Gene expression profiles of progressive pancreatic endocrine tumours and their liver metastases reveal potential novel markers and therapeutic targets

G Capurso¹,², S Lattimore², T Crnogorac-Jurcevic², F Panzuto¹, M Milione¹, V Bhakta², N Campanini⁴, S M Swift³, C Bordi⁴, G Delle Fave¹ and N R Lemoine²

¹Digestive and Liver Disease Unit, II Medical School, University ‘La Sapienza’, Rome, Italy
²Molecular Oncology Unit, Cancer Research UK Clinical Centre, Barts and the London School of Medicine and Dentistry, John Vane Science Building, Charterhouse Square, London EC1 6BQ, UK
³Department of Surgery, University of Leicester, Leicester Royal Infirmary, Leicester, UK
⁴Department of Pathology and Laboratory Medicine, University of Parma, Parma, Italy

(Requests for offprints should be addressed to N R Lemoine; Email: nick.lemoine@cancer.org.uk)

Abstract

The intrinsic nature of tumour behaviour (stable vs progressive) and the presence of liver metastases are key factors in determining the outcome of patients with a pancreatic endocrine tumour (PET). Previous expression profile analyses of PETs were limited to non-homogeneous groups or to primary lesions only. The aim of this study was to investigate the gene expression profiles of a more uniform series of sporadic, non-functioning (NF) PETs with progressive disease and, for the first time, their liver metastases, on the Affymetrix human genome U133A and B GeneChip set. Thirteen NF PET samples (eight primaries and five liver metastases) from ten patients with progressive, metastatic disease, three cell lines (BON, QGP and CM) and four purified islet samples were analysed. The same samples were employed for confirmation of candidate gene expression by means of quantitative RT-PCR, while a further 37 PET and 15 carcinoid samples were analysed by immunohistochemistry. Analysis of genes differentially expressed between islets and primaries and metastases revealed 667 up- and 223 down-regulated genes, most of which have not previously been observed in PETs, and whose gene ontology molecular function has been detailed. Overexpression of bridging integrator 1 (BIN1) and protein Z dependent protease inhibitor (SERPINA10) which may represent useful biomarkers, and of lymphocyte specific protein tyrosine kinase (LCK) and bone marrow stromal cell antigen (BST2) which could be used as therapeutic targets, has been validated. When primary tumours were compared with metastatic lesions, no significantly differentially expressed genes were found, in accord with cluster analysis which revealed a striking similarity between primary and metastatic lesions, with the cell lines clustering separately. We have provided a comprehensive list of differentially expressed genes in a uniform set of aggressive NF PETs. A number of dysregulated genes deserve further in-depth study as potentially promising candidates for new diagnostic and treatment strategies. The analysis of liver metastases revealed a previously unknown high level of similarity with the primary lesions.

Endocrine-Related Cancer (2006) 13 541–558

Introduction

Pancreatic endocrine tumours (PETs) are rare neoplasms arising from pancreatic islet cells, and are classified as functioning (F) or non-functioning (NF), depending on the presence/absence of an associated syndrome due to excessive hormone secretion from cancer cells (Plockinger et al. 2004). Although considered to be ‘indolent’ tumours, at the time of diagnosis almost two-thirds of NF
PETs present with liver metastases, which is the main factor determining the patients’ outcome (Panzuto et al. 2005, Tomassetti et al. 2005). In addition, the different spontaneous behaviour of PETs (stable or progressive disease) plays a crucial role in their natural history. In fact, in some patients, the tumour may remain unchanged for a long period, even without treatment, whereas in others the tumour grows rapidly regardless of anti-proliferative treatment (Kaltsas et al. 2004).

The molecular pathways underlying the development and progression of PETs are poorly understood, with those genes most commonly associated with exocrine neoplasms seemingly uninvolved (Corleto et al. 2002).

Global expression profiling has recently been employed to investigate primary, non-metastatic, well-differentiated NF PETs (Maitra et al. 2003) and a mixed group of F/NF multiple endocrine neoplasia type I (MEN-I)-related PETs compared with pancreatic islets (Dilley et al. 2005). A further mixed group of F/NF well-differentiated PETs has been analysed, in order to compare profiles of primary lesions with and without metastatic ability (Hansel et al. 2004). Others have compared expression profiles of pooled biopsy material of PETs with that obtained from other pancreatic pathologies (Bloomston et al. 2004). However, some of these studies did not provide clinical or histopathological data sufficient to determine the clinical behaviour of the investigated patients, and none of them were specifically aimed at deciphering alterations occurring in patients with progressive disease, or analysed the expression profiles of their liver metastases.

The present study was therefore aimed at investigating the gene expression profiles of a more uniform and aggressive series of sporadic, NF PETs and, for the first time, their liver metastases, on the most comprehensive Affymetrix human genome U133 A (U133A) and U133B GeneChip arrays as, for this well-defined subset of tumours, the biology is poorly understood and new molecular targets are very much needed.

### Materials and methods

#### Tissues and cell lines

Freshly frozen tissue blocks of 15 samples (eight primary lesions and seven liver metastases) from ten individual PET patients undergoing either explorative or radical surgery were obtained from the Digestive and Liver Disease Unit, Rome University with full ethical approval. Sample selection was based on: (1) exclusion of F and MEN-I-associated tumours and (2) inclusion of those with liver metastases and progressive disease (spontaneous tumour growth, according to the World Health Organization (WHO) definition), documented by previously described imaging procedures (Panzuto et al. 2003). According to the WHO (Solcia et al. 2000), there were seven well differentiated endocrine carcinomas (WDEC) and three poorly differentiated endocrine carcinomas (PDEC). (All diagnoses were reviewed by an expert pathologist (CB) through histological/immunohistochemical examination.). All primary lesions were ≥3 cm in diameter and all specimens had a Ki-67 score >2% (Table 1), confirming the homogeneously

### Table 1

Clinical and histopathological features of the PET samples. All patients presented with liver metastases and progressive disease as defined by the WHO. Size and site refer to the pancreatic primary lesion. Outcome indicates that the patient is either dead of disease (DOD), or alive with disease (AWD), at given months from diagnosis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sex/age</th>
<th>Size (cm)</th>
<th>Site</th>
<th>Histology</th>
<th>Ki-67 (%)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>NET-P'1</td>
<td>M/39</td>
<td>7</td>
<td>Tail</td>
<td>WDEC</td>
<td>6</td>
<td>Lost to FU</td>
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<tr>
<td>NET-P2</td>
<td>M/38</td>
<td>4</td>
<td>Body</td>
<td>WDEC</td>
<td>3</td>
<td>DOD 8</td>
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<tr>
<td>NET-M/2</td>
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<td>—</td>
<td>—</td>
<td>4</td>
<td>—</td>
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<td>Tail</td>
<td>PDEC</td>
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<td>F/58</td>
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<td>Body</td>
<td>WDEC</td>
<td>5</td>
<td>AWD 43</td>
</tr>
<tr>
<td>NET-M4</td>
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<td>—</td>
<td>—</td>
<td>5</td>
<td>—</td>
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<td>Tail</td>
<td>WDEC</td>
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<td>DOD 19</td>
</tr>
<tr>
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<td>M/44</td>
<td>3</td>
<td>Tail</td>
<td>WDEC</td>
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<td>DOD 47</td>
</tr>
<tr>
<td>NET-P7</td>
<td>M/72</td>
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<td>Head</td>
<td>WDEC</td>
<td>3</td>
<td>Lost to FU</td>
</tr>
<tr>
<td>NET-P8</td>
<td>F/57</td>
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<td>3</td>
<td>AWD 76</td>
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<tr>
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<td>—</td>
<td>—</td>
<td>3</td>
<td>—</td>
</tr>
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<td>NET-M9</td>
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<td>30</td>
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<tr>
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<td>Tail</td>
<td>PDEC</td>
<td>11</td>
<td>DOD 50</td>
</tr>
</tbody>
</table>

*, Primary lesion; †, liver metastasis; FU, follow-up.
aggressive behaviour of this series. As previously described (Crnogorac-Jurcevic et al. 2003), frozen samples were evaluated by haematoxylin and eosin (H&E)-stained sections of each individual block and, if necessary, macrodissected by trimming in a cryostat to enrich tumour cellularity >70% as confirmed by repeated stained sections during cryosectioning.

One liver metastasis (neuroendocrine tumour (NET)-M5) in which tumour cells accounted for <30% was excluded, as the high cellularity sample obtained after trimming yielded too little RNA. Three human NET cell lines were also used: QGP-1 (metastatic pancreatic somatostatinoma) obtained from Cancer Research UK Cell Services (Clare Hall Laboratories, Potters Bar, UK); BON (metastatic pancreatic carcinoid) obtained from Professor S Rosewicz (Berlin, Germany) and CM (metastatic pancreatic insulinoma) which was a kind gift from Dr M Baroni (Endocrinology Unit, University ‘La Sapienza’, Rome, Italy). QGP and CM cells were cultured in RPMI and BON in E4 media (Cancer Research UK Media Production, Clare Hall Laboratories) supplemented with 10% heat-inactivated FCS (Gibco BRL, Life Technologies, Paisley, Strathclyde, UK). Human pancreatic islet cells, used as a reference throughout the experiments, were provided with appropriate ethical approval from the University of Leicester (Leicester, UK) and purified as described elsewhere (Ricordi et al. 2003). In brief, islets were prepared by digestion with either collagenase P or HI liberase in a Ricordi chamber, followed by separation using Ficoll sodium diatrizoate density gradient on a COBE 2991 cell processor (GAMBRO, BCT, Lakewood, CO, USA). Islets were maintained in a tissue culture incubator overnight in CMRL and 10% FCS, then concentrated in an Eppendorf tube, pelleted briefly using a microfuge for 10 s before removing the supernatant and snap freezing in liquid nitrogen. Each of the four samples of purified islets (1000 to 10000 islets with more than 70% cell purity) originated from different donors.

## RNA preparation

RNA was isolated sequentially from tissue samples and cell lines using Trizol solution (Gibco BRL, Life Technologies Inc., Frederick, MA, USA) using the manufacturer’s recommendations. The quality of RNA from each sample was verified by running an aliquot on the Agilent 2100 bioanalyzer (Agilent Technology, Palo Alto, CA, USA).

## Probe synthesis and hybridization

Double-stranded cDNA was synthesized from 10 µg total RNA with the SuperScript choice system (Invitrogen). Probe preparation, hybridization to a human test array, and subsequently to human U133A and B GeneChip set arrays, and scanning were all performed in accordance with the standard Affymetrix protocols (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

Scanned images were inspected and analyzed using established quality control measures.

## Data analysis

The analysis of the array data was carried out within the R statistical environment, freely available under GNU General Public Licence (http://www.r-project.org) using bioconductor libraries.

After generating log-transformed images from the *.CEL. files, hybridization quality was checked for spatial artefacts. One sample (NET-M6) was excluded at this stage, because of low quality of hybridization, and no further aliquots were available.

The U133A and B chips were independently normalized by the quantiles method (Bolstad et al. 2003) and background corrected using robust multi-array analysis (RMA) (Irizarry et al. 2003). The probe level data were summarized using median polishing, which results in log2 scale transformed data. The quality of the ensuing data was then inspected using a combination of boxplots, histograms and quantile plots to ensure a Gaussian-like distribution.

Differential genes were identified by the Welch two sample t-test, with subsequent P value correction using the false discovery rate (FDR) method (Benjamini & Hochberg 1995). Selected differential genes had an FDR corrected P value <0.05.

When sorted by variance, the top 2500 genes from U133A and B chips were combined to construct a dissimilarity matrix using Euclidean distance. Sample-wise agglomerative hierarchical clustering was performed using average linkage on a dissimilarity matrix, constructed using 1 – p, where p is the sample pair-wise Pearson correlation coefficient. The dissimilarity matrix was also employed for three-dimensional visualization of the data using multidimensional scaling (MDS).

The absolute distance (Euclidean) matrix was used for both the hierarchical clustering and MDS, as the normalization procedure (quantiles) guarantees that all arrays are approximately on the same
scale, thus negating the need to measure a similarity of pattern (correlation).

Data mining

We performed a PubMed search for the differentially expressed genes and the terms ‘Cancer’, ‘Pancreatic Endocrine’, ‘Endocrine Tumo(u)rs’ or ‘Neuroendocrine Tumo(u)rs’ or ‘Pancreas’, and all possible gene aliases through the SOURCE database (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and compared our findings with those of previous manuscripts regarding expression profiles and genomics of pancreatic islets or PETs (Jin et al. 2003, Maitra et al. 2003, Bloomston et al. 2004, Hansel et al. 2004, Wang et al. 2004, Dilley et al. 2005).

Gene ontology of the differentially expressed genes was investigated through the publicly available Amigo database (http://www.godatabase.org/cgi-bin/amigo/go).

Real-time quantitative PCR (QRT-PCR)

QRT-PCR was performed on the same samples employed for the microarray experiments, except for NET-P6 for which there was not enough material, and with the addition of NET-M5 for which there was not enough RNA for the array analysis. cDNAs were synthesized from 1 μg total RNA using random hexamers and Taqman reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. PCR reactions containing 10 ng cDNA, SYBR Green sequence detection master mix reagent (Applied Biosystems) and gene-specific primers were assayed on an ABI 7700 PRISM sequence detection system (Applied Biosystems). The target genes were carried out using five dilutions of reverse transcribed universal human reference RNA (Stratagene, La Jolla, CA, USA) to construct a standard curve. All reactions were performed in triplicate. Gene-specific primers designed using Primer Express (Applied Biosystems) are shown in Table 2. PCR product accumulation was measured in real time by the increase in fluorescence intensity of SYBR green. Data were analysed using the sequence detector program v1.7a (Applied Biosystems). Serial dilutions of cDNA from universal RNA were used to generate a standard curve for each target gene and 18s. The standard curves were then used to determine expression values for each target gene.

Immunohistochemistry

Immunohistochemical analysis (IHC) was performed on formalin-fixed, paraffin-embedded tissue sections from an independent set of 37 PETs (23 primaries, two nodal and 12 liver metastases). All samples except for five insulinomas were from patients with NF PETs. There were eight well-differentiated endocrine tumours, 26 WDECs and three PDECs. Furthermore, 15 non-pancreatic NETs (eight primaries and seven metastases), including five type-3 gastric carcinoids, one duodenal gastrinoma, five ileal and two rectal carcinoids and one poorly differentiated ovarian endocrine carcinoma, were employed. All samples were first stained with H&E to verify the histology, and subsequently with several markers and Ki-67 as previously described (Capurso et al. 2005). Commercial monoclonal antibodies against bridging integrator 1 (BIN1) and LCK were optimized on human brain and tonsil respectively. The immunoreactivity for BIN1 and LCK was evaluated on a semiquantitative scale considering both the extent (score: 0–4 for positive cells respectively <5%, 5–20%, 20–40%, 40–80% and >80%) and the intensity (score: 0–3) of staining. The product of both was used to obtain a final immunostaining score (range: 0–12). Samples with a score >4 were considered positive. The antibodies employed and their working dilutions are reported in Table 3. The immunostaining was visualized using the EnVision polymer method.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>QRT-PCR primer pair sequences and related amplicons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Forward primer</td>
</tr>
<tr>
<td>18s</td>
<td>5’-CGCCGCTAGAGGTGAAATTC-3’</td>
</tr>
<tr>
<td>SerpinA10</td>
<td>5’-AGGAAGGAAAGTCCTTTGAGCTG-3’</td>
</tr>
<tr>
<td>BIN1-nuclear</td>
<td>5’-AACAGTGAACACCGGCTG-3’</td>
</tr>
<tr>
<td>BIN1-cyto</td>
<td>5’-CAGCCCTTTGGAGGAGCTG-3’</td>
</tr>
<tr>
<td>LCK</td>
<td>5’-ATGACGCCCCAATTGCA-3’</td>
</tr>
<tr>
<td>BST2</td>
<td>5’-CCAGAAGGGCTTTCAGGTG-3’</td>
</tr>
</tbody>
</table>
(Dako Cytomation, Hamburg, Germany) followed by haematoxylin counterstaining.

**Statistical analysis**

IHC scores, expressed as mean (95% confidence interval) were evaluated by $t$-test. Proportions of subsets of patients, positive/negative at IHC, were compared by Fisher’s exact test. Correlation between microarray and QRT-PCR data was evaluated by Spearman rank correlation test. A $P$ value $<0.05$ was considered statistically significant.

**Results**

**Differentially expressed genes**

The 20 samples were classified into four groups: purified islets (I; four samples), primary lesions (P; eight samples), liver metastases (M; five samples) and cell lines (three samples). Islets were used as a baseline reference to which both primary and metastases were compared (I vs PM comparison), in an attempt to best capture the transcriptional modifications occurring in aggressive PETs. In addition, primary lesions were compared with metastases in order to decipher changes potentially related to disease progression.

At a significance level delimited by an FDR-corrected $P$ value $<0.05$, we identified 990 individual, annotated genes (794 from U133A and 196 from B chip), of which 667 genes were up-regulated and 323 genes were down-regulated in primary and metastatic tumours compared with purified islets (see Supplementary Table 1; http://erc.endocrinology-journals.org/content/vol13/issue2/).

By comprehensive literature and database searching, we identified only 20 of the 667 up-regulated genes as being previously described in PETs, including tumour protein p53 (TP53) (Lam & Lo 1998), fibronectin (Maitra et al. 2003), elongation of very long chain fatty acid-like 2 (Bloomston et al. 2004), coagulation factor V (Hansel et al. 2004), mitogen activated protein kinase (MAPK3) (Guo et al. 2003), chorionic gonadotrophin-β (Baudin et al. 1999) and cerebral cavernous malformations 1 (Dilley et al. 2005). An additional 177 genes have already been described in cancer types other than PETs, with findings confirming up-regulation for 63 of them, including genes such as NOTCH Homologue 3 (NOTCH3), LCK, SCF, neuropeptide Y receptor Y1 and the apoptosis inhibitors BCL-2 antagonist of cell death (BAD) and baculoviral IAP repeat containing 1 (BIRC1).

The most frequent gene ontology molecular function classifiers for the up-regulated genes are detailed in Fig. 1 and are binding (159 genes, 24% of total) and catalytic activity (114 genes, 17% of total). Of the up-regulated genes with binding activity, 60 had a DNA-binding activity and 49 a protein-binding activity, including receptor binding for nine of them. Almost all the 79 genes related to signal transducer activity had a receptor activity. Amongst those genes with catalytic activity there were 38 with transferase, 32 with hydrolase and 27 with oxidoreductase activity. Of the 26 genes with a structural constituent activity, 15 were extracellular matrix constituents. Moreover, analysing both the gene ontology molecular function and cellular component data, we could classify 26 of 667 up-regulated genes as related to the extracellular matrix, most of which have been described previously in pancreatic adenocarcinoma but not in PETs, thus suggesting a similar cancer–stroma crosstalk.
Out of the 323 down-regulated genes, 17 (including insulin, glucagon and somatostatin) have been reported to be expressed in normal pancreatic islet cells, and 18 (including lipase and elastase) in normal pancreatic acinar cells. These differences are probably due to the loss of functioning islet cells in NF PETs, and to some contamination of purified islets with residual acinar cells, as also suggested by similar findings in studies employing purified islets as a reference (Maitra et al. 2003, Dilley et al. 2005). An additional 21 down-regulated genes have already been described in PETs, including fragile X mental retardation gene/autosomal homologue 1 (Maitra et al. 2003), immediate early response 3 (Dilley et al. 2005), CD44 antigen (Dilley et al. 2005) and growth arrest and DNA damage-inducible α (Maitra et al. 2003) as well as vascular endothelial growth factor (VEGF) whose down-regulation in malignant PETs has been specifically reported (Couvelard et al. 2005). Of note, the finding of down-regulation of topoisomerase-1 could be related to the reported ineffectiveness of topotecan in PETs (Ansell et al. 2004). A further 22 down-regulated genes have already been described as underexpressed in cancer types other than PETs, including the tumour suppressors cylindromatosis (turban tumour syndrome), forkhead box P1 and Kruppel-like factor 6.

The comparison of primary and metastatic lesions applying the same stringent criteria employed for the IvsPM comparison yielded no significant data. This is not surprising given the pattern of expression seen with the multivariate analysis. In fact, a hierarchical dendrogram (Fig. 2A) shows not only
Figure 2 Cluster analysis of the samples employed. Expression profiles of the most variable genes, from both U133A and B chips, visualized by both classical agglomerative hierarchical clustering and multidimensional scaling. (A) Dendrogram representing the similarity in expression of each experimental sample. These can be easily distinguished based upon their origin with all islet specimens clustering together, and primary tumours being placed close to metastatic samples. Paired primary and metastatic samples are shown as linked with a solid red line. CM, QGP and BON cell lines lie on a completely separate branch. (B) Multidimensional scaling of the same data, when projected onto three-dimensional space better preserves the structure of the multidimensional data, with the spatial separation corresponding to the dissimilarities between the samples in the original high dimensional space. Each sphere represents an individual sample and is coloured accordingly: metastases, red; primaries, blue; islets, green; cell lines, orange. Matched primary and metastatic samples 2, 4 and 8 are labelled and linked with a solid black line.
Figure 3 QRT-PCR for SERPINA10. QRT-PCR data confirm the up-regulation of SERPINA10 both in primary (shaded bars) and metastatic (solid bars) lesions, compared with the mean of the four normal islets samples (open bar), while no signal is seen in the cell lines. Relative expression represents the ratio of target gene to 18s expression level in the same sample.
Figure 4  QRT-PCR for BIN1. QRT-PCR data for BIN1 nuclear (open bars) and cytoplasmatic (solid bars) isoforms suggest higher expression of the latter isoform in all samples but the CM cell line. Moreover, the up-regulation of BIN1 is far more evident for the cytoplasmatic isoform.
a high similarity between all the matched primary and metastatic samples, but also an overall similar clustering of primaries and liver metastases. Also PDECs did not cluster differently from WDECs, while there was a distinct dissimilarity between the cell lines and the tumour samples, as is more clearly evident in the MDS plot (Fig. 2B).

**Several genes were selected for further validation**

**Protein Z-dependent protease inhibitor (SERPINA10)**

In the microarray experiments, the expression of SERPINA10 was up-regulated in PETs compared with normal islets, with a fold difference of 6.34 and FDR corrected $P$ value $= 0.005$. SERPINA10 expression did not differ between primary and metastatic lesions, and was not evident in the cell lines. Overexpression of SERPINA10 was confirmed by QRT-PCR as shown in Fig. 3, with a good positive correlation with the array findings ($r = 0.809; \ 95\% \ CI = 0.5227–0.9312; \ P = 0.0017$). No antibodies are presently available for SERPINA10 IHC.

**BIN1**

In the microarray experiments, BIN1 expression was up-regulated in both primary and metastatic lesions, compared with normal islets, with a 4.49-fold difference (FDR corrected $P = 0.02$). BIN1 expression did not differ between primary and metastatic lesions, and was present in the cell lines. As BIN1 encodes a number of isoforms, with different function and intracellular localization, we analysed by QRT-PCR the expression of the most common nuclear isoform (variant 8, NM_004305), as well as that of a cytoplasmic isoform (transcript variant 1, NM_139343), which encodes the longest isoform, also called amphiphysin II, and which binds dynamin, synaptojanin and clathrin (Wechsler-Reya et al. 1997, DuHadaway et al. 2003). Figure 4 illustrates that although both isoforms showed overexpression compared with

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**Figure 5** BIN1 immunostaining in normal pancreatic islet cells and in PETs. (A) BIN1 staining in the normal pancreas, with intense cytoplasmic staining confined to the α (glucagon-producing) cells, sited to the edge of the islet, as confirmed by staining of serial sections with glucagon (data not shown). In (B) a case of pancreatic primary NF PDEC is shown, with diffuse BIN1 staining, and some plasma membrane reactivity. Liver metastases of NF WDECs samples in (C and D) show a similar pattern of staining. Magnification x200.
Figure 6 QRT-PCR for LCK. QRT-PCR data confirm the up-regulation of LCK both in primary (shaded bars) and metastatic (solid bars) lesions, compared with normal islets (open bar). A high signal is seen in the BON cell line, with lower values for QGP-1, and no significant signal for CM.
normal islets, this difference was much higher for the cytoplasmic (mean of 20-fold difference) compared with the nuclear (mean of 8.35-fold difference) isoform. Microarray data showed a good positive correlation with QRT-PCR for both the cytoplasmic ($r = 0.561$; 95% CI = 0.1104–0.8206; $P = 0.0248$) and the nuclear isoforms ($r = 0.777$; 95% CI = 0.4729–0.9157; $P = 0.0019$).

In the healthy pancreas, BIN1 immunostaining was detected in the cytosol of glucagon-positive α cells in islets (Fig. 5A). High levels of BIN1 expression were detected in the cytosol of 23 of 37 (62.1%) PETs, as well as membrane immunoreactivity in some cells (Fig. 5B–D), and in only one of the 15 (6.6%) non-pancreatic NETs evaluated ($P = 0.0004$). Moreover, BIN1 positivity was more frequent in liver metastases (nine of 12, 75%) compared with primary (13 of 23, 56.5%), or lymph node samples (one of two, 50%), although this did not reach statistical significance. In addition, no significant correlation between BIN1 staining and differentiation, proliferation or clinical outcome was found. None of the five F PET samples was BIN1 positive, compared with 23 of the 32 (71.8%) NF PETs samples. Moreover, of the 17 PET samples positive for at least one pancreatic hormone, only seven (41%) were BIN1 positive, compared with 16 of 20 samples negative for all hormones (80%, $P = 0.02$).

**Src-like kinase LCK (p56LCK)**

According to our microarray data, the expression of LCK was similarly up-regulated in both primary and metastatic PETs when compared with normal islets, with a 4.35-fold difference (FDR corrected $P = 0.02$). The overexpression of LCK at the RNA level was confirmed by QRT-PCR (Fig. 6), which showed a good positive correlation with the array findings ($r = 0.732$; 95% CI = 0.35–0.90; $P = 0.006$).

In the healthy pancreas, a diffuse, moderately intense LCK immunoreactivity was detected at the plasma membrane of most normal islet cells (Fig. 7A). In PETs, LCK staining was scored as positive in 18 of 37 samples (48.6%), compared with none of the 15 non-pancreatic NETs. LCK positivity was more frequent in PET liver metastases (eight of 12, 66.6%) compared with primaries (ten of 23, 43.4%) or nodes (neither of two), albeit without significant difference. When compared with that seen in normal islet cells, LCK staining in PETs showed a stronger intensity, and was not limited to the cytoplasmic membrane, being also intracellular.

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**Figure 7** LCK immunostaining in normal pancreatic islet cells and in PETs. (A) LCK staining in the normal pancreas, with diffuse, moderately intense immunoreactivity detected at the plasma membrane of most normal islet cells. In those PET samples positive for LCK, both extension and intensity were higher compared with islets (see the primary WDEC in (B) and its splenic invasion in (C)). Moreover, in PETs, LCK staining was not limited to the cytoplasmic membrane, being also intracellular, or in some cases, such as the malignant insulinoma shown in (D), cytoplasmic only. Magnification ×200.
Figure 8 QRT-PCR for BST2. QRT-PCR data confirm the up-regulation of BST2 both in primary (shaded bars) and metastatic (solid bars) lesions, compared with normal islets (open bar) with a high signal seen in the BON cell line (shaded bar at the right).
In some cases, staining was cytoplasmic only, without membrane positivity (Fig. 7D). No significant correlations between LCK staining and stage, differentiation, proliferation, functional status or clinical outcome were found.

**Bone marrow stromal cell antigen 2 (BST2 or HM1.24 antigen)**

In the microarray experiments, BST2 expression was up-regulated in PETs compared with islets, with a 4.35-fold difference (FDR corrected P value = 0.02), did not differ between primary and metastatic lesions, and was present in all cell lines, with high levels in BON. Overexpression was confirmed by the QRT-PCR data shown in Fig. 8, with a good positive correlation with the array findings (r = 0.772; 95% CI = 0.447–0.917; P = 0.0028). No antibodies were available for BST2 IHC.

**Discussion**

The present report provides a comprehensive dataset of dysregulated genes in highly aggressive NF PETs and their liver metastases. The study was performed with the aim of deciphering alterations potentially related to the progression of this disease, and of providing a source of possible biomarkers and/or molecular targets. The characteristics of our samples (Ki-67 >2, primary size ≥3 cm), together with the presence of liver metastases and the progression of disease in each single patient, were all known to be predictive of poor prognosis (Panzuto et al. 2005, Tomassetti et al. 2005), with both biotherapy and chemotherapy being poorly effective (O’Toole et al. 2004, Oberg & Eriksson 2005), as confirmed by the outcome of five of the seven patients we have had under care in the follow-up period.

Many clinical and biomolecular studies of digestive NETs have previously been limited by the heterogeneity of the series, in terms of site, functional status, stage, clinical behaviour and proliferation index. In this context, and in contrast to other studies (Maitra et al. 2003, Bloomston et al. 2004, Hansel et al. 2004, Dilley et al. 2005), we have investigated uniformly aggressive, NF PET samples and their metastases by employing a complete Affymetrix platform.

Of the 990 individual dysregulated genes (Supplementary Table 1) obtained comparing primary and metastatic lesions to islets, most have never been associated with PETs before. In particular, when comparing our results with those of previous expression profile studies and other publications conducted on PETs, we identified only 41 genes already described in this cancer type, none of them being reported in more than in a single study. The relatively poor concordance between different microarray studies is likely to be related to the different study designs, sample subtype, platforms and analysis employed, and is not a surprising finding, as already highlighted for pancreatic adenocarcinoma (Grutzmann et al. 2004). We have also analysed gene ontology function of the dysregulated genes, which suggests activation of different pathways, from changes in receptors and intracellular signalling to oxidative stress and stromal reaction.

As one of our aims was to provide potential novel biomarkers, we focused on genes previously not associated with PETs, which may be useful diagnostic or therapeutic targets, including genes for which specific inhibitors are already available.

SERPINA10 is a member of the serpin superfamily of proteinase inhibitors, normally synthesised by the liver and secreted into the plasma where it is involved in thrombosis (Han et al. 1998). We found SERPINA10 to be overexpressed in both our microarray and QRT-PCR experiments with 2-fold or higher overexpression in 57% of primary and 100% of metastatic lesions (Fig. 3).

To date, more than 500 serpins have been identified and classified in six subgroups (Van Gent et al. 2003). SERPINA10 shares 33% homology with α-1-antitrypsin (SERPINA1), the main blood plasma anti-proteolytic enzyme, which is the family archetype. SERPINA1 levels have been found to be increased in sera and tissues from different cancer types (Karashima et al. 1990, Higashiyama et al. 1992). SERPINA1 is degraded by matrix metalloproteinases, resulting in production of a cleaved protein which seems to promote tumour progression (Kataoka et al. 1999) and, interestingly, was amongst the up-regulated genes in metastatic PETs (Hansel et al. 2004). While the expression of a number of serpins others than SERPINA1 has been reported in other tumour types, this is the first demonstration of the expression of SERPINA10 in a cancer. While the role of this gene in PETs remains unknown, specific profiles for plasma levels of different serpins have been reported (Wojtukiewicz et al. 1998), and in our experiments α-1-antichymotrypsin (SERPINA3), another serpin commonly dysregulated in cancers (Bernacka et al. 1998), was down-regulated. It would therefore be of interest to evaluate whether specific changes in circulating levels of SERPINA10 (and other SERPINs) occur in PET patients, and
their possible relation with tumour stage and response to therapy.

BIN1 may represent another valuable biomarker for PETs. BIN1 was described initially through its ability to inhibit c-Myc-driven transformation, and since then has been reported to have a tumour suppressor activity, especially in prostate cancer (Ge et al. 2000). However, it has become clear that there are a number of different isoforms of BIN1 which present with specific tissue distributions and distinct functions (Wechsler-Reya et al. 1997, DuHadaway et al. 2003). Nuclear BIN1, as found in the prostate and breast, has a tumour suppressor activity due to an ability to activate caspase-independent cell death. In contrast, cytosolic expression of BIN1 is seen in quiescent brain cells. The latter BIN1 isoform has been alternatively named amphiphysin II, given the similarity with the neuronal protein involved in synaptic vesicle endocytosis. Of note, amphiphysins have been described in other neuroendocrine cells, such as enterochromaffin-like cells (Zanner et al. 2004) and adrenocorticotrophin-secreting cells (Sarret et al. 2004), and seem to play a part in the endocytic processes, but no BIN1 isoforms have been described in the pancreas previously.

Validation of our microarray results by means of QRT-PCR and IHC suggests that cytoplasmic forms of BIN1 related to endocytosis are prevalent in islet cells, and that they are strongly overexpressed in PETs (Fig. 4). By investigation at the protein level we could detect BIN1 only in the subset of α cells in normal islets, while its expression was extended to the vast majority of tumour cells in some two-thirds of PETs (Fig. 5). Notably, only one of the 15 non-pancreatic NETs evaluated stained positive for BIN1. This latter finding, together with the higher positivity for BIN1 observed in PET metastases, suggests a possible clinical utility for BIN1 immunostaining in the diagnosis of distant metastases with neuroendocrine features, as a predictor of a pancreatic origin. Indeed, some 10% of NETs present with liver metastases only, with no primary tumour found at the time of diagnosis, and immunohistochemical markers are employed to facilitate the diagnosis of the unknown primary. The higher percentage of BIN1 positivity in NF PETs, and in those samples negative for pancreatic hormones, makes this prospect as a marker for pancreatic origin even more interesting.

The overexpression of the src-like kinase LCK (p56LCK) is particularly relevant, and offers a potential novel therapeutic target for PETs. LCK is an src-like non-receptor protein tyrosine kinase, expressed mainly in T lymphocytes and thymocytes (Anderson et al. 1994). LCK is also aberrantly expressed in different cancer types and has been associated with a more aggressive tumour behaviour (Veillette et al. 1987, Nakamura et al. 1996). In our microarray experiments, as confirmed by QRT-PCR (Fig. 6), RNA levels of LCK were up-regulated similarly in primary and metastatic PET samples, with some expression also seen in the cell lines analysed. LCK protein expression was seen at the plasma membrane of normal islet cells, with a more prevalent and intense cytoplasmic staining in 43% of primary and 66% of liver metastasis samples. The reasons for the presence of cytoplasmic LCK immunoreactivity in transformed but not in normal islet cells are unclear. One may speculate that this is the result of an overproduction of the protein, with consequent accumulation before targeting to the plasma membrane. Alternatively, it is possible that LCK proteins in PETs are affected by mutations that correct palmitoylation or myristoylation, which are critical for its localization (Bijlmakers et al. 1999). As a number of inhibitors of the src family are available and have been successfully tested in different cancer models, including pancreatic adenocarcinoma (Yezhelyev et al. 2004), LCK and/or other similar kinases may also represent therapeutic targets for PETs. Moreover, the concomitant overexpression of the KIT-ligand stem cell factor (SCF) for which LCK can be a downstream effector (Krystal et al. 1998) is also of potential interest, as KIT expression has previously been described in PETs and therapy with inhibitors proposed (Fjallskog et al. 2003).

BST2, also named HM1.24 antigen, another gene whose expression was validated by QRT-PCR (Fig. 8), is a transmembrane protein expressed by several bone marrow stromal cell lines and fibroblasts (Ishikawa et al. 1995), and overexpressed in myeloma cells and in several solid tumour cell lines showing elevated invasive capacity (Walter-Yohrling et al. 2003). Overexpression of BST2 has also been described in pancreatic adenocarcinoma in a recent meta-analysis of expression profile studies (Grutzmann et al. 2005). Notably, a humanised anti-HM1.24 antibody has been developed and employed in clinical trials of multiple myeloma patients (Ozaki et al. 1999), and immunotherapy against HM1.24 is being developed (Rew et al. 2005). Further investigations of the expression and function of BST2 in PETs are warranted to establish whether it may represent a suitable target for therapy.
As the mechanism by which metastatic disease occurs is far from being clear, a second aim of this study was to explore the genetic abnormalities that differentiate primary from metastatic lesions. The two current hypotheses suggest either additional genetic changes in a subset of cells in primary tumours, which acquire the ability to metastasise (Fidler & Hart 1982), or that the metastatic ability of a tumour is an early event in the transformation process with cells of the primary tumour already carrying the metastatic signatures. Therefore, primary and metastatic lesions should be ‘strikingly similar’ (Bernards & Weinberg 2002). The second theory has been supported by recent microarray studies in colorectal (Koehler et al. 2004) and breast cancer (Hao et al. 2004, Lahdesmaki et al. 2004) where similar profiles for matched primary and metastatic lesions were observed, and which suggest a ‘stable’ genetic portrait for cancer during progression (Lacroix et al. 2004). Our current results are also in keeping with this hypothesis, as no differentially expressed genes able to distinguish primary from metastatic lesions, with all lesions clustering together, were identified (Fig. 2).

In our analysis, we employed isolated islets as a reference, these probably represent the best control one can realistically obtain for PETs. However, the possibility that some transcriptional difference will arise simply from the fact that islets contain different cell types, while PETs stem from a single progenitor cell, has to be considered, and makes validation of any candidate mandatory.

Finally, in the present paper, we have obtained for the first time the expression profiles of the only two human PET cell lines presently available, and of the pancreatic carcinoid cell line BON, and observed their clear separation from the tissue samples, as reported in other tissue/cell systems (Hess et al. 2001). This again raises a caution against extrapolating from data obtained in in vitro studies using cell lines.

In conclusion, we have provided a comprehensive list of differentially expressed genes in a uniform series of malignant NF PETs with very aggressive behaviour. A number of the dysregulated genes, including those validated in the study, deserve further in-depth study as potentially promising candidates for the development of new diagnostic and treatment strategies. The analysis of liver metastases revealed a previously unknown high level of similarity with the primary lesions, suggesting accumulation of most genetic abnormalities in the primary tumour. Confirmation of these results in other series of malignant PETs and primary and metastatic lesions obtained in other NETs, such as carcinoids, are needed to improve our knowledge of these relatively uncommon neoplasms. Furthermore, comparison of the present dataset with expression profiles of other entities that present with similar radiologic and clinical appearance, such as pancreatic adenocarcinoma and chronic pancreatitis, may help in identifying clinically relevant biomarkers for differential diagnosis.

Acknowledgements

The work was funded by grants from Cancer Research UK, the Italian Ministry for the University (MIUR) (2003063877_002), the Italian Ministry of Health, and by the University of Roma, ‘La Sapienza’ (C26A044873). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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