Global Expression Analysis of Well-Differentiated Pancreatic Endocrine Neoplasms Using Oligonucleotide Microarrays

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ABSTRACT

Purpose: **Pancreatic endocrine neoplasms (PENs) are rare, mostly well-differentiated endocrine neoplasms, whose biology has been poorly characterized. Global expression microarrays can document abnormal pathways that impact on tumorigenesis and disease progression.**

Experimental Design: **RNA was extracted from eight well-differentiated PENs and three highly enriched pancreatic islet cell samples (80–90% purity), and examined using the Affymetrix U133A oligonucleotide microarray. Microarray data were normalized using dCHIP (www.dCHIP.org) for identification of differentially expressed genes. PEN tissue microarrays were constructed from 53 archival PENs for immunohistochemical validation of microarray data.**

Results: **Sixty-six transcripts were overexpressed ≥3-fold in PENs compared with normal islet cells, including putative oncogenes (*MLLT10/AF10*), growth factors [insulin-like growth factor-binding protein 3 (*IGFBP3*)], cell adhesion and migration molecules (*fibronectin*), and endothelial elements (*MUC18/MelCAM* and *CD31*). A total of 119 transcripts were underexpressed ≤3-fold in PENs compared with normal islet cells, including cell cycle checkpoint proteins (*p21/Cip1*), the *MIC2* (CD99) cell surface glycoprotein, putative metastasis suppressor genes (*NME3*), and *junD*, a MEN1-regulated transcription factor. Using PEN tissue microarrays, we confirmed the differential up-regulation of IGFBP3 (70%) and fibronectin (22%) and differential down-regulation of p21 (46%) and MIC2 (CD99; 91%) in PENs *versus* normal pancreatic islets. IGFBP3 overexpression was significantly more common in metastatic (93%) *versus* primary PEN lesions (60%), $P = 0.022$. Fibronectin overexpression demonstrated a trend toward significance in lymphatic PEN metastases (55%) compared with primary PEN lesions (24%; $P = 0.14$).**

Conclusions: **Global expression analysis provides insight into tumorigenic pathways in PENs and may identify potential prognostic and therapeutic markers for these uncommon neoplasms.**

INTRODUCTION

Well-differentiated PENs,[7] commonly referred to as islet cell tumors, are a unique group of malignancies often characterized by a clinical neuroendocrine syndrome attributable to the selective overproduction and humoral circulation of pancreas-specific hormones (1, 2). Approximately 2000 new cases of well-differentiated PENs are diagnosed each year in the United States, and of these cases, 60–70% are associated with a clinical syndrome resulting from the secretion of a single functional hormone (3). The remaining one-third of PENs secrete no clinically detectable biologically active hormones and most often present as space-occupying lesions causing obstructive jaundice, upper gastrointestinal luminal obstruction, bleeding, or abdominal pain. Whereas the majority of well-differentiated PENs follow an indolent course, a substantial proportion of nonfunctional tumors are defined by aggressive biology resulting in early locoregional invasion of lymph node basins and adjacent organs, as well as metastases to the liver and beyond (3). Several histopathological parameters, including tumor size, angiolymphatic invasion, mitotic index, Ki-67 labeling index, and ploidy, have been used for predicting long-term outcome after surgical resection (4–7). However, apart from locoregional invasion and distant metastasis, there are no consistent determinants of malignant behavior for these neoplasms.

In contrast to the more common exocrine ductal adenocarcinomas, little is known about the molecular abnormalities or genotype-phenotype correlations that underlie PENs (reviewed in Ref. 8). This is likely a reflection of the small numbers of cases that are accessible for molecular studies. As a result, diagnostic and therapeutic approaches for these neoplasms have seen few advances in the last decade. We have previously demonstrated the power of global expression microarrays to identify a variety of novel tumor markers in exocrine pancreatic

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[7] The abbreviations used are: PEN, pancreatic endocrine neoplasm; IGFBP, insulin-like growth factor-binding protein; TMA, tissue microarray.

ductal adenocarcinomas, with immediate translational potential for patient care (9, 10). We now extend our gene expression studies to well-differentiated PENs; these studies document aberrantly activated pathways that may impact on tumorigenesis, and they identify potential cellular targets in these uncommon tumors. In this study, we identify nearly 200 differentially expressed genes in well-differentiated PENs *versus* enriched normal islet cells and validate a subset of these genes at the protein level using PEN TMAs. The overwhelming majority of differentially expressed genes we have identified have not been described previously in PENs and may serve as potential therapeutic targets and novel tumor markers for this disease.

MATERIALS AND METHODS

Selection of PEN Samples. Permission for this study was obtained through The Johns Hopkins Institutional Review Board (Joint Committee on Clinical Investigation). Snap-frozen tissue was obtained from eight pancreaticoduodenectomy resections at The Johns Hopkins Hospital performed for well-differentiated PENs; none of the tumors selected were high-grade neuroendocrine neoplasms. The clinicopathological features of these eight cases are listed in Table 1. There were four males and four females in the cohort; the median age was 46 years (range, 42–55 years). The median tumor size was 3.6 cm (range, 2.0–6.0 cm). Two patients had concurrent lymphatic metastases; none had associated hepatic metastases. No patient had an associated endocrine hypersecretion syndrome. Tumor samples were collected within 10 min of surgical resection, snap-frozen in liquid nitrogen, and stored at −80°C. H&E-stained sections from adjacent frozen tissue were prepared to confirm the diagnosis (2) and assess neoplastic cellularity. RNA was extracted from PENs containing >80% neoplastic cells and <10% necrosis on frozen section examination.

Human Islet Isolation. Because the normal bulk pancreas contains <2% islets of Langerhans, we used three enriched islet cell samples from cadaveric human pancreata as a normal control; islets were used for research purposes only when insufficient numbers were obtained for transplantation. Cadaveric human pancreata were obtained through the local organ procurement organization affiliated with the University of Pennsylvania School of Medicine (a Juvenile Diabetes Research Foundation islet cell distribution center); to the best of our knowledge, these individuals did not suffer from endocrine pancreatic pathology. Pancreatic islets were isolated using a modification of the automated Ricordi method (11). In brief, collagenase (Liberase; Roche, Nutley, NJ) at a concentration of 1.66 mg/ml in HBSS was infused into the main pancreatic duct using a hand-held syringe and a Webster cannula. Organs were digested at 37°C for 15–25 min in Ricordi chamber, which was agitated with a mechanical shaker to facilitate the digestion process. After digestion, the dispersed pancreatic tissues were washed three times with RPMI 1640 and resuspended in University of Wisconsin solution for 1 h. Then, the liberated islets were separated from exocrine tissues using the COBE 2991 and a top-loaded continuous Ficoll gradient (density range, 1.055–1.120). The isolation process was performed without xenogeneic serum. Islet fractions with highest purity (>80–90%) were snap-frozen for RNA extraction and microarray analysis.

RNA Extraction and Hybridization. Sample preparation and processing procedure were performed at the Roswell Park Cancer Institute Microarray Core Facility, as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA). Briefly, frozen PEN or islet samples were crushed in TRIzol (Invitrogen, Carlsbad, CA) by using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Total RNA was then extracted from the crushed tissue and cleaned using RNeasy columns according to the manufacturer's protocol (Qiagen, Valencia, CA). The integrity of total RNA was confirmed in each case using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Using 5–40 μg of total RNA, double-stranded cDNA was synthesized following SuperScript Choice system (Invitrogen). T7-(dT24) oligomer was used for priming the first-strand cDNA synthesis. The resultant cDNA was purified using Phase Lock Gel phenol/chloroform extraction and precipitated with ethanol. The cDNA pellet was collected and dissolved in appropriate volume. Using cDNA as template, cRNA was synthesized using a T7 MegaScript *in Vitro* Transcription Kit (Ambion, Austin, TX). Biotinylated-11-CTP and 16-UTP ribonucleotides (Enzo Diagnostics, Farmingdale, NY) were added to the reaction as labeling reagents. *In vitro* transcription reactions were carried out at 37°C for 6 h, and the labeled cRNA obtained was purified using RNeasy columns (Qiagen). The cRNA was fragmented in fragmentation buffer [40 mM Tris-acetate (pH 8.1), 100 mM KOAc, and 30 mM MgOAc] for 35 min at 94°C. Fragmented cRNA (10–11 μg/probe array) was used to hybridize to human U133A GeneChip array at 45°C for 24 h in a hybridization oven with constant rotation (60 rpm). The chips were washed and stained using

Table 1 Clinicopathological characteristics of eight patients with PEN

None of the patients had a known endocrine hypersecretion syndrome.

Tumor	Age (yrs)	Sex	Size (cm)	IHC[a]	LN status
PEN 1	54	F	6.5	I, Gl, Ga, Soma−	0/12
PEN 2	47	F	2.0	I, Gl, Ga, Soma−	1/16
PEN 3	55	F	3.7	Soma+; I, Gl, Ga−	0/12
PEN 4	52	M	4.6	Not done	0/12
PEN 5	45	M	6.0	Not done	0/8
PEN 6	42	F	2.0	I, Gl, Ga, Soma−	0/6
PEN 7	45	M	3.5	Not done	0/9
PEN 8	45	M	3.0	I, Gl, Ga, Soma−	1/10

[a] IHC, immunohistochemistry; LN, lymph node; I, insulin; Gl, glucagon; Ga, gastrin; Soma, somatostatin, +, positive labeling, −, negative labeling.

Affymetrix fluidics stations. Staining was performed using streptavidin phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, OR), followed by the addition of biotinylated antibody to streptavidin (Vector Laboratories, Burlingame, CA). Probe arrays were scanned using fluorometric scanners (Hewlett Packard Gene Array Scanner; Hewlett Packard Corp., Palo Alto, CA). The scanned images were inspected and analyzed using established quality control measures.

Data Filtering and Analysis. The 11 .CEL files (3 normal islets and 8 PENs) generated by the Affymetrix Microarray Suite (MAS) version 5.0 were converted into .DCP files using dCHIP[8], as described previously by Li and Wong (12). The 11 .DCP files were normalized, and raw gene expression data were generated using the dCHIP system of model-based analysis. For comparison of global gene expression profiles between normal islet cells and PENs, the three islets were designated as "baseline" (B), and the eight PENs were designated as "experiment" (E) in the dCHIP comparison software. Genes overexpressed ≥3-fold in the PENs *versus* normal islets were then identified by defining the appropriate filtering criteria in the dCHIP software (mean E/mean B $>$ 3; mean E – mean B = 100); similarly, genes underexpressed were identified by reversing the filtering criteria (mean B/mean E $>$ 3; mean B – mean E = 100); *P*s were generated using the *t* test.

Immunohistochemistry. PEN TMAs were generated from 53 archival paraffin-embedded well-differentiated PENs, as described previously (13). Each cancer specimen was represented by one to five 1.4-mm cores on the TMA, and, when available, adjacent normal pancreas parenchyma containing normal islets of Langerhans were also arrayed for comparison. In summary, the 53 arrayed PENs consisted of 44 primary tumors (9 with paired lymph node metastases) and 9 isolated hepatic metastases without corresponding primary tumor; adjacent normal pancreatic parenchyma was arrayed in 43 of 44 primary PENs.

Slides were deparaffinized in fresh xylene and rehydrated through sequential graded ethanol steps. Antigen retrieval was performed by citrate buffer incubation [18 mM citric acid and 8.2 mM sodium citrate (pH 6.0)] using a household vegetable steamer (Black and Decker) for 60 min. Slides were incubated for 5 min with 3% hydrogen peroxide, washed in TBS/T [20 mM Tris, 140 mM NaCl, and 0.1% Tween 20 (pH 7.6)], and incubated in appropriate antibody dilutions for p21(CDKN1A) (1:75; Dako, Carpinteria, CA), CD99 (O13; 1:400; Signet, Dedham, MA), IGFBP3 (1:40; Santa Cruz Biotechnology, Santa Cruz, CA), and fibronectin (1:1200; Dako) for 60 min at room temperature. Normal saline was substituted for the primary antibody in control sections. The avidin-biotin-peroxidase complex method from DAKO (Glostrup, Denmark) was used, and slides were subsequently counterstained with hematoxylin. Assessment of immunohistochemical labeling in the TMAs was performed by two of the authors (D. E. H. and A. M.). Loss of nuclear p21 or membranous CD99 in $>$95% of neoplastic endocrine cells was considered "negative;" similarly, labeling of cytoplasmic IGFBP3 or membranous/cytoplasmic fibronectin in $>$25% of neoplastic cells was considered "positive." The $>$25% cutoff was used based on our extensive previous experience with immunohistochemical evaluation of TMA data (9, 10, 14–16). Because normal islets also express IGFBP3, cases were considered positive when the staining intensity was greater than that in normal islets. We found low to moderate basal IGFBP3 in normal islets, and overexpression in PENs was assessed by comparison with this "basal" level, a task greatly facilitated by the availability of adjacent nonneoplastic islets in 43 of 44 primary tumors on our PEN TMA.

Statistical analysis was performed using the Analyze-it software package for Microsoft. Two-tailed Fisher's exact tests were performed to determine the differences in expression of candidate genes by immunohistochemistry, and $P < 0.05$ was considered significant.

RESULTS

Differentially Expressed Transcripts in PENs. Normalization and comparison of Affymetrix microarray hybridization data were performed using dCHIP (12). Sixty-six transcripts were overexpressed ≥3-fold in PENs compared with enriched islet cells, and the complete list is tabulated in Supplementary Table 1. The list of up-regulated transcripts included putative oncogenes [*MLLT10/AF10* (17, 18)], growth factors [*IGFBP3* (19, 20)], cell adhesion and migration molecules [*fibronectin* (21–23)], and endothelial elements [*MUC18/MelCAM* and *CD31* (24)]. Similarly, 119 transcripts were underexpressed ≤3-fold in PENs compared with enriched islet cells, and the complete list is tabulated in Supplementary Table 2. The list of down-regulated transcripts included cell cycle checkpoint proteins [*p21/Cip1* (25, 26)], the *MIC2* (CD99) cell surface glycoprotein (27), genes involved in DNA damage repair and genomic stability [*O^6-methylguanine-DNA methyltransferase* and *GADD45* (28, 29)], putative metastasis suppressor genes [*NME3/DR-nme23* (30)], and *junD*, a MEN1-regulated transcription factor (31). A literature search of PubMed[9] revealed that the differential expression of several genes in this study has previously been reported (either singly or through global microarray expression analyses) in other cancer types, in principle validating our approach. A second PubMed search using the gene name and either "islet cell" or "pancreatic endocrine" only yielded two genes (*p21/CDKN1A* and *CD99*) that have been previously reported as underexpressed in PENs, by immunohistochemistry (32, 33). Thus, the overwhelming majority of the 185 differentially expressed genes reported in this study represent newly described tumor markers for PENs.

Validation of Selected Differentially Expressed Genes in TMAs. Four differentially expressed genes [two down-regulated (p21 and CD99) and two up-regulated (IGFBP3 and fibronectin) genes] were validated by immunohistochemistry using PEN TMAs (Table 2; Fig. 1). Table 2 also lists the labeling pattern seen in normal islets as a comparison. The denominator (*i.e.*, total numbers of cases evaluated) is variable between the antibodies because of "dropout" of tissue cores during the TMA staining process.

[8] www.dCHIP.org.

[9] www.ncbi.nlm.nih.gov/PubMed.

Table 2 Validation of selected differentially expressed genes in TMAs

Antigen	Abnormal expression pattern in PENs[a] ($N = 53$)[b]	Abnormal expression pattern in primary PENs[a] ($N = 44$)[b]	Abnormal expression pattern in metastatic PENs[a] ($N = 18$)[b]
p21	24/52 (46%)	21/44 (48%)	7/15 (47%)
CD99	48/53 (91%)	39/44 (89%)	17/18 (94%)
IGFBP3	33/47 (70%)	24/40 (60%)[c]	15/16 (93%)[c]
Fibronectin	11/50 (22%)	10/42 (24%)	6/17 (35%)

[a] Abnormal expression pattern denotes loss of labeling for p21 and CD99 and overexpression for IGFBP3 and fibronectin. The criteria for immunohistochemical scoring are detailed in the text.

[b] Total number of cases evaluated (*i.e.*, possible maximum denominator) is 53; this includes 44 primary PEN lesions and 9 isolated hepatic metastases. In addition, 9 matched nodal metastases were also evaluated, such that the total number of metastases (*i.e.*, possible maximum denominator) in this series is 18. As explained in the text, the denominators are variable because of "dropout" during immunohistochemical staining of the tissue microarrays.

[c] Difference is statistically significant ($P = 0.022$, Fisher's exact test).

Normal islet cells demonstrated robust nuclear p21 expression, with labeling seen in 10–25% of nuclei within a given islet (Fig. 1*A*). Fifty-two cases could be evaluated for p21 labeling on the PEN TMA. Overall, loss of p21 expression (*i.e.*, <5% nuclear p21 labeling) was seen 24 of 52 (46%) PENs (Fig. 1*B*), including 21 of 44 (48%) primary tumors and 3 of 8 (38%) isolated hepatic metastases. In seven of eight (88%) lymph node metastases, the pattern of p21 expression was concordant with the corresponding primary tumor, *i.e.*, three of seven matched lesions retained p21, whereas labeling was absent in the remaining four. In only one case, p21 expression was present in the primary tumor, whereas the matched nodal metastasis lacked p21 expression. There were no statistically significant differences in loss of p21 expression between primary (48%) and all metastatic PEN lesions [7 of 15 or 47% ($P = 1.0$, Fisher's exact test)].

Fifty-three cases could be evaluated for CD99 labeling. Intense, diffuse membranous CD99 expression was seen in normal islet cells (Fig. 1*C*), whereas as many as 48 of 53 (91%) PENs, including 39 of 44 (89%) primary tumors and 9 of 9 (100%) isolated hepatic metastases demonstrated loss of CD99 expression (Fig. 1*D*). CD99 expression was concordant in nine of nine (100%) paired primary and lymph node metastases on the PEN tissue array, with loss of CD99 expression demonstrable in eight of nine matched lesions, and retention of labeling was demonstrated in one case. There were no statistically significant differences in loss of CD99 expression between primary (91%) and all metastatic PEN lesions [17 of 18 or 94% ($P = 0.86$, Fisher's exact test)].

IGFBP3 and fibronectin were up-regulated in PENs compared with normal islets on microarray analysis (4.1- and 3.9-fold, respectively). Forty-seven cases could be evaluated for IGFBP3 labeling on the PEN TMA. Immunohistochemical analysis demonstrated weak to moderate granular IGFBP3 labeling in the cytoplasm of almost all normal islets (Fig. 1*E*). Using the staining intensity of normal islets as a baseline, 14 of 47 (30%) cases expressed IGFBP3 equal to or less than the normal islet cells. In contrast, intense, diffuse overexpression of IGFBP3 was seen in 33 of 47 (70%) PENs (Fig. 1*F*), including 24 of 40 (60%) primary PENs and 7 of 7 (100%) isolated hepatic metastases. IGFBP3 expression was concordant in nine of nine (100%) paired primary and lymph node metastases on the PEN TMA, with eight of nine (89%) cases coexpressing IGFBP3, and one matched primary and nodal metastasis demonstrating no labeling. There was a statistically significant difference in IGFBP3 overexpression in all metastatic lesions (15 of 16 or 93%) *versus* primary PENs [24 of 40 or 60% ($P = 0.022$, Fisher's exact test)].

Fibronectin is traditionally considered a stromal marker, and expectedly, intense fibronectin expression was present in regions of peritumoral desmoplasia (data not shown), whereas normal islet cells were negative (Fig. 1*G*). Of note however, diffuse, membranous labeling of the neoplastic cells themselves was present in a significant minority [11 of 50 (22%) cases; Fig. 1*F*]; the latter included 10 of 42 (24%) primary tumors and 1 of 8 (13%) isolated hepatic metastasis. Another notable feature of fibronectin labeling was its propensity to be up-regulated in lymphatic metastases compared with the paired primary tumor; thus, of nine paired primary tumor and lymph node metastases examined, five of nine (55%) demonstrated labeling that was restricted to the metastasis, whereas the primary lesion was negative. There was no significant difference in fibronectin overexpression between primary (24%) and all metastatic PEN lesions [6 of 17 or 35% ($P = 0.55$, Fisher's exact test)]; in contrast, the difference in fibronectin expression between nodal metastases alone (55%) and primary PENs demonstrated a trend but did not reach statistical significance ($P = 0.14$, Fisher's exact test).

DISCUSSION

Well-differentiated PENs, unlike their universally aggressive exocrine counterpart, are comprised of distinct malignant and indolent subsets of tumors. As a consequence, it is imperative to identify and aggressively treat PENs likely to result in adverse clinical outcomes. Characterization of the changes in gene expression and cellular pathway activation that contribute to carcinogenesis of the endocrine pancreas may identify markers for early detection, facilitate accurate prognostic classification of PENs, and lead to new molecular targets for therapy.

We have performed the first global gene expression analysis of PENs and compared this to the gene expression in highly enriched isolated human islets, using the second-generation Affymetrix U133 platform, containing ~22,000 human transcripts. We have identified 66 transcripts overexpressed ≥3-fold and 119 transcripts expressed ≤3-fold in PENs compared with normal islet cells. In addition, we have immunohistochemically validated the differential expression of four genes in TMAs comprised of archival primary and metastatic PEN lesions. A search on the National Library of Medicine's PubMed[9] using the gene name and either "pancreatic endocrine" or "islet cell" revealed that only two transcripts in our combined list of 185 genes, p21 and CD99, have previously been described in the context of PENs (32, 33). Thus, the overwhelming majority of genes we have identified represent novel differentially expressed genes in PENs.

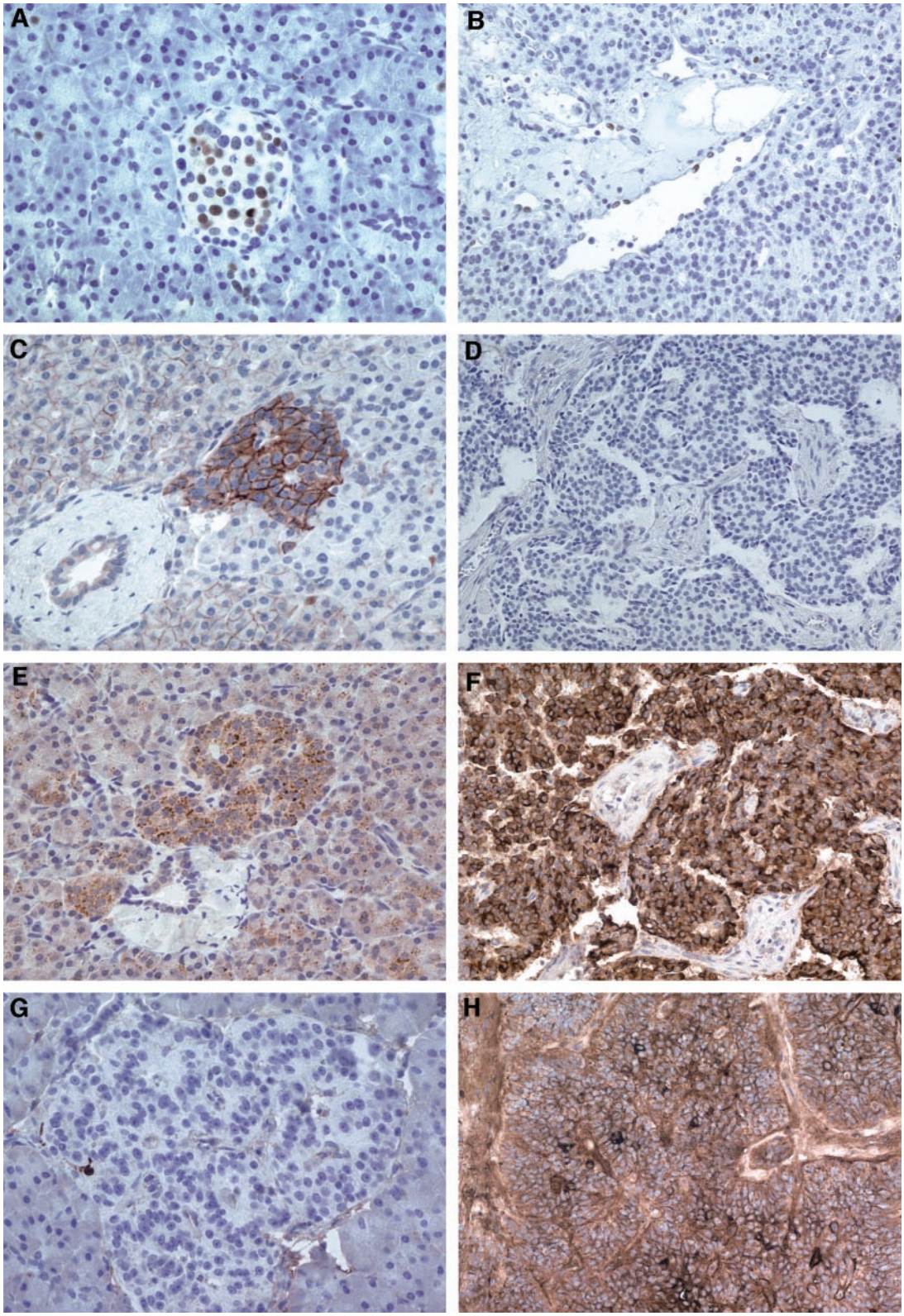

Fig. 1 Immunohistochemical validation of differentially expressed genes in PENs *versus* normal islet cells. *A,* robust nuclear p21 expression is seen in 10–25% of cells in normal islets. *B,* loss of nuclear p21 expression in PEN. Note that interspersed endothelial cells demonstrate nuclear positivity. *C,* robust membranous expression of CD99 is seen in normal islet cells. *D,* loss of CD99 expression in PEN. *E,* weak to moderate granular cytoplasmic labeling of IGFBP3 present in normal islet cells. *F,* intense diffuse IGFBP3 expression in PEN. *G,* normal islet cells do not express fibronectin. *H,* intense diffuse fibronectin expression in PEN.

A particular strength of this study is the use of enriched human islet cells as a normal control, as opposed to bulk pancreas, which contains <2% islets of Langerhans. This approach has permitted an accurate comparison of neoplastic endocrine cells with their normal counterpart, instead of a plethora of nonendocrine tissues. We are aware that this analysis may have generated a minority of "false positive" differentially expressed genes (for example, genes present in tumor-associated endothelium or stromal elements), which would not be expressed by enriched islet cell extracts. Nevertheless, by judiciously parsing the gene lists using known gene function and cellular localization and, more importantly, by validation in tissue sections, we have generated a rational expression profile of neoplastic endocrine cells.

The paramount importance of tissue validation of microarray expression data is best exemplified by two transcripts in the overexpressed gene list: fibronectin and melanoma cell adhesion molecule (MelCAM or Muc18). Fibronectin is classically considered a stromal marker (9). However, on immunohistochemical analysis, a significant minority (22%) of PENs expressed fibronectin on the neoplastic cell surface, a proportion that was even higher in nodal metastasis (see the discussion of fibronectin below). On the contrary, MelCAM/Muc18, a cell adhesion molecule otherwise present in several cancer types (34–36), was not expressed in the neoplastic endocrine cells, although its expression was up-regulated in the microarray analysis (data not shown). MelCAM/Muc18 labeling was restricted to the endothelium only [both normal and neoplasia-associated endothelium (36, 37)], and this transcript was presumably "overexpressed" in PENs due to the rich fibrovascular stroma typically seen in these tumors. As stated above and demonstrated in multiple reports from this group (9, 10, 14–16), global microarray analysis of "bulk" tumor tissues is likely to yield a subset of differentially expressed genes that are due to peritumoral stromal element, rather than the neoplastic cells themselves; therefore, careful parsing of gene function and immunohistochemical/*in situ* validation are critical to avoid "false alarms" in microarray analyses. The ensuing discussion will address some of the genes whose differential expression we have validated in tissue sections of PENs.

As stated previously, we confirmed the differential expression of four genes using PEN TMA. This includes two down-regulated genes (p21 and CD99) and two up-regulated genes (IGFBP3 and fibronectin), respectively. The loss of p21, a cyclin-dependent kinase inhibitor, has been reported in a large number of human neoplasms, including PENs (25, 26). In a study of 109 gastrointestinal carcinoid tumors, including 42 PENs, Canavese *et al.* (33) found a low level of p21 expression in most well-differentiated PENs. The authors used a "p21 labeling index" and reported a median labeling index of 1.29% for "benign tumors" (range, 0–20.48%). Because the scoring criterion was different from the present TMA-based study, we cannot make a direct comparison. In contrast, Choi *et al.* (32) reported loss of p21 in 40% of nonfunctional PENs in their series, which is in excellent agreement with our own findings (46% loss of expression). In our series of PENs, we failed to find a statistically significant difference in p21 expression between primary and metastatic PEN lesions, and p21 expression was concordant in ~90% of matched primary pancreatic and nodal metastatic lesions.

The *MIC2* gene locus is located in the pseudoautosomal (pairing) region of human X and Y chromosomes (38). The protein product of the *MIC2* gene (CD99 or E2 antigen) is a 32-kDa human T-cell surface glycoprotein involved in spontaneous rosette formation with erythrocytes (27). Intense membranous CD99 expression is considered a *sine qua non* of *EWS-FLI1* translocation-positive primitive neuroectodermal tumors/Ewing sarcomas (39). CD99 reactivity has been increasingly recognized in a variety of other mesenchymal tumor types, including poorly differentiated synovial sarcomas and rhabdomyosarcomas, epithelial malignancies (gastric adenocarcinomas), and lymphoblastic lymphoma. In the pancreas, besides pancreatic primitive neuroectodermal tumors/Ewing sarcomas (40), CD99 expression has been uncommonly reported in solid pseudopapillary tumors (41). Choi *et al.* (32) had previously reported absence of CD99 labeling in nonfunctioning islet cell tumors. In accordance with our microarray expression data, we found intense, diffuse CD99 expression in all benign islets, whereas 91% of PENs in our series lacked CD99 expression. The mechanism of CD99 down-regulation in PENs is a matter of speculation. Some studies have suggested a role for the EBV latent membrane protein (LMP1) orchestrating down-regulation of CD99 antigen via a nuclear factor-κβ-mediated pathway in Reed-Sternberg cells (42, 43); however, this is unlikely to be the mechanism of action in PENs. We found no significant correlation between site of lesion and CD99 expression in our series (89% of primary tumors and 94% of hepatic and nodal metastases demonstrated lack of CD99 labeling).

IGFBP3 is a member of the family of insulin-like growth factors (19). The insulin-like growth factors, their receptors, and their binding proteins play key roles in regulating cell proliferation and apoptosis. IGFBP3 is the major carrier protein for IGF1 and IGF2 in the circulation. IGFBP3 possesses both growth-inhibitory and -potentiating effects on cells that are independent of IGF action and are mediated through specific IGFBP3-binding proteins/receptors located at the cell membrane, cytosol or nuclear compartments and in the extracellular matrix (19). For example, in some cancer cells, IGFBP3 has proapoptotic activities both dependent on and independent of p53 (44, 45). On the contrary, elevated serum IGFBP3 may be a predictor for progression and recurrence of breast cancers (20, 46). We have recently demonstrated up-regulation of IGFBP3 in pancreatic ductal adenocarcinomas using the U133 microarray platform.[10] The commonality of IGFBP3 up-regulation in both exocrine and endocrine neoplasms of the pancreas suggests an important, possibly growth-promoting role for this protein in pancreatic tumorigenesis. On immunohistochemistry, we found low to moderate "basal" IGFBP3 in normal islets, and overexpression in PENs was assessed by comparison with this "basal" level, a task greatly facilitated by the availability of adjacent nonneoplastic islets in 43 of 44 primary tumors on our PEN TMA. Using this criterion, 70% of PEN lesions demonstrated robust IGFBP3 overexpression. Although there was an excellent

[10] J. Iacobuzio-Donahue, unpublished data.

correlation between IGFBP3 labeling in matched primary and nodal metastases (9 of 9 or 100% of matched lesions demonstrated a similar pattern of IGFBP3 labeling), we found a significantly higher overall proportion of metastatic foci that labeled with IGFBP3 (15 of 16 or 93%) compared with primary tumors [24 of 40 or 60% ($P = 0.022$)].

Fibronectin is a 430-kDa dimeric glycoprotein that exists in two forms, termed cellular and plasma fibronectin. Cellular fibronectin is the major cell surface glycoprotein of many fibroblast cell lines and serves as a ligand for the integrin family of cell adhesion receptors and regulates cytoskeletal organization (22, 23). Several reports have demonstrated that secretion of this promigratory molecule may be a key event in cancer cells during the progression to a metastatic phenotype. For example, Bittner *et al.* (47) demonstrated overexpression of fibronectin in a series of highly metastatic uveal melanoma cell lines, consistent with an important role for focal contacts in modulating melanoma cell motility. Similarly, Clark *et al.* (48) reported that fibronectin was one of the three consistently up-regulated genes (along with thymosin β4 and RhoC) in pulmonary metastases arising from human and mouse melanoma cell lines. On the contrary, peptides that mimic the cell-adhesive region of fibronectin were shown to inhibit metastasis, indicating that tumor cells must interact with molecules such as fibronectin to metastasize (49). In our series of PENs, we found expression of fibronectin in a small but significant minority of primary tumors (24%). Of interest, however, lymphatic, but not hepatic, metastases of PENs demonstrated a higher proportion of cases with fibronectin expression; in five of nine (55%) PENs, up-regulation of fibronectin was restricted to the nodal metastasis only, whereas the primary lesion was negative ($P = 0.14$). The expression of fibronectin in the majority of lymphatic metastases of PENs also presents an opportunity to potentially use fibronectin-conjugated radionuclides or immunotoxins for the treatment of refractory metastatic disease (50).

In summary, we have performed the first global expression profiling of well-differentiated PENs and identified 119 significantly down-regulated and 66 significantly up-regulated genes compared with enriched human pancreatic islet cells, using the Affymetrix U133 platform. We have validated the differential expression for a subset of these genes using TMAs. The raw gene expression profiling data are available on request and should serve as an effective "launching pad" for future studies aimed at elucidating tumorigenic pathways in endocrine tumors of the pancreas. These gene expression data also have the potential to allow identification of new cellular targets for therapy in these uncommon neoplasms.

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REFERENCES

1. Solcia, E., Rindi, G., Paolotti, D., La Rosa, S., Capella, C., and Fiocca, R. Clinicopathological profile as a basis for classification of the endocrine tumours of the gastroenteropancreatic tract. Ann. Oncol., *10* (Suppl. 2)*:* S9–S15, 1999.

2. Solcia, E., Kloppel, G., and Sobin, L. H. Histological Typing of Endocrine Tumors. New York: Springer-Verlag, 2001.

3. Phan, G. Q., Yeo, C. J., Hruban, R. H., Lillemoe, K. D., Pitt, H. A., and Cameron, J. L. Surgical experience with pancreatic and peripancreatic neuroendocrine tumors: review of 125 patients. J. Gastrointest. Surg., *2:* 472–482, 1998.

4. Kloppel, G., Solcia, E., Capella, C., and Heitz, P. U. Classification of neuroendocrine tumours. Ital. J. Gastroenterol. Hepatol., *31* (Suppl. 2)*:* S111–S116, 1999.

5. La Rosa, S., Sessa, F., Capella, C., Riva, C., Leone, B. E., Klersy, C., Rindi, G., and Solcia, E. Prognostic criteria in nonfunctioning pancreatic endocrine tumours. Virchows Arch., *429:* 323–333, 1996.

6. Lee, C. S., Charlton, I. G., Williams, R. A., Dhillon, A. P., and Rode, J. Malignant potential of aneuploid pancreatic endocrine tumours. J. Pathol., *169:* 451–456, 1993.

7. Rigaud, G., Missiaglia, E., Moore, P. S., Zamboni, G., Falconi, M., Talamini, G., Pesci, A., Baron, A., Lissandrini, D., Rindi, G., Grigolato, P., Pederzoli, P., and Scarpa, A. High resolution allelotype of nonfunctional pancreatic endocrine tumors: identification of two molecular subgroups with clinical implications. Cancer Res., *61:* 285–292, 2001.

8. Gumbs, A. A., Moore, P. S., Falconi, M., Bassi, C., Beghelli, S., Modlin, I., and Scarpa, A. Review of the clinical, histological, and molecular aspects of pancreatic endocrine neoplasms. J. Surg. Oncol., *81:* 45–53, discussion 54, 2002.

9. Iacobuzio-Donahue, C., Maitra, A., Olsen, M., Lowe, A., van Heek, N. T., Rosty, C., Walter, K., Sato, N., Parker, A., Ashfaq, R., Jaffee, E., Ryu, B., Jones, J., Eshelman, J., Yeo, C. J., Cameron, J. L., Kern, S. E., Hruban, R. H., Brown, P. O., and Goggins, M. Exploration of global expression patterns in pancreatic adenocarcinomas using cDNA microarrays. Am. J. Pathol., *162:* 1151–1162, 2003.

10. Iacobuzio-Donahue, C. A., Maitra, A., Shen-Ong, G. L., van Heek, T., Ashfaq, R., Meyer, R., Walter, K., Berg, K., Hollingsworth, M. A., Cameron, J. L., Yeo, C. J., Kern, S. E., Goggins, M., and Hruban, R. H. Discovery of novel tumor markers of pancreatic cancer using global gene expression technology. Am. J. Pathol., *160:* 1239–1249, 2002.

11. Markmann, J. F., Deng, S., Huang, X., Velidedeoglu, E., Desai, N. M., Wolf, B., Matschinsky, F., Barker, F., and Naji, A. Successful human islet transplantation from single donors. Ann. Surg., in press, 2003.

12. Li, C., and Wong, W. H. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. USA, *98:* 31–36, 2001.

13. Luo, J., Zha, S., Gage, W. R., Dunn, T. A., Hicks, J. L., Bennett, C. J., Ewing, C. M., Platz, E. A., Ferdinandusse, S., Wanders, R. J., Trent, J. M., Isaacs, W. B., and De Marzo, A. M. α-Methylacyl-CoA racemase: a new molecular marker for prostate cancer. Cancer Res., *62:* 2220–2226, 2002.

14. Argani, P., Rosty, C., Reiter, R. E., Wilentz, R. E., Murugesan, S. R., Leach, S. D., Ryu, B., Skinner, H. G., Goggins, M., Jaffee, E. M., Yeo, C. J., Cameron, J. L., Kern, S. E., and Hruban, R. H. Discovery of new markers of cancer through serial analysis of gene expression: prostate stem cell antigen is overexpressed in pancreatic adenocarcinoma. Cancer Res., *61:* 4320–4324, 2001.

15. Argani, P., Iacobuzio-Donahue, C., Ryu, B., Rosty, C., Goggins, M., Wilentz, R. E., Murugesan, S. R., Leach, S. D., Jaffee, E., Yeo, C. J., Cameron, J. L., Kern, S. E., and Hruban, R. H. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). Clin. Cancer Res., *7:* 3862–3868, 2001.

16. Hansel, D. E., Rahman, A., Hidalgo, M., Thuluvath, P. J., Lillemoe, K. D., Shulick, R., Ku, J. L., Park, J. G., Miyazaki, K., Ashfaq, R., Wistuba, I. I., Varma, R., Hawthorne, L., Geradts, J., Argani, P., and Maitra, A. Identification of novel cellular targets in biliary tract cancers using global gene expression technology. Am. J. Pathol., *163:* 217–229, 2003.

17. Debernardi, S., Bassini, A., Jones, L. K., Chaplin, T., Linder, B., de Bruijn, D. R., Meese, E., and Young, B. D. The MLL fusion partner AF10 binds GAS41, a protein that interacts with the human SWI/SNF complex. Blood, *99:* 275–281, 2002.

18. Chaplin, T., Ayton, P., Bernard, O. A., Saha, V., Della Valle, V., Hillion, J., Gregorini, A., Lillington, D., Berger, R., and Young, B. D. A novel class of zinc finger/leucine zipper genes identified from the molecular cloning of the t(10;11) translocation in acute leukemia. Blood, *85:* 1435–1441, 1995.

19. Furstenberger, G., and Senn, H. J. Insulin-like growth factors and cancer. Lancet Oncol., *3:* 298–302, 2002.

20. Vadgama, J. V., Wu, Y., Datta, G., Khan, H., and Chillar, R. Plasma insulin-like growth factor-I and serum IGF-binding protein 3 can be associated with the progression of breast cancer, and predict the risk of recurrence and the probability of survival in African-American and Hispanic women. Oncology (Basel), *57:* 330–340, 1999.

21. Danen, E. H., and Yamada, K. M. Fibronectin, integrins, and growth control. J. Cell. Physiol., *189:* 1–13, 2001.

22. Ruoslahti, E. Fibronectin and its integrin receptors in cancer. Adv. Cancer Res., *76:* 1–20, 1999.

23. Labat-Robert, J. Fibronectin in malignancy. Semin. Cancer Biol., *12:* 187–195, 2002.

24. Shih, I. M. The role of CD146 (Mel-CAM) in biology and pathology. J. Pathol., *189:* 4–11, 1999.

25. Waldman, T., Kinzler, K. W., and Vogelstein, B. p21 is necessary for the p53-mediated G_1 arrest in human cancer cells. Cancer Res., *55:* 5187–5190, 1995.

26. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science (Wash. DC), *282:* 1497–1501, 1998.

27. Gelin, C., Aubrit, F., Phalipon, A., Raynal, B., Cole, S., Kaczorek, M., and Bernard, A. The E2 antigen, a 32 kd glycoprotein involved in T-cell adhesion processes, is the MIC2 gene product. EMBO J., *8:* 3253–3259, 1989.

28. Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S. N., Hidalgo, O. F., Vanaclocha, V., Baylin, S. B., and Herman, J. G. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N. Engl. J. Med., *343:* 1350–1354, 2000.

29. Tran, H., Brunet, A., Grenier, J. M., Datta, S. R., Fornace, A. J., Jr., DiStefano, P. S., Chiang, L. W., and Greenberg, M. E. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science (Wash. DC), *296:* 530–534, 2002.

30. Venturelli, D., Martinez, R., Melotti, P., Casella, I., Peschle, C., Cucco, C., Spampinato, G., Darzynkiewicz, Z., and Calabretta, B. Overexpression of DR-nm23, a protein encoded by a member of the nm23 gene family, inhibits granulocyte differentiation and induces apoptosis in 32Dc13 myeloid cells. Proc. Natl. Acad. Sci. USA, *92:* 7435–7439, 1995.

31. Weitzman, J. B., Fiette, L., Matsuo, K., and Yaniv, M. JunD protects cells from p53-dependent senescence and apoptosis. Mol. Cell, *6:* 1109–1119, 2000.

32. Choi, Y. L., Oh, Y. L., Kim, S. H., Park, C. K., and Ahn, G. Comparative study of non-functional islet cell tumors and pancreatic solid and papillary neoplasms: biological behavior and immunohistochemistry. Pathol. Int., *52:* 358–366, 2002.

33. Canavese, G., Azzoni, C., Pizzi, S., Corleto, V. D., Pasquali, C., Davoli, C., Crafa, P., Delle Fave, G., and Bordi, C. p27: a potential main inhibitor of cell proliferation in digestive endocrine tumors but not a marker of benign behavior. Hum. Pathol., *32:* 1094–1101, 2001.

34. Wu, G. J., Varma, V. A., Wu, M. W., Wang, S. W., Qu, P., Yang, H., Petros, J. A., Lim, S. D., and Amin, M. B. Expression of a human cell adhesion molecule, MUC18, in prostate cancer cell lines and tissues. Prostate, *48:* 305–315, 2001.

35. Johnson, J. P. Cell adhesion molecules in the development and progression of malignant melanoma. Cancer Metastasis Rev., *18:* 345–357, 1999.

36. Shih, I. M., Wang, T. L., and Westra, W. H. Diagnostic and biological implications of mel-CAM expression in mesenchymal neoplasms. Clin. Cancer Res., *2:* 569–575, 1996.

37. Sers, C., Riethmuller, G., and Johnson, J. P. MUC18, a melanoma-progression associated molecule, and its potential role in tumor vascularization and hematogenous spread. Cancer Res., *54:* 5689–5694, 1994.

38. Goodfellow, P. J., Darling, S. M., Thomas, N. S., and Goodfellow, P. N. A pseudoautosomal gene in man. Science (Wash. DC), *234:* 740–743, 1986.

39. Ambros, I. M., Ambros, P. F., Strehl, S., Kovar, H., Gadner, H., and Salzer-Kuntschik, M. MIC2 is a specific marker for Ewing's sarcoma and peripheral primitive neuroectodermal tumors. Evidence for a common histogenesis of Ewing's sarcoma and peripheral primitive neuroectodermal tumors from MIC2 expression and specific chromosome aberration. Cancer (Phila.), *67:* 1886–1893, 1991.

40. Movahedi-Lankarani, S., Hruban, R. H., Westra, W. H., and Klimstra, D. S. Primitive neuroectodermal tumors of the pancreas: a report of seven cases of a rare neoplasm. Am. J. Surg. Pathol., *26:* 1040–1047, 2002.

41. Maitra, A., Weinberg, A. G., Schneider, N., and Patterson, K. Detection of t(11;22)(q24;q12) translocation and EWS-FLI-1 fusion transcript in a case of solid pseudopapillary tumor of the pancreas. Pediatr. Dev. Pathol., *3:* 603–605, 2000.

42. Kim, S. H., Shin, Y. K., Lee, I. S., Bae, Y. M., Sohn, H. W., Suh, Y. H., Ree, H. J., Rowe, M., and Park, S. H. Viral latent membrane protein 1 (LMP-1)-induced CD99 down-regulation in B cells leads to the generation of cells with Hodgkin's and Reed-Sternberg phenotype. Blood, *95:* 294–300, 2000.

43. Lee, I., Kim, M. K., Choi, E. Y., Mehl, A., Jung, K. C., Gil, M. C., Rowe, M., and Park, S. H. CD99 expression is positively regulated by Sp1 and is negatively regulated by Epstein-Barr virus latent membrane protein 1 through nuclear factor-κB. Blood, *97:* 3596–3604, 2001.

44. Shen, L., Dean, N. M., and Glazer, R. I. Induction of p53-dependent, insulin-like growth factor-binding protein-3-mediated apoptosis in glioblastoma multiforme cells by a protein kinase Cα antisense oligonucleotide. Mol. Pharmacol., *55:* 396–402, 1999.

45. Collard, T. J., Guy, M., Butt, A. J., Perks, C. M., Holly, J. M., Paraskeva, C., and Williams, A. C. Transcriptional upregulation of the insulin-like growth factor binding protein IGFBP-3 by sodium butyrate increases IGF-independent apoptosis in human colonic adenoma-derived epithelial cells. Carcinogenesis (Lond.), *24:* 393–401, 2003.

46. Goodwin, P. J., Ennis, M., Pritchard, K. I., Trudeau, M. E., Koo, J., Hartwick, W., Hoffma, B., and Hood, N. Insulin-like growth factor binding proteins 1 and 3 and breast cancer outcomes. Breast Cancer Res. Treat., *74:* 65–76, 2002.

47. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., Sampas, N., Dougherty, E., Wang, E., Marincola, F., Gooden, C., Lueders, J., Glatfelter, A., Pollock, P., Carpten, J., Gillanders, E., Leja, D., Dietrich, K., Beaudry, C., Berens, M., Alberts, D., and Sondak, V. Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature (Lond.), *406:* 536–540, 2000.

48. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes R. O. Genomic analysis of metastasis reveals an essential role for RhoC. Nature (Lond.), *406:* 532–535, 2000.

49. Humphries, M. J., Olden, K., and Yamada, K. M. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science (Wash. DC), *233:* 467–470, 1986.

50. Marty, C., Odermatt, B., Schott, H., Neri, D., Ballmer-Hofer, K., Klemenz, R., and Schwendener, R. A. Cytotoxic targeting of F9 teratocarcinoma tumours with anti-ED-B fibronectin scFv antibody modified liposomes. Br. J. Cancer, *87:* 106–112, 2002.