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ORIGINAL REPORT

Pancreatic Endocrine Tumors: Expression Profiling Evidences a Role for AKT-mTOR Pathway

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ABSTRACT

Purpose
We investigated the global gene expression in a large panel of pancreatic endocrine tumors (PETs) aimed at identifying new potential targets for therapy and biomarkers to predict patient outcome.

Patients and Methods
Using a custom microarray, we analyzed 72 primary PETs, seven matched metastases, and 10 normal pancreatic samples. Relevant differentially expressed genes were validated by either quantitative real-time polymerase chain reaction or immunohistochemistry on tissue microarrays.

Results
Our data showed that: tuberous sclerosis 2 (TSC2) and phosphatase and tensin homolog (PTEN) were downregulated in most of the primary tumors, and their low expression was significantly associated with shorter disease-free and overall survival; somatostatin receptor 2 (SSTR2) was absent or very low in insulinomas compared with nonfunctioning tumors; and expression of fibroblast growth factor 13 (*FGF13*) gene was significantly associated with the occurrence of liver metastasis and shorter disease-free survival. TSC2 and PTEN are two key inhibitors of the Akt/mammalian target of rapamycin (mTOR) pathway and the specific inhibition of mTOR with rapamycin or RAD001 inhibited cell proliferation of PET cell lines.

Conclusion
Our results strongly support a role for PI3K/Akt/mTOR pathway in PET, which ties in with the fact that mTOR inhibitors have reached phase III trials in neuroendocrine tumors. The finding of differential SSTR expression raises the potential for SSTR expression to be evaluated as a marker of response to somatostatin analogs. Finally, we identified *FGF13* as a new prognostic marker that predicted poorer outcome in patients who were clinically considered free from disease.

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INTRODUCTION

Pancreatic endocrine tumors (PETs) are heterogeneous diseases in terms of clinical manifestations and behavior.[1] They are clinically classified as functioning (F) or nonfunctioning tumor (NF), based on presence of symptoms caused by hormone secretion.[2] F-PETs are mainly represented by insulinomas. The WHO classification distinguishes three categories: well-differentiated endocrine tumor (WDET) having an indolent clinical course; well-differentiated endocrine carcinoma (WDEC) that are diagnosed based on the presence of invasion or metastasis; poorly differentiated endocrine carcinoma (PDEC) with a survival as poor as that of pancreatic adenocarcinoma.[2] However, the malignant potential of WDECs varies greatly, cannot be predicted by histological appearance and the proliferation rate alone is a valuable predictor of clinical outcome.[3,4]

Radical surgery is the only curative treatment for PETs, while medical treatments including somatostatin analogs, interferon, chemotherapy, and receptor radionuclide therapy are effective only in a portion of patients with progressive disease.[5-8]

Little is known about the molecular pathogenesis of PETs.[1] They occur sporadically or as part of familial cancer syndromes, including multiple endocrine neoplasia type 1 (MEN1), von Hippel Lindau and, less frequently, neurofibromatosis[9] or tuberous sclerosis complex.[10-12] Somatic *MEN1* gene mutations are the most common genetic alterations found, while mutations typically involved in pancreatic adenocarcinoma are uncommon.[13-18]

Gene expression profiling studies have identified several potential biomarkers.[19-25] However,

none of these have shown definite correlation with disease outcome, possibly due to the heteregeneous design of the studies and the low number of cases included, ranging from 8 to 12 cases per category of well differentiated endocrine tumors.

We performed the largest expression profile study of PETs to date, including samples from each clinicopathologic category with the aim of better understanding the molecular basis of this disease, and identifying new prognostic markers and therapeutic targets.

Table 1. Clinicopathologic Information of the Samples Used in This Study

Parameter	Microarray Samples	TaqMan Samples	TMA Samples
No. of patients	72	77	141
Sex			
Male	30	37	60
Female	42	40	81
Age, years	53	54	54
Range	17-78	23-78	17-78
Median tumor size, mm	30	35	30
Surgery			
Complete excision	58	59	113
Paliative/debulking	14	17	25
Explorative	0	1	3
Functional status			
Functioning	16*	20†	34‡
Nonfunctioning	56	57	107
WHO classification			
Well-differentiated tumor benign	39	39	76
Well-differentiated carcinoma	30	34	60
Poorly differentiated carcinoma	3	4	5
Metastasis			
Lymph node	25	26	42
Liver	16	19	30
Invasion			
Vascular	34	37	68
Soft tissue	21	23	39
Perineural	23	27	50
Not specified		3	7
Proliferation index			
Ki67 < 5%	50	55	100
Ki67 ≥ 5%	22	19	37
Not specified		3	4
Median survival			
Median follow-up, years		4.3	4.6
Overall survival		Not reached	Not reached
No. of events		14	23
No. of patients		67	126
Progression-free survival, years		7.7	Not reached
No. of events		25	38
No. of patients		65	125
Disease-free survival		Not reached	Not reached
No. of events		8	18
No. of patients		48	105

Abbreviations: TMA, tissue microarray; ACTHoma, adrenocorticotropic hormone–producing neoplasm.
*Fifteen insulinoma and one gastrinoma.
†Fifteen insulinoma, two gastrinoma, one vipoma, one glucagonoma, and one ACTHoma.
‡Twenty-seven insulinoma, three gastrinoma, two vipoma, one somatostatinoma, and one ACTHoma.

PATIENTS AND METHODS

Primary Tumors

The expression profile study involved 72 primary PETs (Table 1) classified according to WHO criteria,[2] seven matched metastases, five normal pancreas, and five preparations of islets of Langerhans obtained from multiorgan donors as described.[26] The proliferative activity was measured by Ki67 immunohistochemistry, expressed as the percentage of Ki67-positive cells in 2,000 tumor cells within areas of highest immunostaining using the MIB1 antibody (DBA, Milan, Italy). RNA extracted from frozen tissues was assessed for quality using Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

Microarray Analysis and Validation Studies

A total of 95 samples (89 unique plus six technical replicates) were analyzed using an 18.5 K human oligo microarray from the Ohio State University Cancer Center (Appendix, online only). Most analysis and graphics were generated using R software version 2.0.[27] Gene Ontology analysis was performed using the DAVID analysis framework (http://david.abcc.ncifcrf.gov). For validation, quantitative real-time PCR (qRT-PCR; Table 1) was performed on 55 cases of the microarray set and 22 new cases, and immunohistochemistry was applied on tissue microarrays (TMA) containing 141 PETs and 12 normal pancreata (Table 1). Three PET cell lines were treated with Rapamycin (Calbiochem) and RAD001 (Novartis) to inhibit mammalian target of rapamycin (mTOR) pathway. The methods used for validation studies are detailed in Appendix.

Time to Progression and Survival

Patients were monitored every 3 to 6 months with clinical and laboratory evaluation, ultrasound, magnetic resonance imaging or contrast

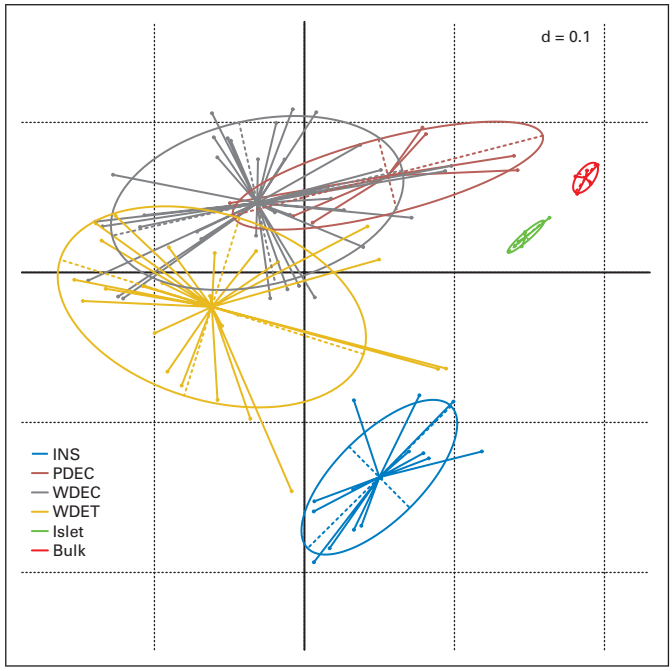

Fig 1. Bidimensional projection of the expression profiles of pancreatic endocrine tumors (PETs) and normal samples by correspondence analysis. Projection of PETs and normal samples into two-dimensional space using the top 1,000 probes with the highest interquartile range. Bulk tissues, islet cells, insulinomas (INS), and nonfunctioning PETs showed a distinctive pattern of expression, which projects them in different regions of the plane. PDEC, poorly differentiated endocrine carcinoma; WDEC, well-differentiated endocrine carcinoma; WDET, well-differentiated endocrine tumor; Islet, normal pancreatic islets of Langerhans; Bulk, normal bulk tissue.

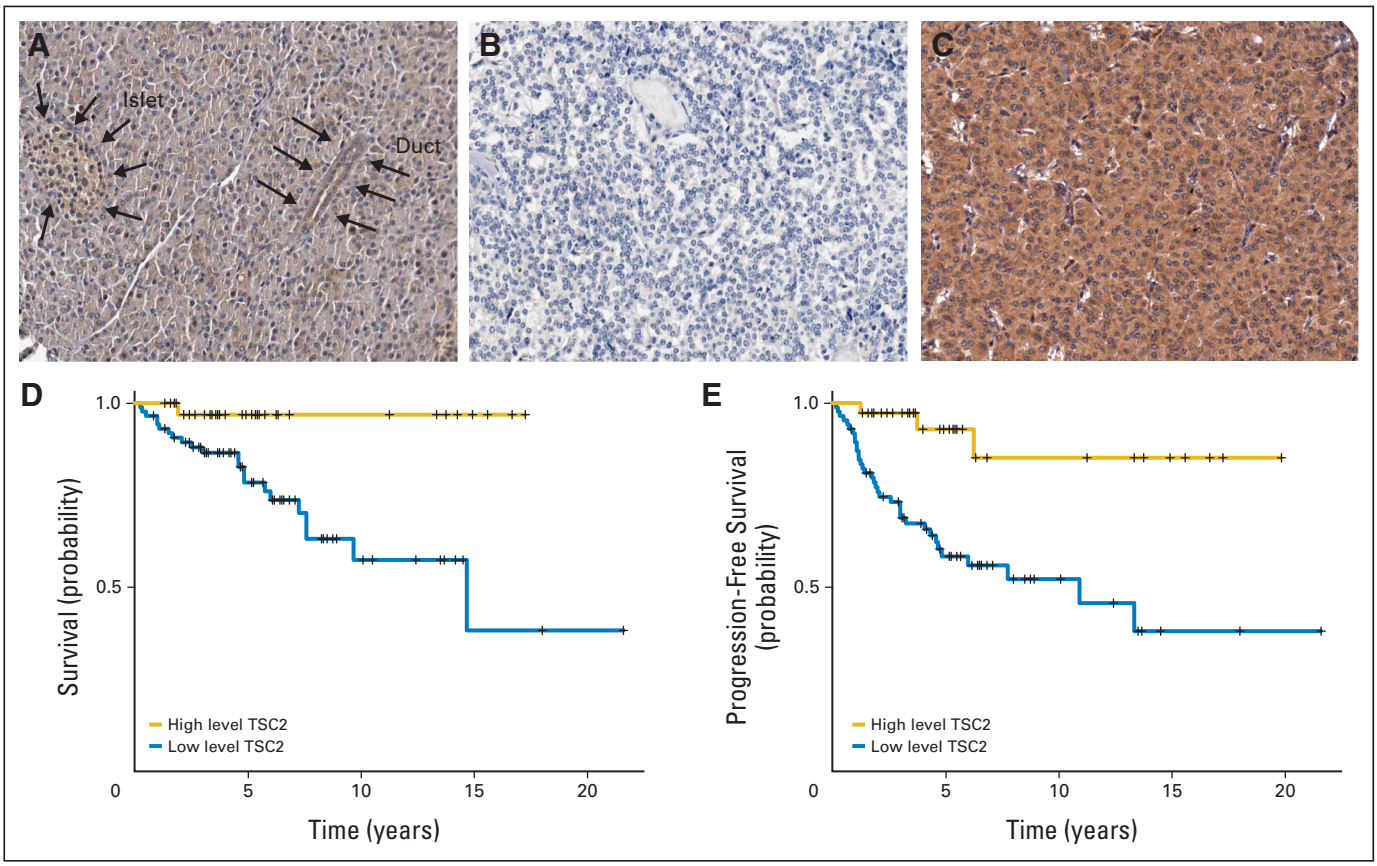

Fig 2. Tuberous sclerosis 2 (TSC2) protein expression and its correlation with survival in pancreatic endocrine tumors (PETs). Immunohistochemistry with antituberin antibody (Novocastra, Newcastle, United Kingdom). Original magnification: ×20. (A) Normal pancreatic tissue with an islet and duct (indicated by arrows), the cells of which show a cytoplasmic staining stronger than that seen in acini; (B) PET tissue with negative staining; (C) PET tissue with strong staining. Correlation of tuberin immunostaining with (D) overall survival and (E) progression-free survival. High level TSC2, staining score higher than 2; low level TSC2, staining score ≤ 2.

enhanced computed tomography (CT) scan when necessary, according to a follow-up protocol established at our center since 1990. Time to progression was defined as the interval from surgery to disease progression.[28] Survival was calculated from the date of diagnosis. Log-rank test was used to compare survival curves. Multivariate survival analysis was performed using Cox's proportional hazard model. The covariates included in the analysis were evaluated using both forward and reverse stepwise methods.

Table 2. Correlation of TSC2 and PTEN Protein Expression With Clinicopathologic Parameters

Parameter	No.	Low TSC2 (%)	*P**	No.	Low PTEN (%)	*P**
WHO classification	137			137		
WDET	73	53.4	< .001	74	25.7	< .001
WDEC	59	88.1		59	57.6	
PDEC	5	100.0		4	75.0	
Functional status	137			137		
F-PET	33	51.5	.015	33	24.2	.027
NF-PET	104	76.0		104	46.2	
Proliferation index	130			130		
Ki67 < 5%	94	64.9	.018	95	32.6	.015
Ki67 ≥ 5%	36	86.1		35	57.1	
Liver metastasis at diagnosis	136			136		
Liver metastasis	30	90.0	.007	28	64.3	.005
Disease free	106	65.1		108	34.3	
Liver metastasis at follow-up†	101			103		
Liver metastasis	15	100.0	.002	16	56.3	.015
Disease free	86	60.5		87	29.9	

Abbreviations: TSC2, tuberous sclerosis 2; PTEN, phosphatase and tensin homolog; WDET, well-differentiated endocrine tumor; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; F-PET, functioning pancreatic endocrine tumor; NF-PET, nonfunctioning pancreatic endocrine tumor.
*Fisher's exact test.
†Patients who had a complete resection of the tumor.

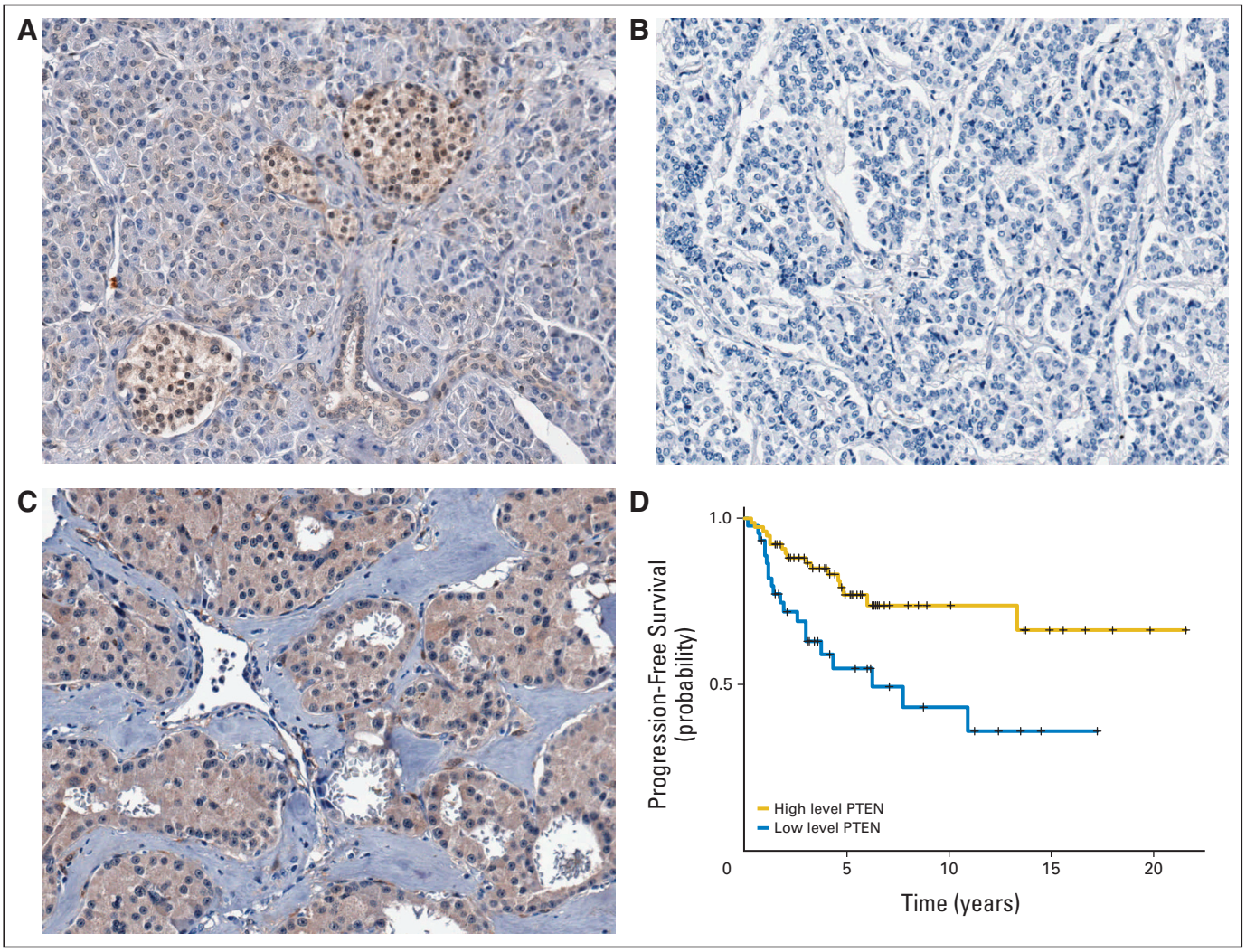

Fig 3. Phosphatase and tensin homolog (PTEN) protein expression and its correlation with survival in pancreatic endocrine tumors (PETs). Immunohistochemistry with anti-PTEN antibody (Cell Signaling Technology, Beverly, MA). Original magnification: ×20. (A) Normal pancreatic tissue with islets showing strongly stained cytoplasm and nuclei; (B) PET tissue with negative staining; (C) PET tissue with strong cytoplasmic and nuclear protein expression. (D) Correlation between PTEN immunostaining and disease-free survival. High level PTEN, staining score ≥ 2; low level PTEN, staining score < 2.

RESULTS

Global gene expression profiles were obtained from 72 primary PETs (39 WDET, 30 WDEC, and three PDEC), seven matched metastases and 10 normal pancreatic samples, using a custom oligomicroarray.

Unsupervised hierarchical clustering analysis and correspondence analysis identified four clusters: normal pancreas, normal islets, insulinoma, and NF-PETs (Fig 1; Data Supplement Fig DS1). These latter fell into three partially overlapping areas reflecting the three WHO categories. Notably, the cluster containing two of the three PDEC, characterized by a ≥ 30% proliferation index, included four well-differentiated carcinomas showing slight to moderate nuclear atypia and a high proliferation index, ranging from 8% to 15%. All the metastasis clustered with their matching primary cancer (Data Supplement).

Class comparison analysis was employed to identify differentially expressed genes between normal and tumor as well as between well-differentiated tumor subtypes, excluding PDECs due to their limited number.

TSC2 Is Downregulated in PETs and Inversely Correlates With Prognosis

Since insulinomas and NF-PETs showed a different expression pattern in the unsupervised analysis, they were independently compared with normal samples (Appendix). This analysis identified 113 upregulated and 25 downregulated genes in insulinomas, while NF-PETs showed 189 upregulated and 55 downregulated genes.

Among the downregulated genes, tuberous sclerosis 2 (*TSC2*) had a consistently decreased expression in both insulinomas and NF-PETs. *TSC2* is an inhibitor of the Akt-mTOR pathway, with a putative oncosuppressor role.[29] Its downregulation has been confirmed using immunohistochemistry on TMAs. In normal pancreata, islet cells had a strong cytoplasmic staining, while nucleus and membrane were negative, whereas in 137 PETs, 35% of tumors had negative or weak

staining, 36% had moderate staining and 30% showed strong TSC2 staining (Fig 2). Patients with a low TSC2 expression (negative to moderate staining) showed a shorter overall survival (Fig 2D; log-rank test n = 123; $P = .005$) as well as a shorter time to progression (Fig 2E; log-rank test n = 122; $P < .001$) and disease-free survival (log-rank test n = 117; $P < .001$). The low level TSC2 expression group was significantly correlated with functional status and tumor aggressiveness (Table 2). More importantly, patients free from liver or lymph node metastasis at diagnosis and low expression of TSC2 had a significantly shorter disease-free survival (log-rank test n = 80; $P = .008$). For instance, the only three WDET patients who had progression of disease showed low TSC2 expression.

Phosphatase and Tensin Homolog Protein Is Downregulated in PETs and Inversely Correlates With Prognosis

As phosphatase and tensin homolog (*PTEN*) is another important inhibitor of the Akt-mTOR pathway,[29] its protein level was also measured using immunohistochemistry on TMAs (Fig 3). The normal pancreatic islet cells showed moderate to strong staining in both the nucleus and cytoplasm, while the membrane was always negative. Tumors showed altered expression in either nuclear, cytoplasmic, or both compartments in 60.6% of the samples. Although a low level of nuclear PTEN (negative to weak staining) was found in 58% of tumors, only cytoplasmic staining showed a significant correlation with clinicopathologic parameters. As observed with TSC2, low cytoplasmic PTEN (negative to weak staining) level was associated with functional status and more aggressive tumors (Table 2). Furthermore, low cytoplasmic expression of PTEN correlated with a shorter time to progression of the disease (Fig 3D; log-rank test n = 122; $P = .002$) and disease-free survival (log-rank test n = 103; $P = .02$).

Combined TSC2 and PTEN Anomalies

Overall, 85% of primary tumors showed altered protein levels of TSC2, PTEN (nuclear or cytoplasmic), or both. Notably, among patients with radical resection of the tumor, of 25 who showed low levels of TSC2 and PTEN expression at both cytoplasmic and nuclear levels, eight (32%) developed liver metastasis and progression of the disease. In contrast, none of the 20 patients with normal level of both TSC2 and PTEN proteins showed liver metastasis and only one had progression of the disease at the primary site. To assess the independent prognostic value of TSC2 and PTEN for both overall and progression-free survival a multivariate analysis has been performed. Selection of the best models was carried out using both forward and backward stepwise methods which gave the same result that is presented in Table 3. Neither TSC2 nor PTEN were independent prognostic indicators at this analysis.

mTOR Inhibitors Induce Growth Arrest in PET Cell Lines

The expression of TSC2 and PTEN protein was also strongly reduced in the three available PET cell lines to the control cells (Hek293T; Fig 4A).

Activity of mTOR was inhibited by both rapamycin and RAD001, even at low concentrations, as suggested by the dephosporylation of its downstream targets rpS6 and 4EBP1 seen in all three cell lines (Fig 4B). As expected, the inhibition of mTOR activated a negative feedback which led to increased AKT phosporylation. Cell proliferation in two cell lines (BON and CM) was significantly affected (Fig 4C), with similar response for both rapamycin and RAD001. Consistent with the expected effect of mTOR inhibition, we observed an increase of cells in G1, and a clear decrease of cells in the S phase, on RAD001 treatment.

SSTR2 Is Upregulated in NF-WDET Compared With Insulinoma

Direct comparison between the 25 NF-WDET and 14 insulinoma identified 161 over-expressed and 101 under-expressed genes.

Among the upregulated genes, the somatostatin receptor 2 (*SSTR2*) showed a median expression 3.5 times higher in NF-WDET compared with insulinoma. The immunohistochemical analysis performed on TMA confirmed this finding (Fig 5). Normal pancreas showed a moderately intense membranous staining of the islet cells with no cytoplasmic or nuclear signals. Among the 25 insulinomas, 10 (40%) showed negative, seven weak (28%) and eight moderate (32%) membranous staining. Conversely, the 47 NF-WDET samples

Table 3. Cox's Regression Analysis of OS and PFS

		OS				PFS		
Parameter*	No. of Cases	HR	95% CI	*P*	No. of Cases	HR	95% CI	*P*
WHO classification				< .001				< .001
Well-differentiated tumor benign	69	1			72	1		
Well-differentiated carcinoma	52	18.8	2.3 to 156		47	10.1	2.8 to 37.2	
Poorly differentiated carcinoma	5	71.9	7.4 to 702		5	15.9	3.1 to 82.1	
Liver metastasis				.003				< .001
No	102	1			103	1		
Yes	24	3.9	1.5 to 9.7		21	3.7	1.7 to 7.8	
Proliferation index				NS				.02
Ki67 < 5%	93				92	1		
Ki67 ≥ 5%	33				32	2.4	1.1 to 5.1	

Abbreviations: OS, overall survival; PFS, progression-free survival; HR, hazard ratio; NS, not significant.
*The best model was selected with the input of the following covariates: WHO classification, presence of liver or nodal metastasis, vascular and perineural and peripancreatic fat invasion, Ki67 (< or ≥ 5%), radical surgery, functional status, tuberous sclerosis 2 and phosphatase and tensin homolog immunohistochemical staining.

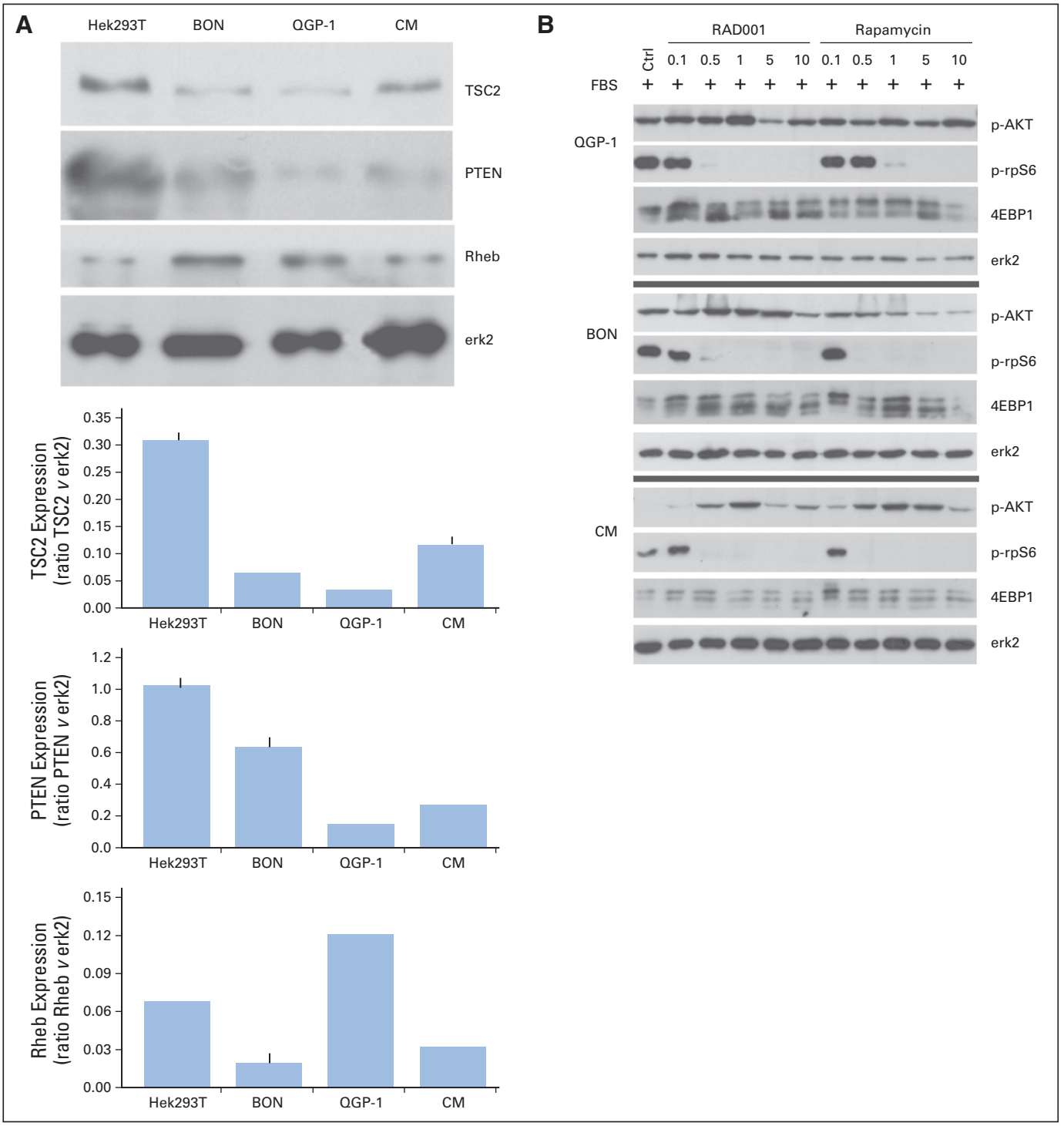

Fig 4. Effect of the treatment of pancreatic endocrine tumor cell lines with mammalian target of rapamycin (mTOR) inhibitors. (A) Protein expression of the three regulators of AKT/mTOR pathway, tuberous sclerosis 2 (TSC2), phosphatase and tensin homolog (PTEN), and Rheb in the pancreatic endocrine cell lines QGP-1, BON, and CM, and relative densitometries (Hek293T cells were used as control). (B) QGP-1, BON, and CM were stimulated with fetal bovine serum for 2 hours and then treated with rapamycin or RAD001 at the indicated concentrations for 24 hours. One representative blot is shown. (Continued on next page.)

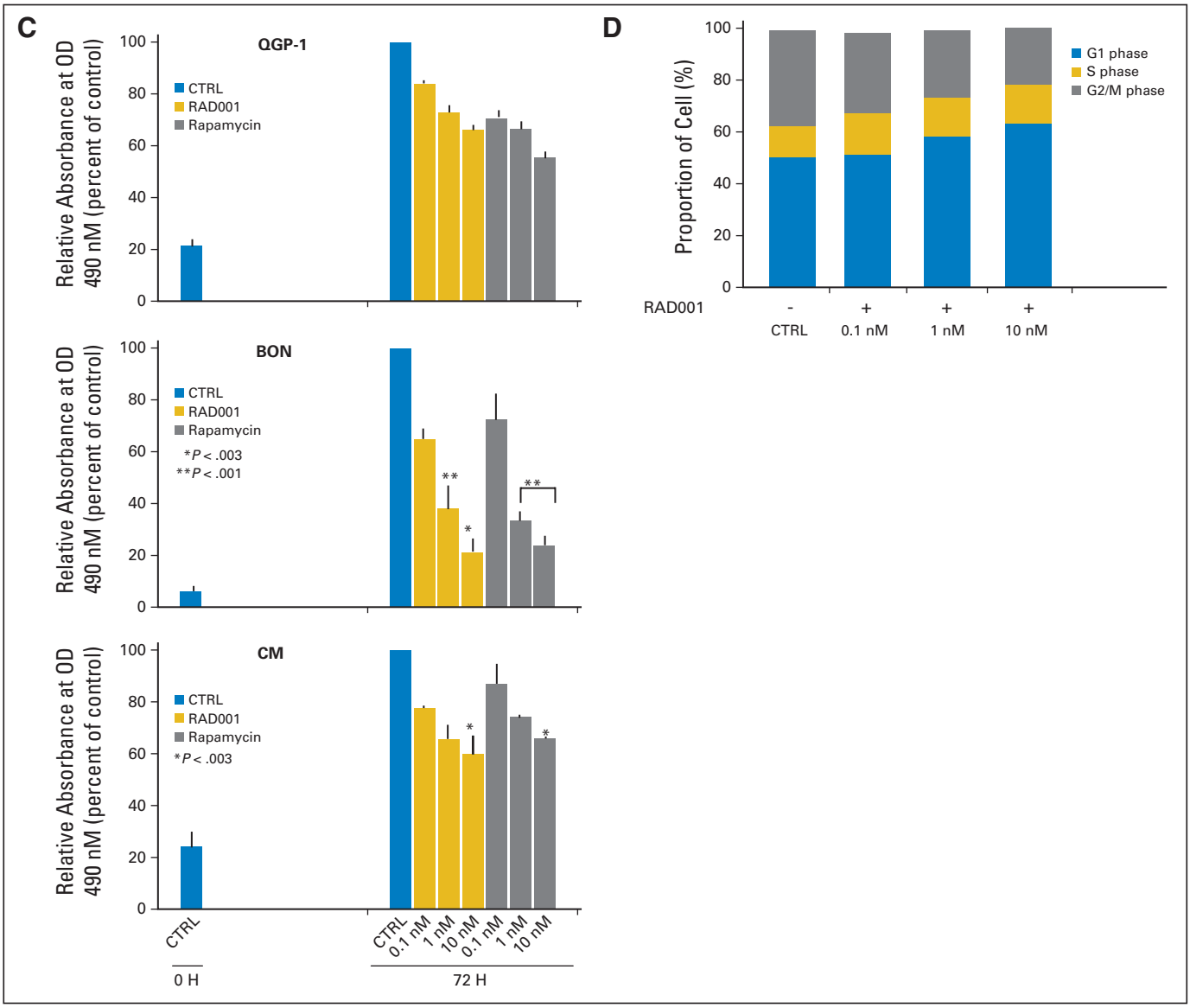

Fig 4. Continued. (C) QGP-1, BON, and CM were treated with rapamycin or RAD001 for 72 hours. The cell proliferation rate was measured by CellTiter 96 kit (Promega, Madison, WI). Means and standard deviations of three independent experiments are reported. *P* represents the significance of each treatment versus untreated control. (D) BON cells were treated for 72 hours with RAD001. Cell cycle was analyzed by flow cytometry. OD, optical density.

showed five negative (11%), 13 weak (28%), 11 moderate (23%), and 18 strong staining (38%). This difference between NF-WDET and insulinoma is highly significant ($P < .001$, Fisher's exact test).

FGF13 *Expression Correlates With Metastatic Potential of NF-PETs*

Taking advantage of the large number of tumors profiled, we divided the population into subgroups based on their clinical-pathological features. It was thus possible to identify several genes that were differentially expressed for samples with different proliferation indexes (cutoff at 5%), WHO classification (WDEC *v* WDET), presence/absence of lymph node metastasis, presence/absence of liver metastasis at diagnosis or follow-up.

Interestingly, a functional classification of genes over-expressed in samples with high proliferation index showed that several Gene Ontology categories related to innate immune response and inflammation were over-represented.

Fibroblast growth factor 13 (*FGF13*) was upregulated in metastatic compared to nonmetastatic primary tumors. Quantitative RT-PCR in a larger set of patients confirmed that *FGF13* expression level was significantly higher in more aggressive tumors (Fig 6A; n = 71; Mann-Whitney U $P < .001$) and in those with a high Ki67 (Fig 6B; n = 77; $P = .003$). Remarkably, high expression of *FGF13* was associated with liver metastasis at diagnosis (n = 75; $P = .001$) or at follow-up (n = 49; $P = .01$), even in patients who had complete primary tumor resection.

Notably, in a multivariate model on well-differentiated neoplasms alone including Ki67 and *FGF13*, high expression of the latter (above median) retained its independent predictive value for shorter progression free survival (Table 4; Cox hazard model, n = 59;

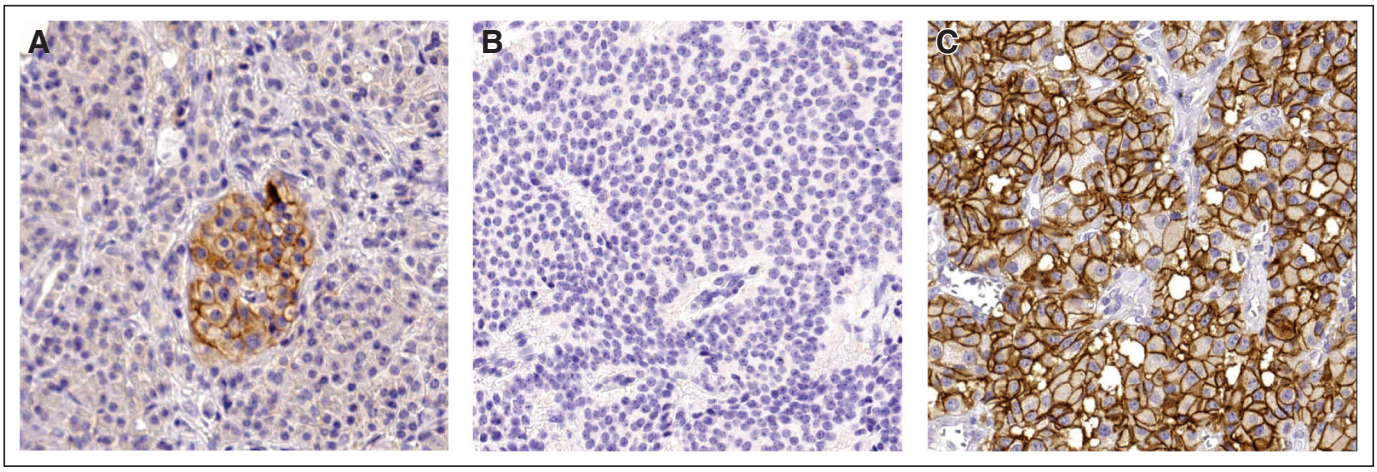

Fig 5. Somatostatin receptor 2 (SSTR2) protein expression in normal pancreatic tissue, nonfunctioning pancreatic endocrine tumors (NF-PETs), and insulinomas. Immunohistochemistry with anti-SSTR2 antibody (Biotrend/Gramsch Laboratories, Schwabhausen, Germany). Original magnification: ×20. (A) Normal pancreatic tissue showing a moderate membranous staining in islet cells; (B) insulinoma tissue with negative membranous staining; (C) NF-PET tissue with strong membranous staining.

$R^2 = 0.34$; $P < .001$). In addition, high *FGF13* predicted a shorter progression-free period (Fig 6C) and disease-free survival in patients considered free from liver or lymph node metastasis at diagnosis (Fig 6D; log-rank test; n = 38; $P = .006$).

Although it was possible to identify genes associated with metastatic potential of primary samples, no relevant difference was observed between the profile of seven metastases (three lymph node and four liver metastases) and their matched primary tumors. The only few genes found downregulated in metastatic tissue were associated with the normal pancreas function, suggesting a high similarity between the expression profiles of primary and metastatic cancer cells (data not shown).

DISCUSSION

Our study takes advantage of the largest set of PET profiles obtained to date to identify biologic traits associated with different clinicopathologic features.

Unsupervised hierarchical clustering of the expression profiles highlighted a distinctive pattern between tumor and normal tissues, and, within the latter, between bulk and islet cells samples, reflecting their different biology and functions. Notably, within PETs, insulinomas expressed a specific pattern of genes that distinguishes them from NF-PETs, while a less clear separation was seen among tumors assigned to different WHO categories. This suggests more subtle differences in terms of gene expression among the nonfunctioning type of PET.

Among the downregulated genes found in PET, *TSC2* is a tumor suppressor of the Akt/mTOR pathway with GTPase activating function that is potentially interesting because of its implication for therapy. *TSC2* mutation leads to tuberous sclerosis complex.[30] Chromosome arm 16p, which contains *TSC2*, has been found to be lost in 37% of PETs.[31,32] Another important tumor suppressor gene involved in the same pathway is *PTEN*, which is also frequently mutated or lost in several sporadic or familiar cancer types[33]; while in PETs the frequency of loss is between 10% and 29%.[31,32,34]

Our immunohistochemical survey on 137 patients showed that TSC2 cytoplasmic protein level was downregulated in 35% of patients, while staining of PTEN was altered in either nuclear, cytoplasmic, or both cell compartments in around 60% of cases. Remarkably, lower cytoplasmic staining of either PTEN or TSC2 correlated with tumor aggressiveness, functional status, proliferation index, presence of liver metastasis at diagnosis or follow-up, and with time to progression. These results held true also when the 5 PDECs contained in the TMA were removed from the analysis (data not shown). Furthermore, TSC2 staining correlated with overall survival and, outstandingly, also with disease-free survival in patients with assumed complete tumor excision. The fact that neither PTEN nor TSC2 were independent prognostic predictors at multivariate analysis does not diminish the value of our observation as its importance resides in the identification of an activated pathway that is pharmacologically targetable.

Several pieces of evidence associated with our findings on TSC2 and PTEN support the hypothesis of involvement of the Akt/mTOR pathway in PET tumorigenesis and progression.

It has been shown that the deficiency of TSC2 or PTEN reduces the inhibition of mTOR activity caused by hypoxia.[35] Although PETs are usually well vascularized, the more aggressive carcinomas show a lower microvascular density compared to the benign form of the tumor.[22,36,37] Notably, we report a lower expression of TSC2 or PTEN associated with the more aggressive subtypes of PETs, where their downregulation may help tumor cells to escape the mTOR inhibition due to lack of oxygen.

Akt/mTOR is involved in the growth and apoptosis of pancreatic β cells.[38] In fact, mice with constitutively active Akt protein or conditional deletion of *TSC2* in the β cells showed similar phenotype, with increased β cell mass and size.[38,39] Guo et al[40] observed the activation of Akt in 14 of 20 PETs and overexpression of cyclin D1 in 13 patients. This latter observation is also important, since *PTEN* mutation or downregulation as well as Akt activation are responsible for the overexpression of cyclin D1,[41] which negatively regulates TSC1-TSC2 function.[42]

It has been shown that lack of TSC2 expression induces impaired PI3K-Akt activation by reducing platelet-derived growth factor receptor[43,44] or insulin receptor substrate level,[45,46] limiting its tumorigenic potential. However, this negative feedback can be overcome by heterozygous *PTEN* inactivation, leading to an active Akt

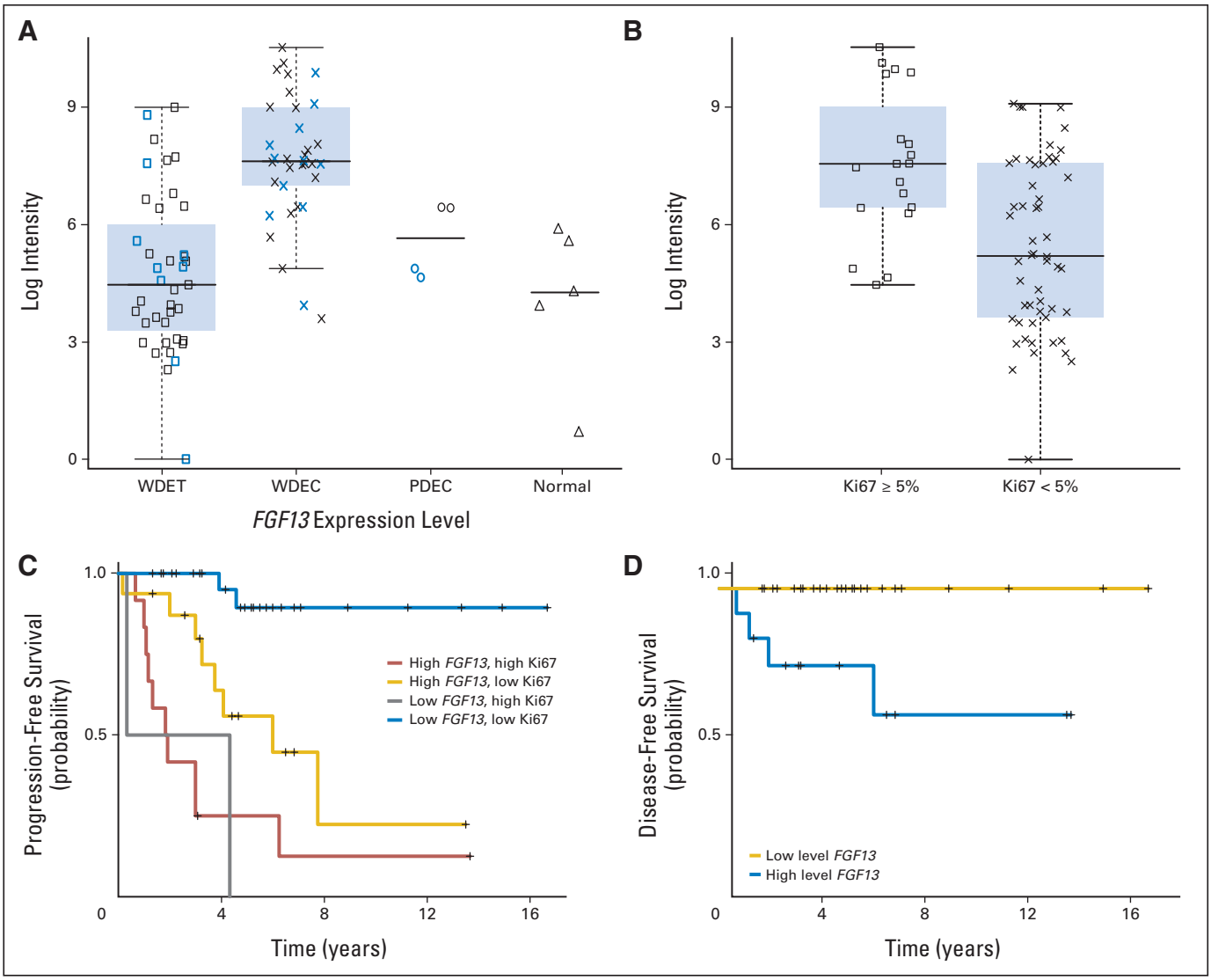

Fig 6. Fibroblast growth factor 13 (*FGF13*) mRNA expression in pancreatic endocrine tumors (PETs). (A) *FGF13* expression measured by quantitative real-time polymerase chain reaction in normal pancreas (two normal bulk pancreas and three islet cell samples) and 77 PETs. These latter comprised 55 cases belonging to the series profiled by microarray (indicated in black) and 22 new cases (indicated in blue). (B) *FGF13* expression in patients with low (Ki67 < 5%) and high (Ki67 ≥ 5%) proliferative index. (C,D) Correlation between *FGF13* mRNA level and progression-free and disease-free survival, respectively. WDET, well-differentiated tumor; WDEC, well-differentiated carcinoma; PDEC, poorly differentiated carcinoma.

protein even in the absence of tyrosine kinase signaling.[47] Partially supporting these observations, we found that low expression of both PTEN and TSC2 was associated with aggressive tumors.

Several new available rapamycin analogs seem to show higher antiproliferative effects in vitro and in vivo on cell lines with lost PTEN expression.[48,49] Our in vitro study performed in three PET cell lines showed inhibition in proliferation and G0/G1 cell cycle arrest on treatment with both rapamycin and RAD001, confirming previous findings.[50,51] Notably, the treatment induced AKT activation, which is considered the molecular mechanism attenuating the therapeutic effects of mTOR inhibitors.[52] Recently, NVP-BEZ235 has been shown to be able to inhibit both PI3K and mTOR, overcoming the above-mentioned negative feedback.[53]

To date, two clinical trials have evaluated the efficacy of mTOR inhibitors in PET. Duran et al analyzed the effect of temsirolimus, a rapamycin analog, in neuroendocrine cancer, and found either a partial response or a stabilization of the disease in 63.9% of patients.[54,55] More recently, Yao et al[56] evaluated the activity of a combined treatment of RAD001 and octreotide. Again, this showed promising results, with 22% of patients showing partial response, while 70% had their disease stabilized.

Our observation that somatostatin receptor 2 is predominantly overexpressed in NF-PET, with almost 40% of the patients having very strong staining, is extremely relevant for PET therapy. In this regard, Butturini et al[28] have recently shown that treatment of NF-PET with somatostatin analog octreotide can stabilize the disease in approximately 40% of these patients.[28,56] The finding of differential SSTR expression raises the potential for SSTR expression to be evaluated as a marker of response to somatostatin analogs.

Table 4. Multivariate Analysis of Progression-Free Survival With *FGF13* Expression and Proliferative Marker Ki67

Variable	Hazard Ratio*	95% CI	*P*
High *FGF13* expression	3.9	1.2 to 12.0	.02
Ki67 ≥ 5%	4.1	1.6 to 10.3	.003

*The hazard ratio and its CI were estimated using a Cox proportional hazard model.

Moreover, we identified *FGF13* as a new marker of progression which has never been described before in PET. Its expression was correlated with tumor aggressiveness and proliferation. More importantly, its overexpression was an independent predictor of a shorter progression-free survival in association with Ki-67 staining, which is the gold standard biologic marker in PET. Furthermore, overexpression was associated with liver metastasis at follow-up and shorter disease-free survival in those patients who underwent complete tumor resection. However, the limited number of events within the analyzed population did not permit the performance of more comprehensive multivariate analyses. Little is known about *FGF13* function and, although FGF13 protein has a high homology with FGFs, it is unable to stimulate FGF receptors.[57] It has been shown that it interacts with Islet brain-2 and function as cofactor to recruit the MAP kinase protein p38.[58]

The expression analysis of primary tumors and matched metastasis clearly showed an almost identical pattern between them, supporting the hypothesis of an early acquisition of genetical features to metastasize by the entire primary tumor.[21,59]

In conclusion, this study suggests an important role of the PI3K-Akt-mTOR pathway in PET tumorigenesis and progression, which support the promising results observed in recent clinical trials involving the use of rapamycin analogs in neuroendocrine tumors. Further in vitro and in vivo studies are required in order to fully understand the efficacy of these drugs in PET, particularly when combined with others potentially able to block *AKT* activation.

Finally, we showed that the overexpression of *FGF13* was a predictor of shorter progression-free survival independent from the proliferation index Ki67. This marker merits further studies on additional case series to validate its clinical applicability.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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