.30634 |
| Sperm-associated antigen 1 | SPAG1 | 1.59 | NM_172218 | Hs.408747 |
| RUN domain containing 1 | RUNDC1 | 1.56 | NM_173079 | Hs.218182 |
| Hypothetical protein FLJ32954 | FAM82A | 1.56 | NA | Hs.9905 |
| Likely ortholog of chicken chondrocyte protein with a poly-proline region | MTFR1 | 1.56 | NM_014637 | Hs.170198 |
| Family with sequence similarity 3, member A | FAM3A | 1.47 | NM_021806 | Hs.289108 |
| **KIAA0476** | **KIAA0476** | **0.69** | **XM_375806** | **Hs.6684** |
| **S-adenosylhomocysteine hydrolase-like 1** | **AHCYL1** | **0.61** | **NM_006621** | **Hs.485365** |
| **L1 element L1.39 p40 and putative p150 genes** | **NA** | **0.61** | **U93574** | **NA** |
| **Similar to histone H2B.n (H2B/n) (H2B.2)** | **H2B.2** | **0.58** | **XM_373001** | **NA** |
| **T-complex 11 (mouse)** | **TCP11** | **0.54** | **NM_018679** | **Hs.435371** |
| **Hypothetical protein FLJ20574** | **FLJ20574** | **0.54** | **NA** | **Hs.123427** |

The functional clustering, the name, symbol, ratio of their expression in benign and malignant tumors, GenBank accession no., and Unigene cluster are indicated for each gene. Genes overexpressed in malignant tumors are indicated in *bold* with a ratio lower than one. The ratios were calculated from values of gene expression in benign and malignant tumors reported to the reference values measured in normal adrenal medulla. Only gene expression differences exhibiting a statistical significance at $P < 0.001$ (Student's $t$ test) are presented. DAZ, Deleted in azoospermia; EGF, epidermal growth factor; FAM, family with sequence similarity; FYVE, Fab1-YOTB/ZK632.12-Vac1-EEA1; KARP-1, Ku86 autoantigen related protein-1; LPS, lipopolysaccharide; MAGUK, membrane-associated guanylate kinases; MYCBP, c-myc binding protein; NA, not applicable; RUN, RPIP8-UNC-14-NESCA; SMC, structural maintenance of chromosomes; SMT, S-adenosyl-methionine-sterol-C-methyltransferase; SOCS, suppressor of cytokine signalling; SUMO, small ubiquitin-related modifier; TAT, transactivating regulatory protein; TPR, tetratricopeptide repeat.

aspect of this disease because of a lack of a reliable diagnostic tools or an effective therapeutic treatment. Gene expression profiling appeared as an attractive approach to gain insights into malignancy of this disease. Therefore, several studies have been initiated to compare the transcriptomes of benign and malignant pheochromocytomas using different tumor collections (11, 12). Analysis of our series of tumors using pangenomic microarrays revealed that the differential expression of a set of approximately 100 genes may distinguish the two tumor types.

Several observations indicate the validity of the microarray results obtained. First, differential expression was confirmed by quantitative PCR for a selection of genes. Second, unsupervised clustering clearly distinguished the two types of tumors based on differential gene expression and aggregated the malignant hereditary cases. Third, although we included mainly sporadic tumors in our initial analysis, the genes selected for validation were found to be differentially expressed in a larger sample collection (31 tumors), further adding a measure of confidence in the data presented here.

One of the most important findings of the present study is that the expression of the 100-gene set could discriminate benign and malignant pheochromocytomas, as revealed by unsupervised clustering of microarray data, while blinded to clinical information. This result indicates that gene expression profiling of pheochromocytoma may represent a potentially useful test to evaluate the prognosis of tumors at the primary site and before metastasis occurrence, and to guide preventive treatment in the case of malignant neoplasms. The case of patient no. 6 is very interesting because this benign tumor was classified by unsupervised clustering among malignant, but close to benign, tumors. This finding suggests that this tumor may have a potential malignant genotype/phenotype and, therefore, that microarray analysis may allow a more accurate classification of pheochromocytoma subtypes. Unsupervised clustering also revealed two groups of benign tumors that segregated separately. Because only sporadic benign tumors have been included in the present study, this segregation is not due to any known hereditary disease. The reason for this separate clustering of benign tumors remains to be identified.

A high number (>80%) of differentially expressed genes was underexpressed in malignant pheochromocytomas. Among these, several encode neuroendocrine factors involved in prominent characteristics of chromaffin cell biology such as catecholamine metabolism, *e.g.* fumarylacetoacetate hydrolase and monoamine oxidase, hormone secretion, like synaptotagmin-like 3 and secretogranin II, and peptide processing, such as QPCT and PAM. The synthesis and release of biologically active peptides are some of the most important features of the neuroendocrine phenotype, and adrenochromaffin cells are known to produce a wide range of such peptides (13). The present study revealed lower expression of three key genes encoding peptide processing and activation factors, *i.e.* PAM, QPCT and neuroserpin, in malignant pheochromocytomas. The instrumental role of PAM in the amidation and, therefore, the activity of peptides regulating adrenal medulla and pheochromocytoma cells, such as NPY, is well known (14). Interestingly, increased expression of PAM and target peptides has been linked to neuroendocrine differentiation in human prostate cancer (15). The expression of the enzyme responsible for cyclization of N-terminal glutaminyl residues in peptides, QPCT, is also down-regulated in malignant tumors, further indicating that malignant transformation of pheochromocytoma is associated with reduced bioactive peptide processing compared

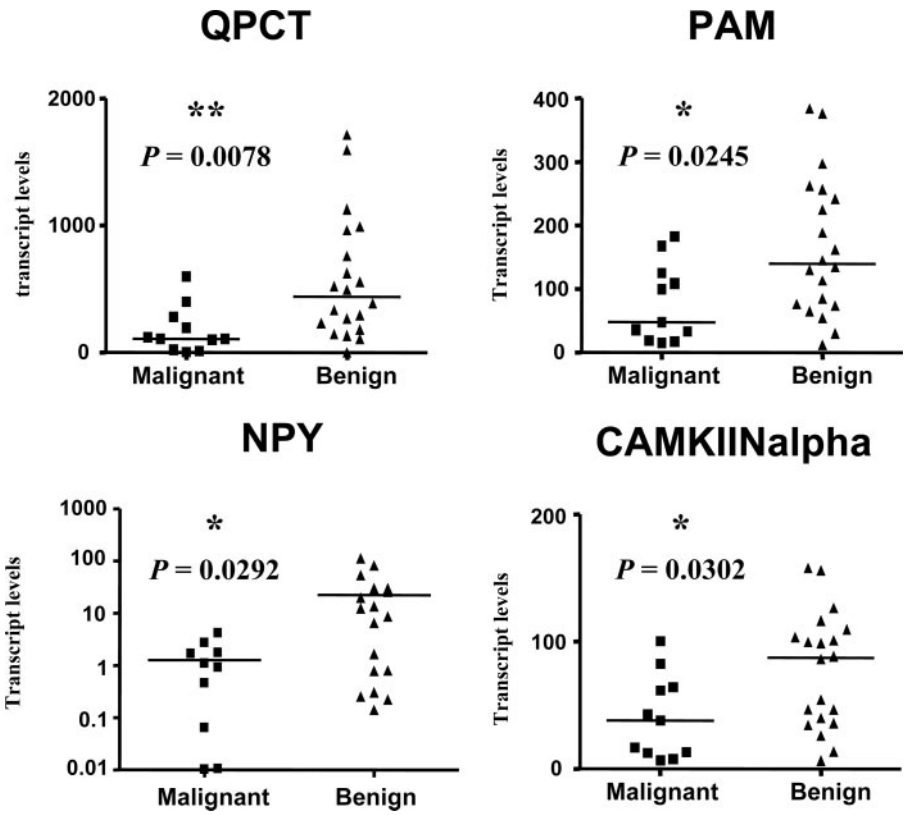


FIG. 2. Comparative quantitation of QPCT, PAM, NPY, and CAMKIIN$\alpha$ gene expression in malignant *vs*. benign pheochromocytomas. A statistical analysis was performed on 11 malignant and 20 benign pheochromocytomas. Results show differential expression of all genes in malignant *vs*. benign tumors. mRNA levels were determined by quantitative real-time PCR as described in *Materials and Methods*.

with benign tumors. Finally, the transcripts of neuroserpin, a serine-protease inhibitor whose expression is associated with neuroendocrine differentiation (16), were also less abundant in malignant pheochromocytomas.

Besides, the expression of characteristic cytoskeleton genes is altered in malignant compared with benign pheochromocytomas. For instance, the $\gamma$-tubulin gene, which encodes a constituent of centrosomes overexpressed in different cancers (17), was more highly expressed in malignant pheochromocytomas. Conversely, the expression of astrotactin and plexin C1, which are involved in cell adhesion (18, 19), was down-regulated in malignant tumors. Finally, the gene expression of occludin, a major component of tight junctions (20), was also repressed in malignant tumors, suggesting a possible diminution of cell-to-cell contacts and an increased permeability in malignant pheochromocytomas.

In conclusion, we have made use of a pangenomic microarray to identify a gene set that may represent a valuable prognostic classifier of pheochromocytomas. This work provides leads for new diagnostic and prognostic markers, and potential therapeutic strategies. The findings have also provided insights into the biology of pheochromocytomas, showing that numerous genes encoding neuroendocrine proteins were less expressed in malignant compared with benign tumors.

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