Microarray analysis reveals differential expression of benign and malignant pheochromocytoma

Jens Waldmann¹, Volker Fendrich¹, Julia Holler⁶, Malte Buchholz², Ernst Heinmöller³, Peter Langer¹, Annette Ramaswamy⁴, Birgit Samans⁵, Martin K Walz⁷, Matthias Rothmund¹, Detlef K Bartsch¹ and Emily P Slater¹

¹Department of Surgery and ²Division of Gastroenterology, Philipps-Universität Marburg, Baldingerstraße, D-35043 Marburg, Germany

³Department of Pathology, Klinikum Kassel, 34125 Kassel, Germany

⁴Department of Pathology, Philipps-Universität Marburg, Baldingerstraße, D-35043 Marburg, Germany

⁵Institute of Molecular Biology and Tumor Research, Philipps-University Marburg, 35037 Marburg, Germany

⁶Department of Surgery, University Giessen, 35392 Giessen, Germany

⁷Department of Surgery, Center for Minimally Invasive Surgery, Kliniken Essen-Mitte, 45136 Essen, Germany

(Correspondence should be addressed to J Waldmann; Email: jwaldman@med.uni-marburg.de)

Abstract

The diagnosis of a malignant pheochromocytoma (PC) can only be established by the presence of distant metastases, but a subset of apparently benign PCs develop metastases. We have employed a microarray analysis to identify a typical gene expression profile which distinguishes malignant from benign PC. Total RNA was isolated from fresh-frozen tissue of five benign and five malignant PCs. The reference consisted of laser microdissected tissue from normal adrenal medulla. After generating Cy3- and Cy5-fluorescently labeled cDNAs, F-chips containing 11 540 spots were hybridized. Data were analyzed with the IMAGEGENE 3.0 software. Gene expression levels were validated by real-time (RT)-PCR and immunohistochemistry (IHC). The analysis revealed a more than twofold difference in expression between benign and malignant PCs in 132 genes: 19 were up-regulated and 113 were down-regulated. Expression differences of six genes (calsequestrin, NNAT, neurogranin, secreted protein acidic and rich in cysteine (SPARC), EGR2, and MAOB) were confirmed by RT-PCR in 25 PCs. IHC for calsequestrin revealed an overexpression in malignant PCs (7/10 vs 1/10, $P=0.03$). Comparative analysis by microarray of all ten PCs (benign/malignant) versus normal adrenal medulla revealed a more than twofold expression difference in 455/539 and 491/671 genes respectively. Several of these genes are known to participate on adrenal tumorigenesis, potential tumor suppressor genes, and oncogenes. Comprehensive gene expression analysis of malignant and benign PCs revealed different gene profiles, which could be used to discriminate between malignant and benign PCs. Based on these findings, the strategy for further follow-up and treatment could be modified accordingly.

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Introduction

Pheochromocytomas (PCs) are rare neoplasms of the adrenal medulla, which are derived from chromaffin cells of the neural crest. Extra-adrenal PC or paraganglioma (PG) arise from closely related chromaffin cells in sympathetic or parasympathetic paraganglia. The annual incidence is $\sim 1/100\,000$ patient years, and PC causes secondary hypertension in about 1% (Beard *et al.* 1983, Sinclair *et al.* 1987, Omura *et al.* 2004),

although a prevalence of 0.05% is reported in autopsy studies (Platts *et al.* 1995, McNeil *et al.* 2000, Khorram-Manesh *et al.* 2004). Sporadic PCs are three times more frequent than the familial form of PC, which is a result of germline mutations affecting cancer susceptibility genes. Germline mutations affect the oncogene *RET* and the oncosuppressors *VHL*, *SDHB*, *SDHD*, and *NF1*. These familial PCs represent $\sim 25\%$ of cases, and are part of the MEN2 syndrome, the PG

syndromes 1, 3, and 4, the von Hippel–Lindau disease, and neurofibromatosis (Neumann *et al.* 2002). In the case of an inherited tumor syndrome, one must consider the higher rate of malignancy (most commonly associated with SDHB mutation), recurrence, and bilateral disease before patients are scheduled for surgery, which emphasizes the need for genetic testing in every patient with PC.

The surgical treatment of unilateral sporadic PC is well established, and laparoscopic adrenalectomy has become safe in the background of an adequate α -blockage. The management of patients with bilateral disease is still unsettled. An indefinite follow-up is suggested by the NIH consensus conference for familial cases, and at least a 10-year follow-up is warranted for sporadic cases as recurrent and malignant diseases occur in about 17% (Pacak *et al.* 2001, Amar *et al.* 2005). The major problem regarding the clinical management is the identification of patients with malignant disease before they develop distant metastases (DM) or recurrence as malignant PC is incurable.

The prevalence of malignancy in PC ranges from 2.4 to 26%, depending on the definition of malignancy (Melicow 1977, Proye *et al.* 1992). Although the study of Proye *et al.* apparently overestimated the rate of malignancy by defining microscopic venous thrombi and regional invasion as true signs of malignancy. Only 14/91 (15%) patients revealed DM. The highest rate of malignancy is observed in patients with SDHB mutations, and reaches 50% (Gimenez-Roqueplo *et al.* 2003). However, the histological distinction of benign from malignant PC is not reliable. Malignancy is only proven by the presence of distant or lymph node metastases. Efforts have been made to establish molecular markers for malignancy such as over-expression of HSP90, human telomerase reverse transcriptase, HIF-2 α , tenascin, N-cadherin, COX-2, and others. Comparative genomic hybridization experiments in malignant and benign PC revealed losses of 1p, 3q, and 6q, and gains of 9q and 17q. Progression to malignant PC was associated with deletions on 6q and 17p (Dannenberg *et al.* 2000).

A recent microarray analysis of rat PC compared with normal adrenal medulla revealed numerous differentially expressed genes and ~60% of these overlap with a human PC database (Elkahloun *et al.* 2006). Following the study from Giordano who initially assessed gene expression profiles of malignant and benign PC, five other groups have described similar studies (Eisenhofer *et al.* 2004a,b, Dahia *et al.* 2005, Brouwers *et al.* 2006, Thouennon *et al.* 2007). These clearly identified a set of genes that could be

used as multimarkers for malignancy. Over 80% of differentially expressed genes were down-regulated, which led to the hypothesis that the malignant potential is related to a less differentiated expression pattern. Interestingly, Tischler found that PCs in Nf1 knockout mice express genes which are typical for neural progenitor cells (Powers *et al.* 2007).

Despite these data, the development of malignancy, tumorigenesis, and its underlying molecular basis are poorly understood.

In this study, we compared five benign and five malignant PCs to normal human adrenal medulla by cDNA microarrays to identify differentially expressed genes that discriminate malignant PC from benign PC and to improve our knowledge of underlying molecular mechanisms.

Patients and methods

Patients and tumors

All ten patients with PC included in this study underwent adrenalectomy in the Department of Surgery, University of Marburg between 1998 and 2006. The tumor tissue was frozen in liquid nitrogen 10–20 min after surgical removal, and stored in our collection of fresh-frozen adrenal tissue.

This study was performed according to the local ethics committee guidelines, and all patients gave their informed consent in written form.

For the purpose of this study, we chose five benign PCs and five malignant PCs. We only used sporadic benign PCs where a germline mutation had been excluded by mutation analysis (see below). Malignant PC tumor tissue was only available in five cases. The diagnosis of a malignant PC was based on the presence of DM. Frozen tumor samples were formalin-fixed, embedded in paraffin, and sections were evaluated with hematoxylin and eosin (HE) staining to determine the diagnosis and the neoplastic cellularity. To exclude contamination with normal adrenal medulla or cortical tissue, only tumor samples with a neoplasticity of at least 90% were subjected to further analysis.

In total, we analyzed 36 different primary PC samples: 10 in the microarray analysis, 25 by real-time (RT)-PCR, and 20 by immunohistochemistry (IHC). Adrenal tumors were classified as PCs, and extradrenal tumors were classified as PG.

Normal control

Normal adrenal medulla that served as the normal control consisted of 60 fresh-frozen sections from ten different patients who underwent adrenal surgery for

reasons other than PC (five Conn's syndrome and five Cushing's syndrome). They were chosen randomly from our tissue bank that contains over 200 frozen samples of adrenal tumors and normal adrenal tissue. From ten slides of each normal adrenal tissue, we collected the adrenal medulla cells by laser microdissection (LMD) using a P.A.L.M. MicroBeam (Bernried, Germany).

Laser microdissection

Owing to the irregular distribution of adrenal medulla and cortex in an adrenal specimen, we performed a LMD to prevent cortical contamination in the normal medulla control. We stained six frozen sections (10- μ m thickness) of each normal adrenal tissue in methylene blue (1:10) in DEPC-H₂O under RNase-free conditions. Staining was followed by LMD with a P.A.L.M. Microlaser System. Tissue fragments were collected in the adhesive lid of a microfuge tube. Immediately following LMD, tissue was removed from the lid and immersed in 100- μ l lysis buffer (Macherey Nagel, Düren, Germany; Heinmoller *et al.* 2003, Niyaz *et al.* 2005).

RNA extraction

Total RNA from frozen tumor samples and from the LMD normal controls was isolated by homogenization of tissue in lysis buffer and purification by DNase treatment. RNA was recovered using the RNA Kit NucleoSpin (Macherey Nagel) according to the manufacturer. RNA integrity was ensured by RT-PCR of chromogranin A (CgA) and β -actin with the Qiagen one-step RT-PCR Kit by the standard protocol. CgA was used to assure the medullar origin of the normal control, as it is expressed in adrenal medulla and PC, but not expressed in adrenal cortex. Primer sequences for CgA (GenBank accession number: BT006869) and β -actin (Genebank accession M10277) were as follows:

CgA: forward 5'-GAGTGGGAGGACTC-CAAACG-3'
reverse 5'-CCACTTTCTCCAGCTCTGCC-3' (Amplicon: 340 bp)
 β -actin: forward 5'-GATGATGATATCGCCGCGCTCGTCGTC-3'
reverse 5'-GTGCCTCAGGGCAGCGGAACCGCTCA-3' (Amplicon: 778 bp)

After visualization of the product from the one step RT-PCR, only samples which showed expression of both were included in the further analysis.

Amplification of RNA

Two micrograms of each tumor RNA and 2 μ g of the pooled normal adrenal medulla RNA (10 $\times$ 200 ng) were amplified with the Message Amp aRNA Kit. Amino allyl cDNA was synthesized with 2 μ g aRNA, and then labeled and purified with the CyScribe Post-Labeling Kit (Amersham Biosciences). The amino allyl-modified cDNA was chemically labeled with CyDye NHS esters. The coupling reactions of amino allyl-modified cDNA were performed separately with Cy3 and Cy5. The labeled probes were purified with Qiagen spin columns and combined before hybridization. Tumors were labeled with Cy3 and normal adrenal medulla with Cy5.

Microarray analysis

The combined labeled samples were hybridized to the cDNA microarray for 16 h at 55 °C and washed at a stringency of 0.1 $\times$ SSC/0.1% SDS and 0.1 $\times$ SSC. The microarray contains 11 551 DNA spots from the human cDNA library 'Human Sequence-Verified cDNA UniGene Gene Sets gf200, gf201u, and gf202' (Invitrogen). Each experiment was performed as a sandwich hybridization using two arrays. cDNA microarrays were analyzed using Scan Array Express and Software from Perkin Elmer (Waltham, MA, USA).

To account for spot differences, the background corrected ratio of the two channels was calculated and then log₂-transformed. Raw data were standardized to balance intensities to both dyes and to achieve comparable expression levels across experiments. A spatial and intensity-dependent standardization (Yang *et al.* 2002) was used to correct for inherent bias on each chip (the lowest scatter plot). The chips also contain standardized controls, which were employed to normalize and thus control for bias selected differences.

Mean log ratios were calculated from replicates, as each spot was measured twice due to the sandwich hybridization. Spots were excluded if replicates differed more than the maximum of threefold and 75% of the calculated average log ratio, or if the background intensity was higher than the signal intensity.

Differentially expressed genes were selected based on an absolute value of the *t*-statistic of 1.96 and a fold-change difference of at least 2. Prior to the cluster analysis, the expression profile of each gene was centered by subtracting the mean observed value. We performed an average linkage hierarchic clustering for genes as well as for chips with the Euclidean distance metric as implemented in the program Genesis (Sturn *et al.* 2002). Expression data and gene annotations

were stored in Array Express (<http://www.ebi.ac.uk/arrayexpress/>) (accession number E-MEXP-2137), which complies with minimal information about a microarray experiment (MIAME) guidelines.

RT-PCR

The mRNA was reverse transcribed into cDNA with oligo-dT primers using the Superscript 1st Strand System for RT-PCR (Invitrogen) at 42 °C for 50 min. All PCRs were carried out on a 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA) over 40 cycles, with denaturation for 15 s at 95 °C and combined annealing/extension at 60 °C for 1 min. Following an activation step at 95 °C for 10 min, determination of early growth response factor 1 and 2 (*EGR 1* and 2), calsequestrin, monoaminooxidase B (*MAOB*), *SPOCK 2*, neurogranin, and neuronatin (*NNAT*) mRNA expression was performed over 40 cycles with 15 s of denaturation at 95 °C and annealing/extension/data acquisition at 60 °C for 60 s using the Power SYBR Green PCR kit (Applied Biosystems). Primer sequences were as follows:

<i>EGR1</i> :	forward 5'-TGGGCAAGAACAC-CATGATG-3'
	reverse 5'-AGTTTCTCCAGAG-CTGGGTTGT-3'
<i>EGR2</i> :	forward 5'-CCCTAAAATGGTG-AATCAGAGCAT-3'
	reverse 5'-CGTTCCTCCCATCA-CATTGC-3'
<i>Calsequestrin</i> :	forward 5'-CTGCTAGTGAGCC-TTCCCATT-3'
	reverse 5'-TATTGTTGCCCTGGC-CAATT-3'
<i>MAOB</i> :	forward 5'-GAGCCACAATAAGC-CACTGGTAT-3'
	reverse 5'-AGGGAGTGAAGGAG-GATAATTGG-3'
<i>SPOCK2</i> :	forward 5'-TGGATTTCCGTCTG-TAGATTAACTG-3'
	reverse 5'-GGATGACTCACGG-GACTCTTG-3'
<i>NNAT</i> :	forward 5'-AGCGGATCTCGG-CAAACC-3'
	reverse 5'-AGCCGATGATGAG-CAGTTCAG-3'

Relative fold mRNA expression levels were determined using the $2^{-(\Delta\Delta C_t)}$ method (Livak & Schmittgen 2001) with ribosomal protein, large, P0 (*RPLP0*) as housekeeping control. *RPLP0* primer

sequences were as follows: forward 5'-TGGGCAA-GAACACCATGATG-3' and reverse 5'-AGTTTCTC-CAGAGCTGGGTTGT-3'.

Immunohistochemistry

For immunostaining, formalin-fixed and paraffin embedded archival tumor samples and corresponding normal tissues were stained as previously described (Waldmann et al. 2008). Briefly, slides from archived ACCs were heated to 60 °C for 1 h, deparaffinized using xylene, and hydrated by a graded series of ethanol washes. Antigen retrieval was accomplished by microwave heating in 10 mM sodium citrate buffer, pH 6.0 for 10 min. For IHC, endogenous peroxidase activity was quenched by a 10-min incubation in 3% H₂O₂. Nonspecific binding was blocked with 10% serum for 1 h. Sections were then probed with a primary antibody against calsequestrin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a working dilution of 1:100 overnight at 4 °C. For IHC, bound antibodies were detected using the avidin–biotin complex (ABC) peroxidase method (ABC Elite Kit, Vector Labs, Burlingame, CA, USA). Final staining was developed with the Sigma FAST DAB peroxidase substrate kit (Sigma). As a positive control, we used murine heart tissue with every batch, as suggested by the manufacturer.

Statistical analysis

For analyzing proportions, the Mann–Whitney *U* test was applied. In addition, a discriminant analysis (leave-one-out) was performed to estimate the diagnostic value of RT data. *P* values <0.05 were considered to be statistically significant. Data were analyzed using SPSS (Chicago, IL, USA), version 14.0 for Microsoft Windows.

Results

Patients

The clinical data of five patients with benign PC and five patients with malignant PC (with DM) are shown in Table 1. For all benign PCs, a hereditary origin was excluded by prior DHPLC analyses of the *RET*, *VHL*, *SDHB*, and *SDHD* genes.

We conducted a gene expression profile comparing five benign and five malignant PCs to normal adrenal medulla. After background subtraction and data normalization, the ratios were averaged for all tumors.

Table 1 Clinical data of PC which were used for microarray, RT-PCR, and IHC

Patient ID	Histology	Age (years)	Size (mm)	Syndrome	Ki 67 (%)	DM
P1	Malignant PC	44	50	None	1	y LN
P2	Malignant PC	42	95	None	1	y LN
P3	Malignant PC	65	130	None	5	y LN
P4	Malignant PC	78	50	None	3	y LN
P5	Malignant PC	30	20	MEN2B	1	y LN
P6	Benign PC	54	35	None	ND	n
P7	Benign PC	20	33	None	5	n
P8	Benign PC	47	95	None	1	n
P9	Benign PC	53	50	None	1	n
P10	Benign PC	35	45	None	1	n
P11	Benign PC	21	35	MEN2A	<1	n
P12	Benign PC	71	60	None	<1	n
P13	Benign PC	48	75	None	<1	n
P14	Benign PC	48	40	None	1	n
P15	Benign PC	60	60	None	<1	n
P16	Benign PC	41	40	None	1	n
P17	Benign PC	41	100	None	2	n
P18	Benign PC	46	40	MEN2A	<1	n
P19	Malignant PG	33	30	None	10	y LG/LN
P20	Benign PC	43	80	None	ND	n
P21	Benign PC	38	40	MEN2A	<1	n
P22	Benign PC	42	45	MEN2A	<1	n
P23	Benign PC	43	60	None	ND	n
P24	Benign PC	55	70	None	<1	n
P25	Benign PG	47	40	None	1	n
P26	Benign PC	34	70	None	<1	n
P27	Benign PC	78	40	None	<1	n
P28	Benign PC	62	75	None	<1	n
P29	Benign PC	48	30	None	<1	n
P30	Benign PC	46	35	None	<1	n
P31	Malignant PG	39	130	PGS (SDHB)	5	y LM
P32	Benign PC	47	95	None	<1	n
P33	Malignant PC	20	45	PGS (SDHB)	3	y BN, P
P34	Malignant PC	57	75	None	<1	y BN
P35	Malignant PC	82	30	None	1	y LM
P36	Malignant PG	37	20	PGS (SDHD)	15	y HM

DM, distant metastases; PC, pheochromocytoma; PG, paraganglioma; n, no; y, yes; PGS, paraganglioma syndrome; LM, liver metastases; LN, lymph node metastases; LG, lung metastases; BN, bone metastases; P, peritoneum; HM, heart metastases; RT, real-time PCR; IHC, immunohistochemistry. For microarray $n=10$: P1–P10; for RT $n=25$: P1–P25; and for IHC $n=20$: P1, P3–5, P19, P31, P33–36 versus P9, P12, P15, P24, P26–30, P32.

Differentially expressed genes in benign and malignant PC compared with normal adrenal medulla

In the analysis, we found 455 spots in benign PC and 539 spots in malignant PC that were up-regulated more than twofold, which represent 344 and 389 unique genes respectively. A similar number of cDNA probes were down-regulated in benign (491) and malignant (674) PCs, which correspond to 411 and 572 unique genes respectively.

Among these, we identified several genes which are known to play a role in the tumorigenesis of adrenal tumors (e.g. *IGF2*, *RGS*, *CDK 5*, and *ubiquitin*).

One of the main goals of microarray analysis is to identify potential tumor suppressor genes (TSGs), which led us to focus on clearly down-regulated genes. Interestingly, *MGST1* and *GSTA 1,3,4* were down-regulated 62/52- and 131/84-fold in both malignant/benign PC respectively. Other potential TSGs were *NPY1R*, *MAX*, *IGFB6*, and *Ezrin*.

Genes up-regulated more than twofold and which potentially promote tumorigenesis were identified in malignant and benign PCs, and are enlisted in Table 2. One of the most interesting candidates was small ubiquitin-like modifier (SUMO1), which is known to be involved in the by SUMOylation of several TSGs and transcription factors, such as p53, c-jun, and NF- κ B.

Table 2 Gene sets which show different expressions in both benign and malignant pheochromocytoma (PC) compared with normal adrenal medulla

Gene symbols	Gene title/definition	Gene ID	Chromosomal location	Fold change	Direction
<i>MGST1</i>	Microsomal glutathione S-transferase 1	4257	12p12.3-p12.1	61.98/131.73	Down
<i>GSTA3</i>	Glutathione S-transferase α 3	2940	6p12.1	52.33/83.61	Down
<i>GPI</i>	Glucose phosphate isomerase	2821	19q13.1	24.69/55.37	Down
<i>MAX</i>	MYC associated factor X	4149	14q23	18.72/27.15	Down
<i>FDX1</i>	Ferredoxin 1	2230	11q22	17.39/27.15	Down
<i>NPY1R</i>	Neuropeptide Y receptor Y1	4886	4q31.3-q32	14.89/25.70	Down
<i>NUPR1</i>	Nuclear protein transcriptional regul. 1	26471	16p11.2	12.07/15.92	Down
<i>MAOA</i>	Monoamine oxidase A	4128	Xp11.3	5.05/13.48	Down
<i>LAMA2</i>	Laminin, α 2	3908	6q22-q23	7.69/9.00	Down
<i>MT1M</i>	Metallothionein 1M	4499	16q13	6.87/10.20	Down
<i>GSTA4</i>	Glutathione S-transferase α 4	2941	6p12.1	6.31/8.74	Down
<i>IGFBP6</i>	IGF-binding protein 6	3489	12q13	7.05/8.30	Down
<i>VIL2</i>	Villin 2 (ezrin)	7430	6q25.2-q26	6.32/7.40	Down
<i>GSTT1</i>	Glutathione S-transferase θ 1	2952	22q11.23	8.22/6.99	Down
<i>DNASE1L3</i>	Deoxyribonuclease I-like 3	1776	3p21.1-p14.3	7.45/6.34	Down
<i>NGFR</i>	Nerve growth factor receptor	4804	17q21-q22	3.55/5.08	Down
<i>EGFR</i>	Epidermal growth factor receptor	1956	7p12	3.17/4.51	Down
<i>SP100</i>	SP100 nuclear antigen	6672	2q37.1	2.40/4.15	Down
<i>CTNNB1</i>	Catenin (cadherin-associated protein)	1499	3p21	3.25/3.30	Down
<i>SUMO1</i>	SMT3 suppressor of mif two 3 homolog 1	7341	2q33	14.31/9.99	Up
<i>IGF2</i>	IGF2 (somatomedin A)	3481	11p15.5	12.90/9.65	Up
<i>RGS5</i>	Regulator of G-protein signaling 5	8490	1q23.1	6.45/4.52	Up
<i>CDK5R2</i>	Cyclin-dependent kinase 5, regulatory subunit 2 (p39)	8941	9q33	4.63/3.81	Up
<i>UBE2D3</i>	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	7323	4q24	3.86/3.58	Up
<i>MUM1</i>	Melanoma associated antigen (mutated) 1	84939	19p13.3	3.74/4.12	Up
<i>IGFBPL1</i>	IGF-binding protein-like 1	347252	9p13.1	3.70/3.58	Up
<i>CSNK1G3</i>	Casein kinase 1, γ 3	1456	5q23	2.72/3.20	Up
<i>MARK3</i>	MAP/microtubule affinity-regulating kinase 3	4140	14q32.3	2.90/3.13	Up
<i>TRAF5</i>	TNF receptor-associated factor 5	7188	1q32	3.65/3.01	Up
<i>CDC42BPA</i>	CDC42-binding protein kinase α (DMPK-like)	8476	1q42.11	3.24/4.69	Up

The direction of expression difference 'Down' and 'Up' indicates underexpression (Down) and overexpression (Up). The first number in the 'Fold-change' column represents malignant PC, and the second number represents benign PC. Abbreviations and full names of genes are listed as well as the chromosomal location and gene ID (NCBI online database).

Differentially expressed genes in malignant compared with benign PC

Nineteen spots were detected to be up-regulated, and 113 spots to be down-regulated with at least a twofold difference at the mRNA level in malignant PC compared with benign PC. This represents 15 and 102 unique genes. A selection of these genes is shown in Table 3.

Unsupervised hierarchical cluster

Transcriptional profiles distinguished between malignant and benign PCs and identified several differentially expressed transcripts as demonstrated by cluster analysis (as shown in Fig. 1). The majority of the differentially expressed genes were down-regulated.

Table 3 Gene sets which distinguish between malignant and benign pheochromocytoma (PC) identified by microarray analysis

Gene symbols	Gene title/definition	Gene ID	Chromosomal location	Fold change	Direction	Comparison (Brouwers <i>et al.</i>)
<i>FOSB</i>	FBJ murine osteosarcoma viral oncogene homolog B	2354	19q13.32	14.36	Down	NS
<i>NRGN</i>	Neurogranin (protein kinase C substrate, RC3)	4900	11q24	9.63	Down	NS
<i>ARC</i>	Activity-regulated cytoskeleton-associated protein	23237	8q24.3	7.21	Down	ND
<i>QPCT</i>	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	25797	2p22.2	6.01	Down	S
<i>CCL4L2</i>	Chemokine (C-C motif) ligand 4-like 2	388372	17q12	6.35	Down	S
<i>EGR2</i>	Early growth response 2 (Krox-20 homolog, <i>Drosophila</i>)	1959	10q21.1	5.56	Down	S
<i>RGS1</i>	Regulator of G-protein signaling 1	5996	1q31	5.48	Down	S
<i>PLAT</i>	Plasminogen activator, tissue	5327	8p12	4.84	Down	NS
<i>MAO B</i>	Monoamine oxidase B	4129	Xp11.23	4.95	Down	NS
<i>PLAUR</i>	Plasminogen activator, urokinase receptor	5329	19q13	4.60	Down	NS
<i>SPOCK2</i>	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	9806	10pter-q25.3	4.42	Down	S
<i>GLDN</i>	Gliomedin	342035	15q21.2	4.17	Down	ND
<i>CCL2</i>	Chemokine (C-C motif) ligand 2	6347	17q11.2-q12	4.38	Down	S
<i>EGR1</i>	Early growth response 1	1958	5q31.1	4.05	Down	S
<i>NNAT</i>	Neuronatin	4826	20q11.2-q12	3.56	Down	NS
<i>CD163L1</i>	CD163 molecule-like 1	283316	12p13.3	3.49	Down	S
<i>PCDH20</i>	Protocadherin 20	64881	13q21	3.31	Down	S
<i>H19</i>	H19, imprinted maternally expressed untranslated mRNA	283120	11p15.5	3.15	Down	S
<i>FABP5</i>	Fatty acid-binding protein 5 (psoriasis-associated), E-FABP	344332	8q21.13	3.15	Down	NS
<i>CITED2</i>	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	10370	6q23.3	3.04	Down	NS
<i>JUNB</i>	Jun B proto-oncogene	3726	19p13.2	3.03	Down	S
<i>DUSP5</i>	Dual specificity phosphatase 5	1847	10q25	2.85	Down	S
<i>SERPING1</i>	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	710	11q12-q13.1	2.82	Down	NS
<i>MAOA</i>	Monoamine oxidase A	4128	p11.3	2.67	Down	NS
<i>BIN1</i>	Bridging integrator 1	274	2q1	2.40	Down	S
<i>AIFM1</i>	Apoptosis-inducing factor, mitochondrion-associated, 1	9131	Xq25-q26	2.38	Down	NS
<i>CDH2</i>	Cadherin 2, type 1, N-cadherin (neuronal)	1000	18q11.2	2.22	Down	S
<i>DKK3</i>	Dickkopf homolog 3 (<i>Xenopus laevis</i>)	27122	11p15.2	2.12	Down	NS
<i>CDC26</i>	Cell division cycle 26 homolog (<i>Saccharomyces cerevisiae</i>)	246184	9q32	2.11	Down	S
<i>FOS</i>	V-fos FBJ murine osteosarcoma viral oncogene homolog	2353	14q24.3	2.11	Down	S
<i>IGFBP2</i>	IGF-binding protein	3485	2q33-q34	2.11	Down	ND
<i>CTSO</i>	Cathepsin O	1519	4q31-q32	2.09	Down	ST
<i>SEZ6</i>	Seizure-related 6 homolog (mouse)	124925	17q11.2	5.57	Up	NS
<i>CASQ2</i>	Calsequestrin 2 (cardiac muscle)	845	1p13.3-p11	3.63	Up	NS
<i>STXBP6</i>	Syntaxin-binding protein 6 (amisyn)	29091	14q12	2.91	Up	NS
<i>RNF32</i>	Ring finger protein 32	140545	7q36	2.47	Up	S
<i>MAN1A2</i>	Mannosidase, α , class 1A, member 2	10905	1p13	2.27	Up	NS
<i>DTWD1</i>	DTW domain containing 1	56986	15.q21.2	2.22	Up	ST
<i>MINA</i>	MYC-induced nuclear antigen	84864	3q11.2	2.10	Up	S

The direction of expression difference 'Down' and 'Up' indicates underexpression (Down) and overexpression (Up). Abbreviations and full names of genes are listed as well as chromosomal location and gene ID (NCBI online database). S, significant; NS, not significant; ST, statistical trend ($P < 0.1$).

Analysis of dataset with gene set enrichment analysis (GSEA), DAVID, and EASE

We identified 50 gene sets, which discriminate between malignant and benign PC with a P value <0.01 . If we reset the significance level to a P value of 0.05, the number of gene sets increased to 135. A core enrichment was shown for eight gene sets: *ZNF198*, *UBE2N*, *CHR17P11*, *BRACXUP*, *CSNK1A1*, *WNT* pathway, and *SUFU*. Related to the Wnt pathway, TSGs (*CREBBP*), kinases (*GSK3 β* , *NLK*, and *MAP3K7*), oncogenes (*CTNNB1*, *MYC*, and *CCND1*), and transcription factors (*TCF1*, *HDAC1*, and *PPARD*) were shown to be significantly overexpressed in malignant PC.

Validation of microarray analysis by RT-PCR

Some of the genes which were differentially expressed in malignant and benign PC were subjected to further analysis to ensure the validity of the microarray. Expression levels of *calsequestrin*, *neurogranin*, *NNAT*, *SPOCK2*, *EGR2*, and *MAOB* were confirmed by RT-PCR in 6 malignant and 19 benign PCs (P1–P25) as shown in Fig. 2. ERCC1, EGR1, and MAOA did not reveal statistically different levels of expression in malignant and benign PC. *P* values were calculated by the Mann–Whitney test, and are shown in Fig. 2. The discriminant analysis (leave-one-out) showed that 78% of cross-validated grouped cases were classified correctly.

Comparison with the study of Brouwers *et al.*

A comparison of the gene lists from both microarrays revealed a total of 4709/15233 (30.9%) genes that matched. Brouwers *et al.* found a total of 1126 genes that were up-regulated, and 1633 that were down-regulated in all assessed malignant PC compared with benign PC. In primary metastatic/malignant PC, Brouwers *et al.* found a total of 1263 genes that were up-regulated, and 1808 genes that were down-regulated versus benign PC. The overall conformance of Brouwers *et al.* and the present microarray analysis is illustrated by a Venn diagram in Fig. 3. The group of

Figure 1 Supervised hierarchical cluster in malignant and benign pheochromocytomas. On the left, malignant PCs (P1, P2, P7, P9, and P10) and on the right, benign PCs (P3, P4, P5, P6, and P8) are shown. Expression differences are indicated by the color (red, overexpressed (threefold difference); green, underexpressed (threefold difference) compared with the average expression of all samples). Genes that revealed a differential expression in malignant and benign PC by the microarray analysis are listed in the right margin. Full colour version of this figure available via <http://dx.doi.org/10.1677/ERC-09-0118>.

all malignant PCs is referred as Brouwers_1 in Fig. 3 (yellow), while the primary malignant PCs are referred as Brouwers_4 in Fig. 3 (purple). This takes into account that a subgroup of PC patients present with DM at diagnosis (Brouwers_4), while others develop metastases in further follow-up (these are included in Brouwers_1). The meaning of the color code is explained in detail in the legend of Fig. 3. Details on the comparison with Brouwers data for distinct genes are given in Table 3, which either shows confirmation (S, significant) or difference (NS, not significant) of the two datasets. Eighteen of the 39 genes (present on both chips) were significantly differentially expressed in both analyses (see also Table 3). Interestingly, 5/11 down-regulated genes that overlapped are cation binders (secreted protein acidic and rich in cysteine (*SPARC*), *SP100 nuclear antigen*, *vav2 oncogene*, *EMR3*, *RCHY1*), and 5/11 genes are known to play role in the cell cycle (*SPARC*, *SP100 nuclear antigen*, *vav2 oncogene*, *NFARC1*, *p78*). It has to be kept in mind that only 704/1962 (35.9%) of up-regulated genes and 886/2691 (32%) of down-regulated genes of the total NIH dataset (number in parentheses) were found on the chip used for the present study.

IHC for calsequestrin

IHC revealed an increased expression of calsequestrin at the protein level in ten malignant PCs (P1, P3–5, P19, P31, and P33–36) compared with ten benign PCs (P9, P12, P15, P24, P26–30, and P32). Whereas only one of the ten benign PCs showed strong calsequestrin expression, seven of ten malignant PCs did ($P=0.01$). Clinical data are summarized in Tables 1 and 3. Positive staining was localized to the cytoplasm as in the positive control (murine heart muscle). Figure 4 shows the typical expression difference in a benign and a malignant PC. Sustenacular cells stained positive in malignant and benign PC, and were used as internal control.

Discussion

Malignancy in PC can only be established accurately if DM are present. Neither histology nor clinical patterns can distinguish between patients with truly benign PC and patients who will develop DM in further follow-up.

Figure 2 Comparative quantification of calsequestrin (CASQ2), neurogranin (NGRN), Sparc, EGR2, MAOB, and Sez6 in 25 PCs (6 malignant/19 benign) by RT. The relative expression levels of the above mentioned genes are shown as whisker plots. The left bar of each diagram stands for benign PC, and the right bar stands for malignant PC. The statistical significance (t -test) is included in each diagram.

Figure 3 Venn diagram with the overlap between the microarray of the present study and the NIH dataset (Brouwers *et al.* 2006). On the left diagram underexpressed genes in malignant PC, and on the right overexpressed genes in malignant PC are shown. Brouwers 1 and 4 represent all malignant tumors (1) and all primary malignant tumors (4). Slater represents data of the present study. Colors (yellow, red, and blue) are assigned to genes, which are underexpressed or overexpressed without an intersection between the three groups (Slater, Brouwers_1 and 4). Overlapping genes are indicated by the colors orange, green, and blue. The number of genes which are assessed by both chips (Slater/Brouwers) are shown without parentheses. If genes were only on the array of Brouwers *et al.*, number is given in parentheses. Full colour version of this figure available via <http://dx.doi.org/10.1677/ERC-09-0118>.

DM in patients with apparently benign PC occur in up to 7% of cases (Khorram-Manesh *et al.* 2005). The treatment of metastatic PC is an unresolved problem as response to chemotherapy, radiotherapy,

and I131-MIBG is very limited and results in 5-year survival rate of ~40% (Remine *et al.* 1974, Safford *et al.* 2003, Khorram-Manesh *et al.* 2005). However, long-term survival of more than 20 years has been reported, reflecting the different biological subtypes of malignant PCs (Jarvelainen & Viikari 2001, Yoshida *et al.* 2001).

Gene expression profiling is an attractive approach to obtain detailed insights into the biology of PC. It offers a possibility to identify both molecular markers for malignancy and potential targets for novel therapies. Therefore, several studies have assessed malignant and benign PC by gene expression profiling (Anouar *et al.* 2006, Brouwers *et al.* 2006, Thouennon *et al.* 2007).

In our study, we identified a set of 130 genes, which could distinguish between malignant and benign PC. To validate our results, we confirmed the differential expression by RT-PCR, and we increased the sample size to 29 PCs, including the ten PCs, which were subjected to the microarray analysis.

In addition, an unsupervised clustering clearly could discriminate malignant from benign PC while blinded to clinical characteristics. This indicates that gene expression profiling may serve as an approach to classify PC and to evaluate prognosis. Based on expression data, patients can be subjected to closer follow-up or adjuvant therapy in the future.

Our study shows that the majority of genes were under-expressed in malignant PC (119/132), which is in the line with previous studies (Anouar *et al.* 2006, Brouwers *et al.* 2006, Thouennon *et al.* 2007). Interestingly, several of these genes encode neuro-endocrine factors (*neurogranin* and *NNAT*), enzymes involved in catecholamine metabolism (*MAOA*) and peptide processing (*QPCT*, *SERPING1*). The decreased expression may reflect a loss of differentiation in malignant PC, which possibly arises from immature neuroendocrine precursor cells. According to Nakamura & Kaelin (2006), neural crest precursor cells fail to undergo apoptosis during embryonic development. This is supported by the findings of Powers *et al.* (2007) who reported a neural progenitor profile in NF1 knock out mice PCs.

With SPARC, protocadherin 20 (*PCDH20*), and N-cadherin, we identified matricellular proteins that are known regulators of metastasis in the context of epithelial-to-mesenchymal transition (EMT). SPARC was down-regulated in malignant PC, which is consistent with its role in the inhibition of angiogenesis and tumor growth in neuroblastomas (Chlenski *et al.* 2002, 2006). Powers found SPARC to be up-regulated

Figure 4 Immunostaining for calsequestrin in two malignant and two benign PCs. Immunostaining for calsequestrin in two different benign and malignant PCs. Note the intense staining (brown) in malignant PC compared with benign PC. Non-neoplastic sustentacular cells were stained in both malignant and benign PCs. Full colour version of this figure available via <http://dx.doi.org/10.1677/ERC-09-0118>.

in PC compared with normal adrenal medulla (11.1-fold) as did we in our study (3.9-fold, data not shown; Powers *et al.* 2007). N-cadherin was also found to be down-regulated in a neuroblastoma cell line, and is a target gene of RASSF1A (Agathangelou *et al.* 2003). *PCDH20* belongs to a subfamily of the cadherin superfamily, displays features of a TSG, and is found to be down-regulated in small cell lung cancer (Imoto *et al.* 2006). Also, an activation of the WNT pathway might indicate a role of EMT in the malignant transformation of PC, as activation of the WNT/ β -catenin pathway is known to provoke EMT in colorectal cancer (Brabletz *et al.* 2005).

Seizure-related 6 homolog (*SEZ6*) was one of the few up-regulated genes in malignant PC, and is reported to serve as a prognostic marker for lung cancer (Ishikawa *et al.* 2006). Calsequestrin was also up-regulated in malignant PC, as confirmed by RT and IHC (Fig. 4). It is normally expressed in heart muscle, and plays role in subcellular calcium storage. The function in the context of PC is unknown, but one could speculate that an impaired calcium homeostasis might influence intracellular signaling.

Two candidates for TSGs are BIN1 and NNAT, which are both under-expressed in malignant PC. NNAT is overexpressed in neuroblastoma with a favorable diagnosis, while BIN1 expression is reported to be reduced in neuroblastomas with unfavorable biological features (Tajiri *et al.* 2003, Higashi *et al.* 2007). Interestingly, we found SUMO, a small-ubiquitin-like modifier, to be up-regulated in benign and malignant PC. SUMO controls diverse cellular functions of protein targets including transcription factors and other regulators that maintain cellular homeostasis. Additionally, SUMO plays important roles in genomic stability and DNA repair (Kim & Baek 2006).

Chromosomal losses of 6q and 17p are frequently observed in malignant PC (Dannenberg *et al.* 2000). Focusing on chromosomal locations of genes that showed decreased expression in malignant PC, we identified CITED 2 and phosphatase and actin regulator 2 (PHACTR2) on chromosome 6q, but none on chromosome 17p.

A limitation of this study is the fact that some of the apparently benign PCs that were used for the microarray may turn out to be malignant in further follow-up. As reported in the literature, DM can develop 20 years after surgery (Tanaka *et al.* 1993), and only lifelong follow-up can assure that the PC is truly benign. Another limitation is the small sample number, which underlines the ultimate need for

multicenter studies. To reevaluate our data, we compared it with the data of Brouwers *et al.* (2006). Thirty-nine of 191 (20%) down-regulated genes were also decreased expressed in Brouwers analysis (see Fig. 3), while only 4/55 (7%) of up-regulated genes were confirmed by Brouwers. The limited comparability (different array platforms) must be carefully considered when interpretate these results. Only ~30% of genes assessed by our array platform were also found on the array chip used by Brouwers *et al.* Different array platforms (affymetrix versus agilent) were applied, and therefore, their numeric results could not be directly combined to select significant markers. The resulting gene sets were therefore generated by simple intersection on their Unigene symbol annotation. At least, the normal adrenal medulla was obtained from patients who underwent adrenalectomy due to adrenocortical disease (primary hypercortisolism/primary hyperaldosteronism). Thus, effects of cortisol on gene expression in the adrenal medulla have only been reported so far for tyrosinhydroxylase and catecholamin synthesizing enzymes, this may have influenced our data (Kvetnansky *et al.* 1995). Under different circumstances, where adrenal medulla could be obtained (Organ donor, cancer-associated nephrectomy), changes in gene expression appear to be likely, as even histological have been described (Gelfman 1964, Cuschieri 1969, Masterson & Mostafa 1998).

Future studies should validate marker genes and elaborate pathways by functional studies. The development of tissue microarrays with paraffin-embedded PC samples would provide an instrument for testing different prognostic markers when clinical data on follow-up, especially the development of DM, is available.

In conclusion, we performed a microarray analysis to discriminate between malignant and benign PC and identified a set of genes that clearly classify PC. This study offers a useful database for further elaboration of prognostic markers in PCs, and provides insights into the biology of this disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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