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Defining molecular classifications and targets in gastroenteropancreatic neuroendocrine tumors through DNA microarray analysis

Eva-Maria Duerr[1,2], Yusuke Mizukami[1,2], Aylwin Ng[4], Ramnik J Xavier[1,2,4], Hirotoshi Kikuchi[1,2], Vikram Deshpande[5], Andrew L Warshaw[6], Jonathan Glickman[7], Matthew H Kulke[8] and Daniel C Chung[1,2,3]

[1]Gastrointestinal Unit, [2]Department of Medicine, [3]Cancer Center, [4]Center for Computational and Integrative Biology, [5]Department of Pathology and [6]Department of Surgery, Massachusetts General Hospital, Boston, Massachusetts 02114, USA
[7]Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02114, USA
[8]Department of Adult Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02114, USA

(Correspondence should be addressed to D C Chung, GI Unit, GRJ 825, Massachusetts General Hospital, 50 Blossom Street, Boston, Massachusetts 02114, USA; Email: dchung@partners.org)

Y Mizukami is now at Asahikawa Medical College, Asahikawa, Japan

Abstract

Current classifications of human gastroenteropancreatic neuroendocrine tumors (NETs) are inconsistent and based upon histopathologic but not molecular features. We sought to compare a molecular classification with the World Health Organization (WHO) histologic classification, identify genes that may be important for tumor progression, and determine whether gastrointestinal NETs (GI-NETs) differ in their molecular profile from pancreatic NETs (PNETs). DNA microarray analysis was performed to identify differentially expressed genes in PNETs and GI-NETs. Confirmation of expression levels was obtained by quantitative real-time PCR. Immunoblotting and mutational analysis were performed for selected genes. Hierarchical clustering of 19 PNETs revealed a 'benign' and 'malignant' cluster that corresponded well with the WHO categories of well-differentiated endocrine tumor (WDET) and well-differentiated endocrine carcinoma (WDEC) respectively. *FEV*, adenylate cyclase 2 (ADCY2), nuclear receptor subfamily 4, group A, member 2 (*NR4A2*), and growth arrest and DNA-damage-inducible, beta (GADD45b) were the most highly up-regulated genes in the malignant group of PNETs. Platelet-derived growth factor receptor (PDGFR) was expressed in both WDETs and WDECs, and phosphorylation of PDGFR-β was observed in 83% of all PNETs. Malignant ileal GI-NETs exhibited a distinctive gene expression profile, and extracellular matrix protein 1 (ECM), vesicular monoamine member 1 (VMAT1), galectin 4 (*LGALS4*), and RET Proto-oncogene (*RET*) were highly up-regulated genes. Gene expression profiles reflect the current WHO classification and can distinguish benign from malignant PNETs and also PNETs from GI-NETs. This suggests that molecular profiling may enhance tumor classification schemes. Potential gene targets have also been identified, and PDGFR and RET are candidates that may represent novel therapeutic targets.

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Introduction

Pancreatic neuroendocrine tumors (PNETs) and gastrointestinal neuroendocrine tumors (GI-NETs) are tumors of neuroendocrine origin that share many common biological features. Based on criteria including tumor size, mitotic rate, Ki-67 index, angioinvasion, and distant metastases, the World Health Organization (WHO) classifies these tumors into three groups: well-differentiated NETs (WDET), well-differentiated neuroendocrine carcinomas

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(WDEC), and poorly-differentiated neuroendocrine carcinomas (PDEC; Kloppel *et al.* 2004). The WDETs are further subdivided into WDETs of benign behavior and WDETs of low-grade malignant/uncertain behavior (Table 1). Whether these two subgroups are distinct entities or related entities within a spectrum is unknown. It also remains a challenge to distinguish WDETs from WDECs because histologic criteria are imperfect and the only definitive criterion for malignancy is the presence of metastases. Although our understanding of the cellular biology and clinical behavior of NETs has increased in sophistication, insights into their underlying molecular genetics have lagged behind. A number of candidate genes have been implicated in the pathogenesis of PNETs (reviewed in (Duerr & Chung 2007)), including multiple endocrine neoplasia type 1 (*MEN1*; Shan *et al.* 1998, Wang *et al.* 1998), retinoic acid receptor-β (House *et al.* 2003*b*), *hMLH1* (human mutL homologue 1; House *et al.* 2003*a*), *RASSF1* (Ras association domain family 1; House *et al.* 2003*b*), Her2/neu (herstatin; Evers *et al.* 1994, Goebel *et al.* 2002), and the cell cycle regulators cyclin D1 (Chung *et al.* 2000, Guo *et al.* 2003), $p16^{INK4a}/p14^{ARF}$ (Muscarella *et al.* 1998), $p18^{INK4c}$, and $p27^{Kip1}$ (Guo *et al.* 2001), as well as tyrosine kinase receptors (Fjallskog *et al.* 2003). However, the genetics of tumor progression are poorly defined.

It is also uncertain how similar the genetic alterations are that underlie PNETs and GI-NETs, and whether they can be distinguished on a molecular level. Although there are important differences in their clinical behavior, they are still classified in a similar manner by the WHO criteria. DNA microarray technology is a promising tool to better understand gene expression patterns that underlie tumor development. Thus far, only a few studies have investigated gene expression profiles in PNETs. Most of these have focused on differences between tumors and normal tissue (Maitra *et al.* 2003, Bloomston *et al.* 2004, Capurso *et al.* 2006). In the present study, we sought to compare molecular classifications with the WHO histologic classification, identify genes that may be important for tumor progression, and determine whether GI-NETs differ in their molecular profile from PNETs. This was accomplished with the use of DNA microarrays. With such an approach, novel genes that are expressed in a highly differential manner can be identified, and expression patterns of candidate genes that may have biological relevance to neuroendocrine tumorigenesis can be easily defined. This strategy can therefore identify genes of potential interest in both a non-targeted and targeted manner, and confirmation can be obtained through protein analysis.

Materials and methods

Patient samples

Fresh frozen tissue samples of 24 PNETs (5 benign WDETs, 11 low-grade malignant WDETs, and 8 WDECs) and 6 malignant GI-NETs were obtained as surgical discards from Massachusetts General Hospital and Brigham and Women's Hospital/Dana-Farber Cancer Institute respectively. Tumors were classified according to the WHO 2004 criteria. All of the PNET samples were primary tumors. Clinical characteristics are summarized in Tables 2 and 3. This protocol was approved by the institutional review board of each institution.

RNA extraction

RNA was extracted from frozen tumors following dissection from normal surrounding tissue using Trizol

Table 1 The World Health Organization classification of neuroendocrine tumors of the pancreas

Category	Histology	Localization	Size (cm)	% Ki-67 positive cells	Angioinvasion
Well-differentiated neuroendocrine tumor					
Benign behavior	Well-differentiated	Confined to pancreas	<2	≤2%	No
Well-differentiated neuroendocrine tumor					
Low-grade malignant (or uncertain behavior)	Well-differentiated	Confined to pancreas	≥2	>2%	Yes
Well-differentiated neuroendocrine carcinoma	Well-differentiated	Invasion of adjacent organs and/or metastases	≥2	>2%	Yes
Poorly-differentiated neuroendocrine carcinoma	Poorly-differentiated	Invasion of adjacent organs and/or metastases	Any	>30%	Yes

Table 2 Clinical characteristics of pancreatic neuroendocrine tumor (PNET) samples included in DNA microarray and western blot analyses

ID no.	**Age**	**Sex**	**Tumor type**	**WHO classification**	**Specimen type**	**Tumor size** (cm)	**Ki-67 index** (%)	**Micro-array**	**PDGFR WB**
2	62	M	PTHRP-secreting tumor	WDEC	Primary	8.5	3.9	Yes	No
53	69	F	Non-functioning PNET	WDET LM	Primary	13.0	5.7	Yes	Yes
55	70	M	Glucagonoma	WDET LM	Primary	3.0	2.0	Yes	No
56	73	M	Insulinoma	WDEC	Primary	2.5	1.8	Yes	No
57	46	M	Non-functioning PNET	WDEC	Primary	10.0	<1	No	Yes
59	65	M	ACTHoma	WDEC	Primary	6.5	2.0	Yes	Yes
61	58	F	Gastrinoma	WDEC	Primary	12.0	16.8	Yes	Yes
63	57	F	Insulinoma	WDET B	Primary	1.5	2.3	No	Yes
69	48	F	Gastrinoma	WDEC	Primary	3.0	nd	Yes	No
70	36	F	Insulinoma	WDET B	Primary	1.5	nd	Yes	Yes
71	54	F	Insulinoma	WDET LM	Primary	2.5	1.1	Yes	Yes
72	43	F	Insulinoma	WDET B	Primary	1.2	1.2	Yes	Yes
74	24	F	Insulinoma	WDET LM	Primary	3.0	4.0	Yes	Yes
76	15	F	Insulinoma	WDET LM	Primary	1.8	1.8	Yes	No
77	62	F	Glucagonoma	WDEC	Primary	7.0	7.5	No	Yes
78	51	F	Insulinoma	WDET B	Primary	1.8	3.9	No	Yes
79	51	F	Insulinoma	WDET LM	Primary	5.0	<1	No	Yes
80	54	M	Insulinoma	WDET LM	Primary	2.2	<1	Yes	No
81	74	F	Non-functioning PNET	WDET LM	Primary	4.0	4.2	Yes	Yes
82	60	M	Insulinoma	WDET B	Primary	1.8	<1	Yes	Yes
83	47	F	Non-functioning PNET	WDEC	Primary	7.0	<1	Yes	Yes
84	59	M	Recurrent gastrinoma	WDEC	Primary	nk	nd	Yes	Yes
85	44	M	Non-functioning PNET	WDET LM	Primary	3.0	6.5	Yes	Yes
86	83	F	Insulinoma	WDET LM	Primary	3.0	<1	Yes	Yes

WDET B, well-differentiated endocrine tumor – benign; WDET LM, well-differentiated endocrine tumor – low-grade malignant; WDEC, well-differentiated endocrine carcinoma; nd, not done (no tumor sample available); nk, not known.

following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA).

DNA microarrays

RNA analyses were performed at the DNA Microarray Core Facility at Massachusetts General Hospital Cancer Center. Amounts, purity, and integrity of RNA were evaluated by u.v. spectrophotometry and an RNA-nano Bioanalyzer (Agilent, Palo Alto, CA, USA). Probe synthesis and hybridization of human U-133A GeneChip DNA microarrays (Affymetrix, Santa Clara, CA, USA) were performed following the manufacturer's instructions.

Microarray data analysis

Data analysis was performed using DChip software (www.dchip.org). CEL files (primary Affymetrix array data files) were loaded and normalized at the probe cell level by the Invariate Set Normalization method (Li & Hung Wong 2001). The model-based method (Li & Hung Wong 2001) was used for probe selection and computing expression values. These expression values were attached with standard errors as measurement accuracy. The lower confidence intervals of fold changes were conservative estimates of real fold changes. The ANOVA test was carried out using a P value <0.05 in order to define a set of significantly up- or down-regulated genes. The resulting genes were filtered for gene presence calls of >20 in $>50\%$ of samples. Two-group comparison was employed selecting for increased or decreased gene expression by more than 1.5-fold. Hierarchical clustering analysis (Eisen *et al.* 1998) was performed on the genes that met the above criteria.

Gene ontology

Enrichments of gene ontology (GO) categories were computed using the hypergeometric probability distribution, which identifies GO molecular function categories overrepresented in the set of differentially induced genes relative to their representation on the Affymetrix U133A array. The analysis was performed using Onto-Tools (Draghici *et al.* 2003) and GO

Table 3 Clinical characteristics of gastrointestinal (GI) neuroendocrine tumor samples included in the DNA microarray analysis

ID	Age	Sex	Primary site	Specimen type	Primary tumor size (cm)	Stage	Carcinoid syndrome
2974-1	72	nk	Ileum	Primary ileal tumor	0.4	Metastatic	Yes
11898-1	52	F	Ileum	Liver met lesion	2.0	Metastatic	Yes
33762-1	67	M	Ileum	Liver met lesion	1.5	Metastatic	Yes
53456-1	59	F	Colon	Primary colon tumor	nk	Metastatic	nk
67494-1	53	F	Ileum	Primary ileal tumor	2.5	T3N1M0	No
80670-1	70	F	Ileum	Primary ileal tumor	3.5	Metastatic	No

nk, not known.

molecular function categories with P value <0.05 are considered significantly overrepresented.

Protein interaction network

The network was constructed by iteratively connecting interacting proteins, with protein interaction data obtained from the Human Protein Reference Database (Peri *et al*. 2004). The network uses graph theory, which represents components (gene products) as nodes and interactions between components as edges. Graph layout descriptions were written in the Dot language (Gansner & North 2000) that implements a multi-dimensional scaling heuristic, which creates a virtual physical model (Spring model; Kamada & Kawai 1989) and is coupled to an iterative solver (Newton–Raphson algorithm) that searches for low-energy configurations to optimize the graph layout.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR of RNA from the 24 tumor samples used in the microarray analysis and 3 normal pancreas samples was performed utilizing the SuperScript III platinum Two-Step qRT-PCR Kit (Invitrogen). The 18S rRNA served as an endogenous control. Primer sequences and PCR conditions for *FEV*, adenylate cyclase 2 (*ADCY2*), nuclear receptor subfamily 4, group A, member 2 (*NR4A2*), growth arrest and DNA-damage-inducible, beta (*GADD45β*), extracellular matrix protein 1 (*ECM1*), vesicular monoamine member 1 (*VMAT1*), *LGALS4*, *RET*, and *18S* are available upon request. A fluorogenic SYBR Green and MJ research detection system were used for real-time quantification. Relative mRNA expression was calculated using the parameter threshold cycle (C_T) values. ΔC_T was the difference in the C_T values derived from the specific gene being assayed and the 18S rRNA. $\Delta\Delta C_T$ represented the difference between the paired samples, as calculated by the formula ΔC_T of a sample $-\Delta C_T$ of a reference (the average ΔC_T of three normal pancreas samples). The amount of target, normalized to 18S and the reference, was calculated as $2^{-\Delta\Delta CT}$.

Protein lysates and western blot analysis

Protein lysates were prepared from 18 snap-frozen PNET samples and 3 normal tissues (two from pancreas and one from duodenum). Thirteen samples were from the same PNETs used for the microarray studies (Table 2). Total cell lysate (150 μg) was separated by SDS-PAGE (NuPAGE, Invitrogen) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Immunoblotting was performed with anti-platelet-derived growth factor receptor-β (PDGFR-β), anti-PDGFR-α, anti-phospho PDGFR-β Tyr716 (Upstate, Billerica, MA, USA), anti-phospho PDGFR-β Tyr751 (Sugen), and anti-β-Actin (Sigma, St Louis, MO, USA).

RET sequencing

The cDNA of seven WDECs and six GI-NETs was PCR amplified using three different primer sets spanning codons 573–666 (exons 10 and 11), 729–826 (exons 13 and 14), and 858–940 (exons 15 and 16). PCR products were purified (QIAquick gel extraction kit, Qiagen) and sequenced on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). Primer sequences are available upon request.

Ret immunohistochemistry

Formalin-fixed paraffin-embedded samples of 21 cases of small intestinal NETs from Brigham and Women's Hospital and 65 cases of PNETs from Massachusetts General Hospital were assembled as part of a tissue microarray. Multiple independent cores from each sample were placed onto the microarray (range 2–6). Ret expression was assessed by immunohistochemistry with a Ret antibody (Santa Cruz, Santa Cruz, CA, USA) at 1:50 dilution after treatment with formic acid, as previously described (Lee *et al*. 2005). Ret staining was scored from 0 to 3^+, and each core sample was scored separately. A papillary thyroid cancer sample was included as a positive control.

Statistical analysis

The P values were calculated utilizing the Wilcoxon rank-sum test with a P value <0.05 considered statistically significant.

Results

Differentially expressed genes in PNETs identified by cDNA microarray

Based upon the WHO criteria, 19 PNET samples were initially classified into 3 histologic groups: WDETs of benign behavior ($n=3$), WDETs of low-grade malignant behavior ($n=9$), and WDECs ($n=7$; Table 2). When comparing benign and low-grade malignant WDETs with WDECs, 112 genes were differentially expressed by at least 1.5-fold with a P value <0.05. Hierarchical clustering revealed two distinct clusters (Fig. 1): the 3 benign WDETs clustered together with 8/9 low-grade malignant WDETs and 1 WDEC (‘benign cluster’), and 6/7 WDECs clustered together with the 1 remaining low-grade malignant WDET (‘malignant cluster’).

In the ‘malignant cluster’, 71 genes were up-regulated and 41 genes were down-regulated (Fig. 1). Supplementary Tables 1 and 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/, contain lists of genes over- and under-expressed in the ‘malignant cluster’. GO analysis revealed that among the up-regulated genes in this cluster, the most frequent and statistically significant molecular function classifiers were ‘transcription regulator’ (11 genes, $P=0.006$) and ‘binding’ (46 genes, $P=0.008$; Fig. 2a). Of the genes with binding activity, 28 had protein-binding activity, 19 had ion-binding activity, 16 had nucleic acid-binding activity, 8 had nucleotide-binding activity, 4 had chromatin-binding activity, and 1 had antigen-binding activity (Fig. 2b).

Hierarchical clustering also revealed that genes located on chromosomes 11 and 17 were over-represented in the 112 differentially regulated genes. Specifically, 8/112 (7.1%) of these genes were located on chromosome 11; 2 were up-regulated in the ‘malignant cluster’; and 6 were up-regulated in the ‘benign cluster’. Of these genes 10/112 (8.9%) were located on chromosome 17, and 8 were up-regulated in the ‘malignant cluster’ but only 2 were up-regulated in the ‘benign cluster’. Most of these genes (80%) were located on chromosome 17q.

Of note, a correlation was observed between mRNA expression and the hormonal profile of these tumors. Insulin mRNA levels were 18.6-fold higher in insulinomas compared with non-insulinomas, gastrin mRNA levels were 31.6-fold higher in gastrinomas compared with non-gastrinomas, and glucagon mRNA levels were 26-fold higher in glucagonomas compared with non-glucagonomas.

Validation of selected genes with quantitative real-time-PCR

The four most highly up-regulated genes in the ‘malignant cluster’ of PNETs (*FEV*, *ADCY2*, *NR4A2*, *GADD45β*) were selected for further validation by qRT-PCR. In the microarray studies, *FEV* was up-regulated 11.61-fold, *ADCY2* was up-regulated 4.47-fold, *NR4A2* was up-regulated 4.45-fold, and *GADD45β* was up-regulated 3.28-fold in the ‘malignant’ cluster. Statistically significant overexpression of all four genes was confirmed by qRT-PCR (*FEV*: 37-fold, $P=0.007$; *ADCY2*: 55-fold, $P=0.026$; *NR4A2*: 15.2-fold, $P=0.0006$; *GADD45β*: 5-fold, $P=0.002$; Fig. 3). In all cases, the microarray studies underestimated the extent of up-regulation.

Analysis of potential candidate genes

In addition to the identification of *FEV*, *ADCY2*, *NR4A2*, and *GADD45β* as novel genes that may play a role in tumor progression, we were curious whether specific candidate oncogenes and tumor suppressor genes such as MEN1, retinoic acid receptor-β, hMLH1, RASSF1, Her2/neu, cyclin D1, p16^{INK4a}/p14^{ARF}, p18^{INK4c}, and p27^{Kip1} were differentially regulated. However, none of these genes was differentially regulated in our microarray study. In addition, angiogenic factors including aFGF, bFGF, or VEGF were not differentially regulated.

Another group of candidate genes are the receptor tyrosine kinases, which are frequently activated in human cancers. These are particularly attractive candidates, as tyrosine kinase inhibitors are promising as molecularly targeted agents. There was no statistically significant difference in expression of PDGFR-α or PDGFR-β, although there was a trend towards higher expression levels (2.3-fold increase) of PDGFR-α in WDECs compared with WDETs. However, given their potential clinical importance and potential biological relevance in neuroendocrine tumorigenesis, we performed immunoblot analysis to evaluate protein expression levels (Fig. 4). PDGFR-α was expressed in 94% of PNETs. It was present in 4/5 (80%) benign WDETs, 8/8 (100%) low-grade malignant WDETs, and 5/5 (100%) WDECs. It was also detected in 1/3 (33%) normal

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samples, although at much lower levels. PDFGR-β was expressed in 17/18 (94%) PNETs with no difference among tumor stages, and it was also expressed in 2/3 (66%) normal pancreatic samples.

To evaluate PDGFR-β activation, the phosphorylation status at Tyrosine 751 or Tyrosine 716 was determined. Thirteen of eighteen (72.2%) PNETs were phosphorylated at Tyr751 (3/5 benign WDETs, 6/8 low-grade malignant WDETs, and 4/5 WDECs) and no phosphorylation was detected in any of the three normal samples (Fig. 4). Phosphorylation of Tyr716 was observed in 13/18 (72.2%) PNETs, occurring in 20% of benign WDETs, 87.5% of low-grade malignant WDETs, and 80% of WDECs. It was also seen in one of the three normal tissues. Overall, 15/18 (83%) PNETs demonstrated phosphorylation at one or both of these sites. Thus, the PDGFR-β subunit was frequently expressed and activated in PNETs, suggesting that tyrosine kinase inhibition of PDGFR may be a successful therapeutic approach.

Differentially expressed genes in GI-NETs versus PNETs identified by cDNA microarray analysis

The 25 malignant tumor samples were grouped into 6 GI-NETs and 19 PNETs (for sample details see Tables 2 and 3). Between the two groups 385 genes were differentially expressed by at least 1.5-fold with a P value <0.05. Hierarchical clustering revealed that GI-NETs clustered together in one group and PNETs in another (Fig. 5), indicating that gene expression patterns can indeed distinguish these NET subtypes. When compared with PNETs, 157 genes were up-regulated and 228 genes were down-regulated in the GI-NETs (Fig. 5). Supplementary Tables 3 and 4, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/, illustrate the genes over- and under-expressed in GI-NETs. We also performed an analysis excluding samples in which only a liver metastasis was available and confirmed that GI-NETs did differ in their genetic signature from PNETs. The four remaining primary GI endocrine tumors clustered together with one WDEC and the other 18 PNETs represented another cluster (data not shown).

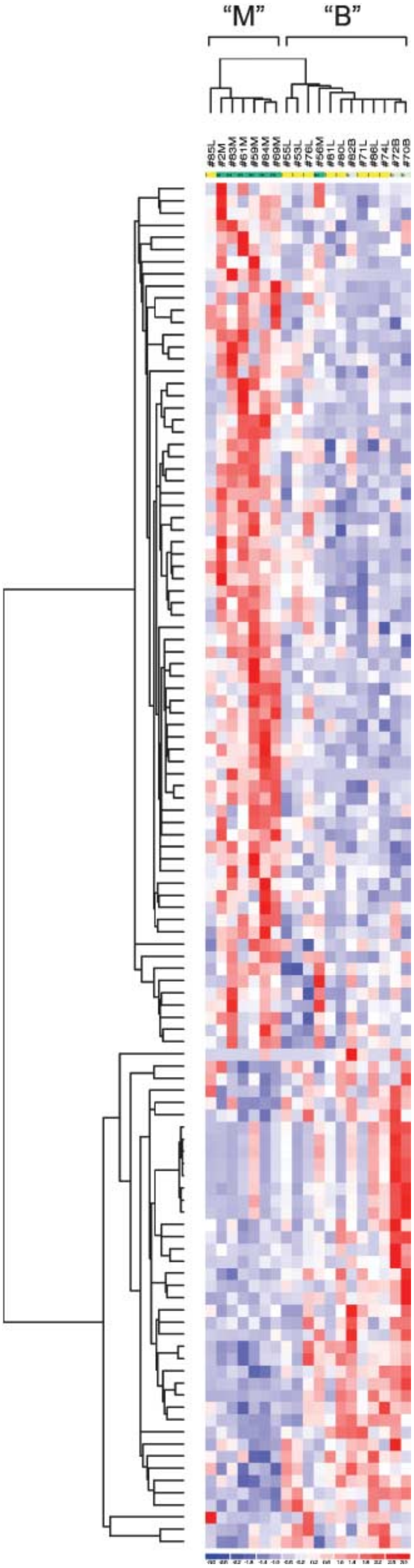

Figure 1 Hierarchical clustering of genes in PNET pathogenesis reveals that malignant PNETs cluster apart from benign and low-grade malignant PNETs. Each row represents a cDNA clone on the Affymetrix chip and each column represents an individual tumor mRNA sample. Red represents overexpressed genes, and blue represents underexpressed genes. Benign WDET: samples 70, 72, 82; low-grade malignant WDET: samples 53, 55, 71, 74, 76, 80, 81, 85, 86; and malignant WDEC: samples 2, 56, 59, 61, 69, 83, 84.

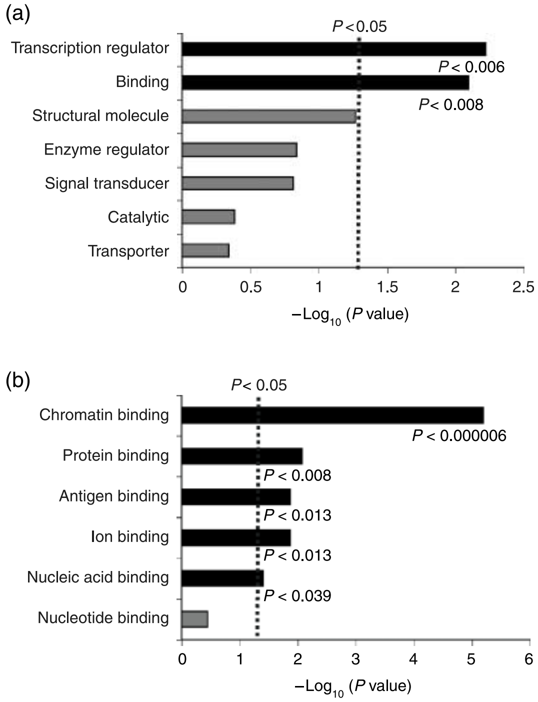

Figure 2 (a) Gene ontology analysis reveals that the molecular function categories 'transcription regulation' and 'binding' are significantly overrepresented ($P<0.05$) among the set of up-regulated genes in the 'malignant' cluster of PNETs. (b) Detailed GO analysis for the molecular function category 'binding' among the set of up-regulated genes in the 'malignant' cluster of PNETs reveals several significantly overrepresented sub-categories ($P<0.05$, dark gray bars). The graphs display the negative log-transformed P values.

GO analysis revealed that among the up-regulated genes in GI-NETs, the most statistically significant molecular function classifiers were 'transporter' (19 genes, $P=0.00078$) and 'motor activity' (3 genes, $P=0.018$; Fig. 6a). Of the up-regulated genes with transporter activity, there were 12 with ion transporter activity, 8 with channel or pore class transporter activity, 3 with ATPase activity, 1 with neurotransmitter transporter activity, and 1 with drug transporter activity (Fig. 6b). This is in sharp contrast to the group of malignant PNETs, where genes involved in 'binding' and 'transcription regulation' were the most statistically significant.

Hierarchical clustering also revealed that chromosomes 1, 2, 5, 8, 9, 12, 14, 15, 18, and X were overrepresented among the 385 genes. Specifically, more genes on chromosomes 1, 5, 8, and 14 were overexpressed in GI-NETs compared with PNETs, and more genes on chromosomes 2, 9, 12, 15, 18, and X were overexpressed in PNETs compared with GI-NETs.

Validation of selected genes with quantitative real-time-PCR

The three most highly up-regulated genes in GI-NETs identified by the microarray studies (*ECM1*, *VMAT1*, and *LGALS4*) were verified by qRT-PCR. In addition, we analyzed *RET* because it is critical in the pathogenesis of medullary thyroid cancer, another NET type. In the microarray studies, *ECM1* protein was up-regulated 28-fold, *VMAT1* by 25-fold, galectin 4 (*LGALS4*) by 24-fold, and *RET* by 3.62-fold in GI-NETs compared with PNETs. qRT-PCR confirmed the up-regulation of all these genes in GI-NETs (*ECM1*: 39-fold, $P=0.0011$; *VMAT1*: 523-fold, $P=0.0029$; *LGALS4*: 43-fold, $P=0.012$; *RET*: 28-fold, $P=0.012$; Fig. 7). *VMAT1* was not detectable in normal pancreatic tissue and most WDECs. Immunohistochemistry was performed for Ret on a larger series of small intestinal NETs and PNETs. There were 21 cases of small intestinal NETs (8 WDETs and 13 WDECs) and 65 cases of PNETs (14 benign WDETs, 43 low-grade malignant WDETs, and 8 WDECs) on the tissue microarrays. Among the intestinal NETs, 11% of the samples displayed weak or no Ret staining ($0–1^{+}$), whereas 89% exhibited strong staining ($2^{+}–3^{+}$). By contrast, 65% of PNETs exhibited weak staining ($0–1^{+}$) and only 35% exhibited strong staining ($2^{+}–3^{+}$; Fig. 8).

RET sequencing

RET is mutated in the MEN2 syndrome and familial medullary thyroid cancer. Because of this critical role in another NET type and the high levels of expression in GI-NETs, we sought to determine whether mutations in *RET* may also underlie GI-NET pathogenesis. Mutations occur primarily at three 'hotspot' regions within the cysteine-rich domain and the tyrosine kinase domains 1 and 2. DNA sequencing of these 'hotspot' regions in six GI-NETs and seven WDECs did not reveal any mutations. Incidentally, we identified two single base pair polymorphisms (CTT to CTA at codon 769 and TCG to TCC at codon 904), neither of which resulted in an amino acid change.

Discussion

The present study provides a comprehensive dataset of dysregulated genes in human PNETs and GI-NETs. We sought to identify differences in gene expression patterns between pancreatic WDETs and WDECs and to determine whether GI-NETs differ in their genetic profile from PNETs. Such a strategy may enhance our understanding of tumor pathogenesis and progression,

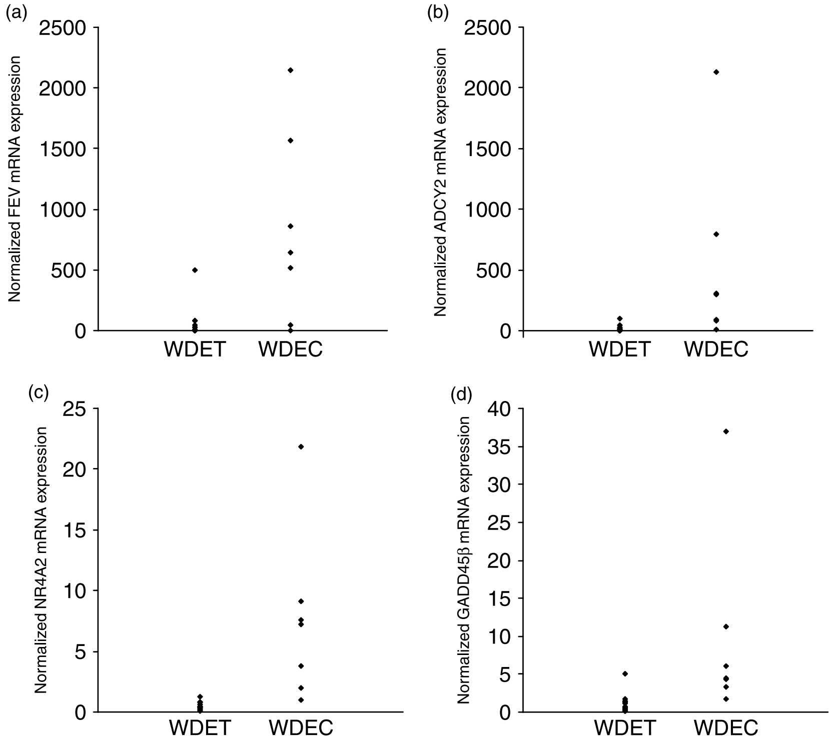

Figure 3 Analysis of the four most up-regulated genes in WDECs (n=7) relative to WDETs (n=12) by qRT-PCR: (a) FEV, (b) ADCY2, (c) NR4A2, and (d) GADD45β. Values are normalized to three normal pancreas samples.

as well as identify novel diagnostic markers and molecular targets for therapy.

Hierarchical clustering revealed that PNETs could be segregated on a molecular level into two groups. The 'benign cluster' comprised all benign WDETs, 8/9 low-grade malignant WDETs, and 1/7 WDECs. The 'malignant cluster' comprised 1/9 low-grade malignant WDETs and 6/7 WDECs. This is the first demonstration that the histologic subgroup of low-grade malignant WDETs shares more molecular similarities

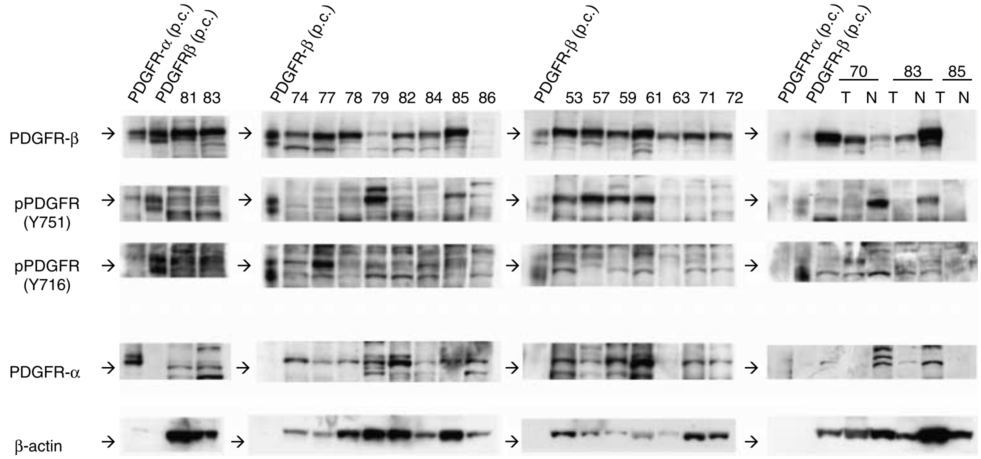
PDGFR-α (p.c.)
PDGFRβ (p.c.)
81
83
PDGFR-β (p.c.)
74
77
78
79
82
84
85
86
PDGFR-β (p.c.)
53
57
59
61
63
71
72
PDGFR-α (p.c.)
PDGFR-β (p.c.)
70
83
85
T
N
T
N
T
N
PDGFR-β
pPDGFR (Y751)
pPDGFR (Y716)
PDGFR-α
β-actin

Figure 4 Western blot analysis reveals that expression of PDGFR is common in PNETs and is observed both in early and late tumor stages. Furthermore, the PDGFR-β subunit is frequently activated by phosphorylation in PNETs. Benign WDETs: samples 63, 70, 72, 78, 82; low-grade malignant WDETs: samples 53, 57, 71, 74, 79, 81, 85, 86; malignant WDECs: samples 59, 61, 77, 83, 84; PDGRFα (p.c.), positive control for PDGRF-α; PDGFRβ (p.c.), positive control for PDGFR-β; pPDGFR, phospho-PDGFR-β; N, normal pancreatic tissue; T, tumor tissue; arrow, position of specific band.

with benign WDETs than with WDECs and provides a molecular correlation of the WHO classification scheme. The clinical behavior of low-grade malignant WDETs is generally good and consistent with this clustering result. We cannot completely exclude an influence of the heterogeneity in the tissue samples on our results. However, due to the rarity of PNETs, it was unfeasible to perform gene expression analyses for each individual hormonal subtype, and it was hypothesized that there may be fundamentally similar mechanisms that underlie all tumor subtypes. Of note, within the group of low-grade malignant WDETs, only 55% were insulinomas, indicating that there was a diversity of tumor types at each stage analyzed.

GO analysis revealed that the molecular functions of ‘binding’ and ‘transcriptional regulation’ were significantly overrepresented in the malignant cluster, possibly reflecting novel pathways that are critical for tumor progression. In addition, genes on chromosomes 11 and 17 were overrepresented in PNETs. This is consistent with published comparative genomic hybridization (CGH) literature, which has shown that genomic gains are common on chromosome 17 (Terris *et al*. 1998, Speel *et al*. 1999, Stumpf *et al*. 2000) and frequently associated with malignant behavior (Speel *et al*. 2001).

The four most highly up-regulated genes in WDECs (*FEV*, *ADCY2*, *GADD45β*, and *NR4A2*) have not previously been implicated in the pathogenesis of PNETs. Two of these, *GADD45β* and *NR4A2*, regulate apoptosis. GADD45β can block apoptosis induced by IL-1β (interleukin-1β) in cultured islet cells (Larsen *et al*. 2006). FEV is a member of the ETS family of oncogenic transcription factors (Peter *et al*. 1997). Further functional studies will be necessary to determine the specific roles these genes may play in PNET pathogenesis and whether they may ultimately serve as novel therapeutic targets that have an impact upon patient management.

We then investigated whether certain target genes that have been previously implicated in PNET pathogenesis were differentially expressed. Immunoblot analysis revealed that PDGFR-α and -β were expressed in PNETs regardless of stage. More importantly, PDGFR-β was activated by phosphorylation in the majority of PNETs. Others have reported high levels

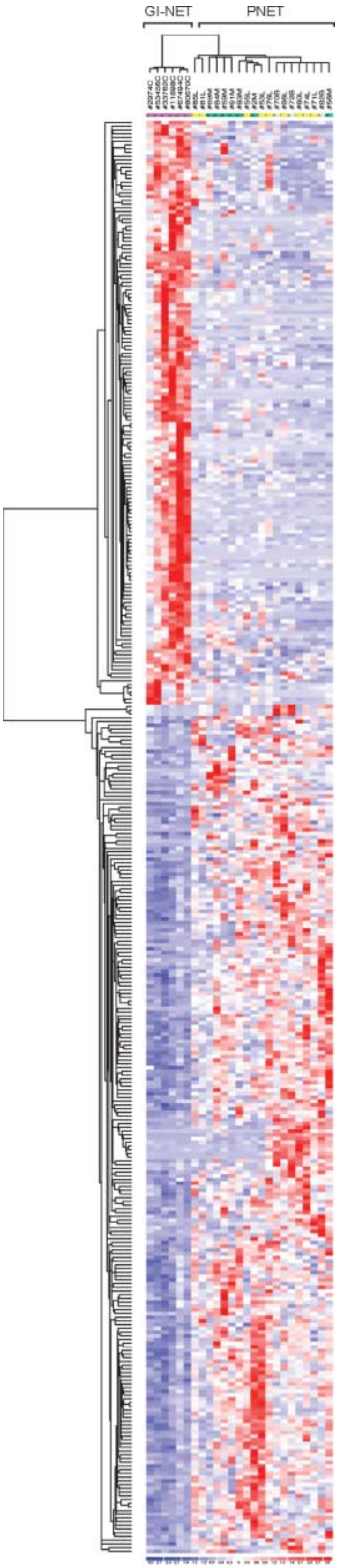

Figure 5 Hierarchical clustering of genes differentiates GI-NETs and PNETs. Each row represents a cDNA clone on the Affymetrix chip and each column represents a tumor mRNA sample. Red represents overexpressed genes, and blue represents underexpressed genes. Sample numbers for PNETs are the same as in Fig. 1, GI-NETs: samples 2974, 11898, 33762, 53456, 67494, 80670.

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of expression of PDGFR-α, PDGFR-β, and c-Kit in PNETs, but no assessment of receptor activation has been previously performed (Fjallskog *et al.* 2003). The possibility that PDGFR is expressed in mesenchymal components such as fibroblasts or pericytes (Pietras *et al.* 2003) cannot be excluded. Nevertheless, a specific PDGFR tyrosine kinase inhibitor may be a promising option for the treatment of PNETs. Observations of antitumor activity associated with receptor tyrosine kinase inhibitors further support a potential role for PDGFR in PNETs. In a multi-institutional study, treatment with sorafenib, a small molecule inhibitor with a spectrum of activity that includes VEGFR-2 and PDGFR-β, was associated with objective radiologic partial responses in 11% of PNET patients (Hobday *et al.* 2007). In a second study, treatment with sunitinib, which targets a similar spectrum of receptor tyrosine kinases, was associated with a 13% partial response rate in PNETs (Kulke *et al.* 2005). These observations, combined with our findings of PDGFR-β activation in PNETs, support further investigation of specific PDGFR-β inhibition as a clinical strategy in this tumor type. Interestingly, construction of a protein–protein interaction map revealed a novel connection between PDGFR-β and two highly expressed genes in PNETs, GADD45β, and NR4A2 (Supplementary Figure 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/). Activation of PDGFR-β may therefore be involved in the regulation of apoptosis in PNETs.

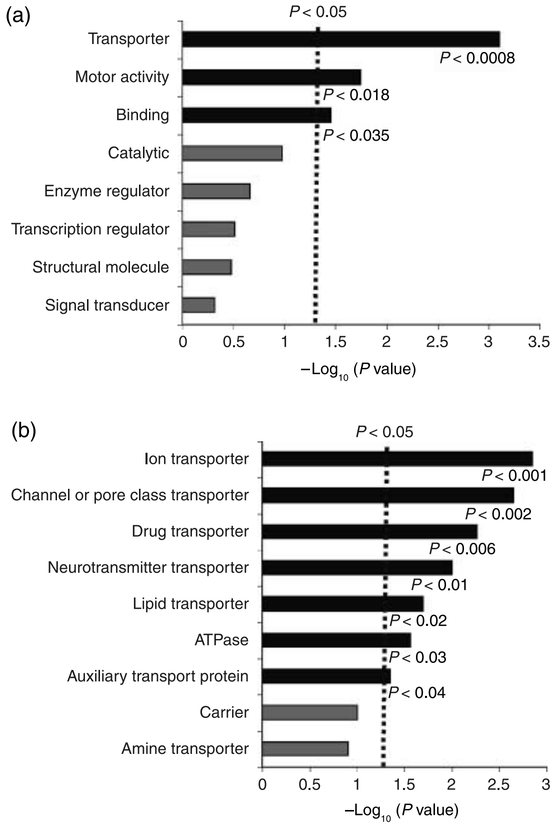

Figure 6 (a) Gene ontology analysis reveals that the molecular function categories 'transporter', 'motor activity' and 'binding' are significantly overrepresented ($P<0.05$) among the set of up-regulated genes in GI-NETs. (b) Detailed GO analysis for the molecular function category 'transporter' among the set of up-regulated genes in GI-NETs reveals several significantly overrepresented sub-categories ($P<0.05$, dark gray bars). The graphs display the negative log-transformed P values.

We provide the first description that GI-NETs cluster separately from PNETs by microarray analysis. Although the GI-NETs in this study comprised both primary tumors ($n=4$) and metastases ($n=2$), we can exclude an influence of the heterogeneity of samples on our clustering result, as GI-NETs still clustered independently from PNETs when metastases were excluded from the analysis (data not shown). GO analysis revealed that in contrast to PNETs, genes involved in ion transport, channel transport, and neurotransmitter transport were significantly over-represented in GI-NETs. This may provide new insights into the pathogenesis of GI-NETs. Hierarchical clustering also revealed that genes on chromosomes 9 and 18 were underexpressed in GI-NETs, possibly reflecting chromosomal deletions that have been reported in CGH and LOH (loss of heterozygosity) studies of these tumors (Kytola *et al.* 2001, Tonnies *et al.* 2001, Wang *et al.* 2005).

Of the three most highly up-regulated genes in GI-NETs (*VMAT1*, ECM1, and *LGALS4*), *VMAT1* and *LGALS4* have been previously described in this context (Nilsson *et al.* 2004, Vikman *et al.* 2005, Rumilla *et al.* 2006). Galectin 4 is expressed in the alimentary tract, where it is a component of adherens junctions or lipid rafts in the microvillus membrane (Huflejt & Leffler 2004) and is strongly expressed in ileal carcinoids (Rumilla *et al.* 2006). *ECM1* is expressed in highly vascularized organs (Mongiat *et al.* 2003) and over-expressed in a number of malignant epithelial tumors (Kebebew *et al.* 2005). Finally, we demonstrated that *RET*, an oncogene encoding a transmembrane receptor tyrosine kinase, is up-regulated in GI-NETs. This observation was confirmed by immunohistochemistry of a large panel of intestinal and PNETs. Ret binds glial cell line-derived neurotrophic factor family members and activates MAPK/ERK, PI3K, JNK, p38MAPK, and phospholipase C γ (Arighi *et al.* 2005). Although no somatic mutations were identified, the high expression of *RET* in GI-NETs suggests that it may be an attractive therapeutic target. SU11248 is an inhibitor of multiple tyrosine kinases including RET

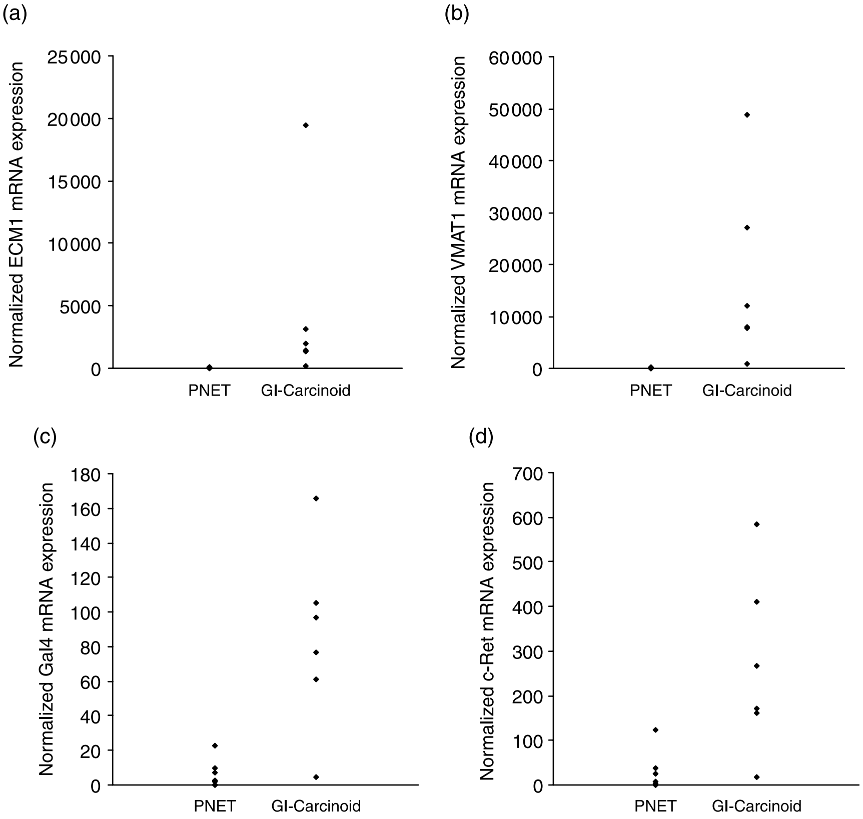

Figure 7 Analysis of the three most up-regulated genes and RET in GI-NETs ($n=6$) compared with malignant PNETs ($n=7$) by qRT-PCR. (a) ECM1, (b) VMAT1, (c) LGALS4, and (d) RET. Values are normalized to three normal pancreas samples.

(Kim *et al.* 2006), and a phase II study of SU11248 as a single agent in 39 patients with advanced GI-NETs revealed a 5% partial response rate (Kulke *et al.* 2005). *RET* may therefore play a pathogenic role in GI-NETs, but further investigation of targeted agents is required.

In comparison to published reports of gene expression profiles in NETs, our study has provided several new insights. Previous studies compared WDETs with normal islet controls (Maitra *et al.* 2003), MEN-1 associated NETs with normal islets (Dilley *et al.* 2005), PNETs with normal pancreas, pancreatitis, and pancreatic adenocarcinoma (Bloomston *et al.* 2004), non-functioning PNETs and their metastases with normal islets (Capurso *et al.* 2006), and metastatic with non-metastatic PNETs, primarily non-functioning (Hansel *et al.* 2004, Couvelard *et al.* 2006). Interestingly, there was no significant overlap between the identified genes in these studies and our current analysis. We hypothesize that this poor concordance is most likely a reflection of the different study designs, software platforms, data analysis parameters, and sample subtypes. In contrast to three reports that are most similar to ours (Hansel *et al.* 2004, Capurso *et al.* 2006, Couvelard *et al.* 2006), we studied a broader mix of PNET subtypes, not exclusively non-functioning PNETs, and this may potentially explain the disparity. With respect to technical differences, we utilized an Affymetrix platform, whereas Couvelard *et al.* obtained microarray chips from the Sanger center. In addition, we utilized the DChip program for data analysis, whereas Couvelard *et al.* performed their analysis with GeneSpring. Capurso *et al.* also utilized Affymetrix chips. However, their study differed significantly in that they compared PNETs with normal islets, whereas our comparison was between PNETs of different stages. Although Affymetrix chips were also used by Hansel *et al.* their analysis comprised only 12 tumors, whereas our analysis included 24 tumors. Nevertheless, it should be noted that there were some similarities, as one study also identified an up-regulation of PDGFR-β in WDECs (Couvelard *et al.* 2006). In addition, GO analysis in one study also revealed the molecular function classifier ‘binding’ as the most frequent in their up-regulated genes (Capurso *et al.* 2006). In aggregate, our results enhance the spectrum of genes implicated in NET pathogenesis.

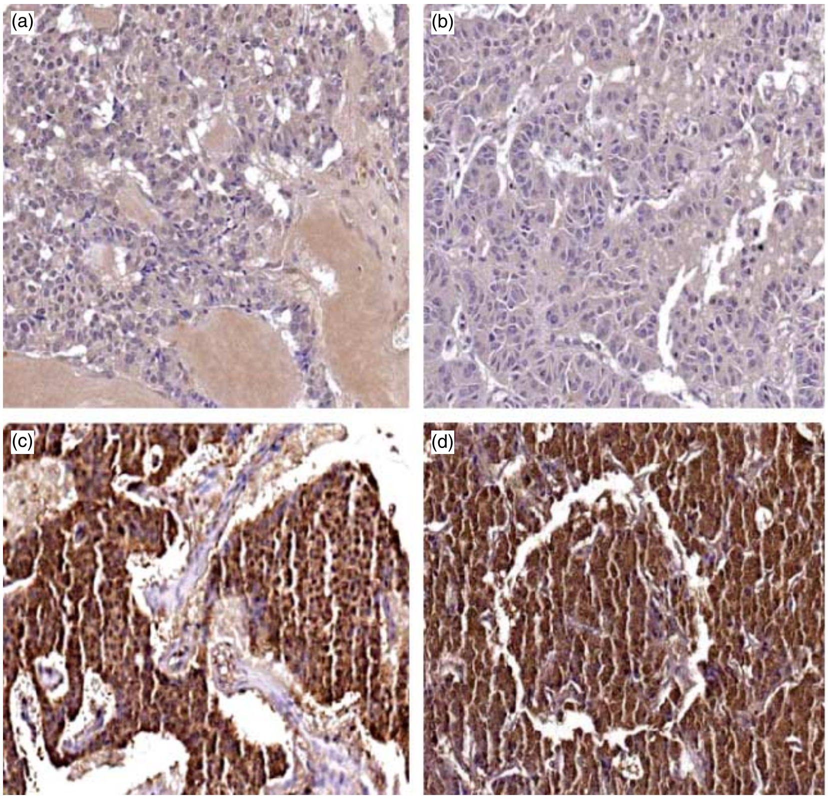

Figure 8 Representative immunohistochemical staining for Ret in PNETs (a ad b) and small intestinal NETs (c and d). In these PNET cases, Ret staining was scored as 0, and in the small intestinal NETs, Ret staining was scored as 3+.

In summary, we have identified a novel set of genes that may play a role in the pathogenesis and progression of PNETs and GI-NETs. Our results reveal a correlation with the WHO histologic classification on a molecular level. Furthermore, there are molecular signatures that distinguish PNETs from GI-NETs, reinforcing the principle that these two groups must be studied separately. By improving the molecular classification of these tumor subtypes, we may ultimately enhance our ability to predict tumor behavior, provide important new insights into the molecular biology and tumor pathogenesis, and design the next generation of targeted therapies. In this context, a potentially important role for PDGFR in the pathogenesis and treatment of PNETs has been revealed.

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