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

Erwan Thouënnon, Abdel G. Elkahloun, Johann Guillemot, Anne-Paule Gimenez-Roqueplo, Jérôme Bertherat, Alice Pierre, Hafida Ghzili, Luca Grumolato, Mihaela Muresan, Marc Klein, Hervé Lefebvre, L'Houcine Ouafik, Hubert Vaudry, Pierre-François Plouin, Laurent Yon, and Youssef Anouar

Institut National de la Santé et de la Recherche Médicale U413 (E.T., J.G., A.P., H.G., L.G., H.L., H.V., L.Y., Y.A.), Laboratory of Cellular and Molecular Neuroendocrinology, European Institute for Peptide Research (Institut Fédératifde Recherche Multidisciplinaires sur les Peptides 23), University of Rouen, 76821 Mont-Saint-Aignan, France; Genome Technology Branch (A.G.E.), National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892; Department of Genetics (A.-P.G.-R.), Hôpital Européen Georges Pompidou, 75015 Paris, France; Department of Endocrinology (J.B.), Institut National de la Santé et de la Recherche Médicale U567, Institut Cochin, 75014 Paris, France; Department of Endocrinology (M.M., M.K.), Hôpital de Brabois, 54511 Nancy, France; Equipe Mixte Institut National de la Santé et de la Recherche Médicale EMI 0359 (L.O.), Laboratory of Experimental Cancerology, Université de la Mediterranée, Aix-Marseille II, 13015 Marseille, France; and Hypertension Unit (P.-F.P.), Hôpital Européen Georges Pompidou, AP-HP, University of Paris-5, 75015 Paris, France

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 α-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 α-amidating monooxygenase (PAM), neuropeptide Y (NPY), and calcium/calmodulin-dependent protein kinase II (CAMKIINα) 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 CAMKIINα (Fig. 2).

Discussion

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

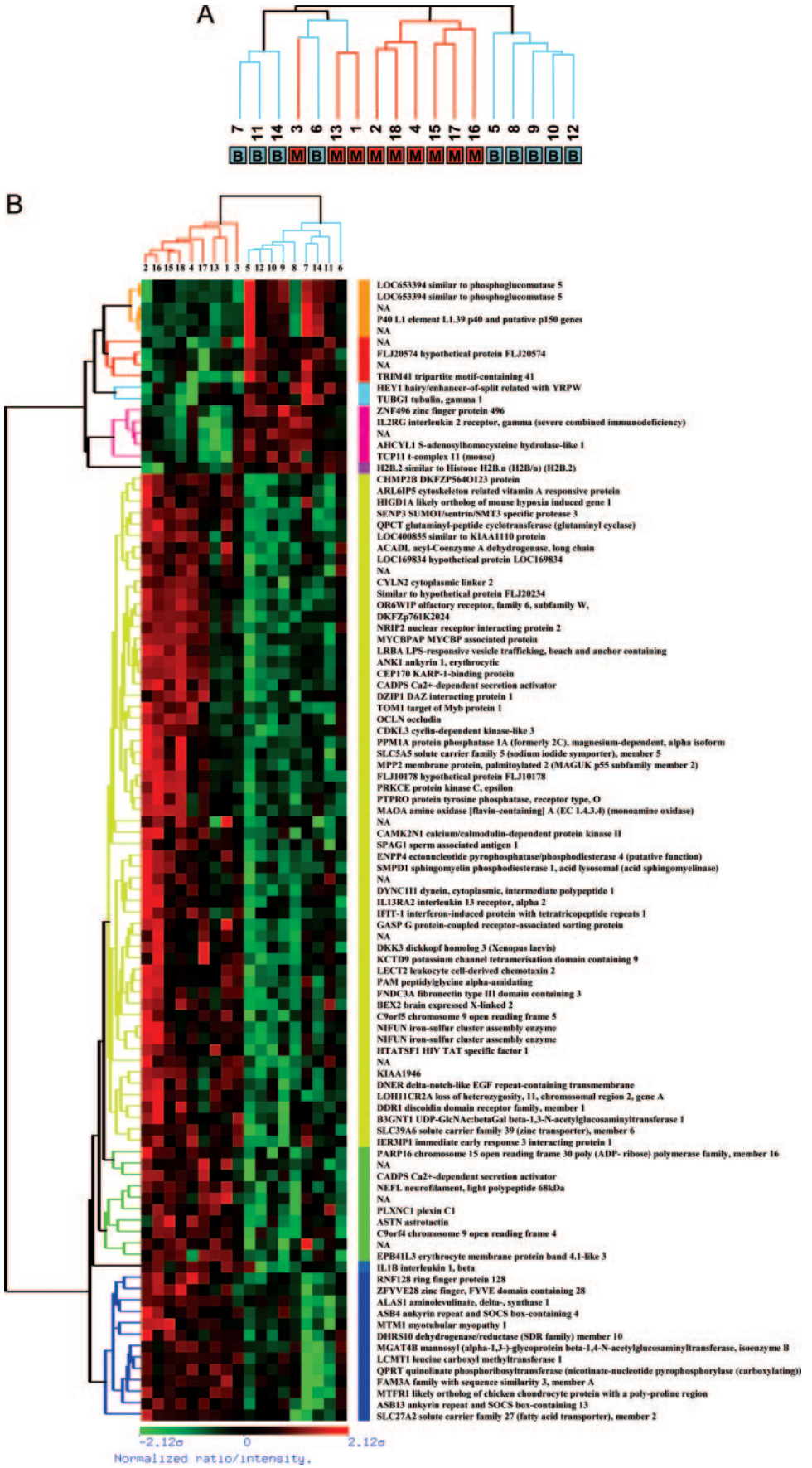


FIG. 1. A, Unsupervised hierarchical clustering of benign (B) and malignant (M) pheochromocytoma samples based on gene expression profiling. B, Supervised hierarchical clustering of pheochromocytomas (*columns*) and genes identified by microarray analysis (*rows*) on the basis of their expression profile. 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, γ 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, δ-, 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 β-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 (α-1,3-)-glycoprotein β-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 α-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, α 2	IL13RA2	2.63	NM_000640	Hs.336046
Protein phosphatase 1A (formerly 2C), magnesium-dependent, α isoform	PPM1A	2.56	NM_177951	Hs.130036
Neuropeptide Y	NPY	2.38	NM_000905	Hs.1832
IL1, β	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, ϵ	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, γ (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
δ-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

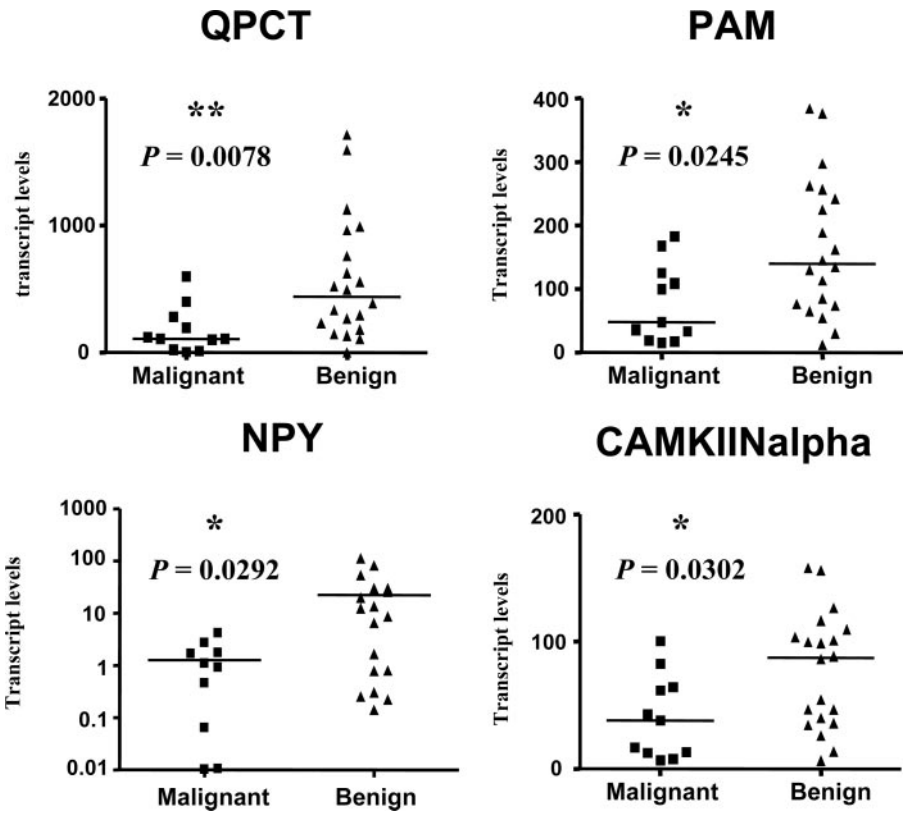


FIG. 2. Comparative quantitation of QPCT, PAM, NPY, and CAMKIINα gene expression in malignant *vs*. benign pheochromocytomas. A statistical analysis was performed on 11 malignant and 20 benign pheochromocytomas. Results show differential expression of all genes in malignant *vs*. benign tumors. mRNA 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 γ-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.

Acknowledgments

Received June 5, 2007. Accepted September 10, 2007.

Address all correspondence and requests for reprints to: Dr. Y. Anouar, Institut National de la Santé et de la Recherche Médicale U413, Laboratory of Cellular and Molecular Neuroendocrinology, Institut Fédératif de Recherche Multidisciplinaires sur les Peptides 23, University of Rouen, 76821 Mont-Saint-Aignan, France. E-mail: youssef.anouar@univ-rouen.fr.

This work was supported by Institut National de la Santé et de la Recherche Médicale (U413), Conseil Régional de Haute-Normandie, Fédération des Maladies Orphelines, Ligue de Recherche Contre le Cancer de Haute-Normandie, Association pour la Recherche sur le Cancer, and the grant PHRC AOM 02068 from Institut National de la Santé et de la Recherche Médicale and Ministère Délégué à la Recherche et aux Nouvelles Technologies for the COMETE Network.

Disclosure Statement: The authors have nothing to disclose.

References

1. **Amar L, Bertherat J, Baudin E, Ajzenberg C, Bressac-de Paillerets B, Chabre O, Chamontin B, Delemer B, Giraud S, Murat A, Niccoli-Sire P, Richard S, Rohmer V, Sadoul JL, Strompf L, Schlumberger M, Bertagna X, Plouin PF, Jeunemaitre X, Gimenez-Roqueplo AP** 2005 Genetic testing in pheochromocytoma or functional paraganglioma. J Clin Oncol 23:8812–8818
2. **Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K, Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peczkowska M, Szmigielski C, Eng C** 2002 Germ-line mutations in nonsyndromic pheochromocytoma. N Engl J Med 346:1459–1466
3. **Eisenhofer G, Huynh TT, Pacak K, Brouwers FM, Walther MM, Linehan WM, Munson PJ, Mannelli M, Goldstein DS, Elkahloun AG** 2004 Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel-Lindau syndrome. Endocr Relat Cancer 11:897–911
4. **Woodward ER, Maher ER** 2006 Von Hippel-Lindau disease and endocrine tumour susceptibility. Endocr Relat Cancer 13:415–425
5. **Koch CA, Pacak K, Chrousos GP** 2002 The molecular pathogenesis of hereditary and sporadic adrenocortical and adrenomedullary tumors. J Clin Endocrinol Metab 87:5367–5384
6. **Lenders JW, Eisenhofer G, Mannelli M, Pacak K** 2005 Phaeochromocytoma. Lancet 366:665–675
7. **Manger WM** 2006 Diagnosis and management of pheochromocytoma–recent advances and current concepts. Kidney Int 70(Suppl 1):S30–S35
8. **Chen Y, Kamat V, Dougherty ER, Bittner ML, Meltzer PS, Trent JM** 2002

Ratio statistics of gene expression levels and applications to microarray data analysis. Bioinformatics 18:1207–1215
9. **Allander SV, Nupponen NN, Ringner M, Hostetter G, Maher GW, Goldberger N, Chen Y, Carpten J, Elkahloun AG, Meltzer PS** 2001 Gastrointestinal stromal tumors with KIT mutations exhibit a remarkably homogeneous gene expression profile. Cancer Res 61:8624–8628
10. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F** 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034
11. **Anouar Y, Yon L, Guillemot J, Thouennon E, Barbier L, Gimenez-Roqueplo AP, Bertherat J, Lefebvre H, Klein M, Muresan M, Grouzmann E, Plouin PF, Vaudry H, Elkahloun AG** 2006 Development of novel tools for the diagnosis and prognosis of pheochromocytoma using peptide marker immunoassay and gene expression profiling approaches. Ann NY Acad Sci 1073:533–540
12. **Brouwers FM, Elkahloun AG, Munson PJ, Eisenhofer G, Barb J, Linehan WM, Lenders JW, De Krijger R, Mannelli M, Udelsman R, Ocal IT, Shulkin BL, Bornstein SR, Breza J, Ksinantova L, Pacak K** 2006 Gene expression profiling of benign and malignant pheochromocytoma. Ann NY Acad Sci 1073:541–556
13. **Ait-Ali D, Turquier V, Grumolato L, Yon L, Jourdain M, Alexandre D, Eiden L, Vaudry H, Anouar Y** 2004 The proinflammatory cytokines tumor necrosis factor-α and interleukin-1 stimulate neuropeptide gene transcription and secretion in adrenochromaffin cells via activation of extracellularly regulated kinase 1/2 and p38 protein kinases, and activator protein-1 transcription factors. Mol Endocrinol 18:1721–1739
14. **Eipper BA, Milgram SL, Husten EJ, Yun HY, Mains RE** 1993 Peptidylglycine α-amidating monooxygenase: a multifunctional protein with catalytic, processing, and routing domains. Protein Sci 2:489–497
15. **Rocchi P, Boudouresque F, Zamora AJ, Muracciole X, Lechevallier E, Martin PM, Ouafik L** 2001 Expression of adrenomedullin and peptide amidation activity in human prostate cancer and in human prostate cancer cell lines. Cancer Res 61:1196–1206
16. **de Groot DM, Martens GJ** 2005 Expression of neuroserpin is linked to neuroendocrine cell activation. Endocrinology 146:3791–3799
17. **Kronenwett U, Huwendiek S, Castro J, Ried T, Auer G** 2005 Characterization of breast fine-needle aspiration biopsies by centrosome aberrations and genomic instability. Br J Cancer 92:389–395
18. **Zheng C, Heintz N, Hatten ME** 1996 CNS gene encoding astrotactin, which supports neuronal migration along glial fibers. Science 272:417–419
19. **Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM** 1999 Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell [Erratum (2001) 104: following 320] 99:71–80
20. **Li Y, Fanning AS, Anderson JM, Lavie A** 2005 Structure of the conserved cytoplasmic C-terminal domain of occludin: identification of the ZO-1 binding surface. J Mol Biol 352:151–164