Attenuation of the Retinoblastoma Pathway in Pancreatic Neuroendocrine Tumors Due to Increased Cdk4/Cdk6

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Abstract

Purpose: In mice, genetic changes that inactivate the retinoblastoma tumor suppressor pathway often result in pancreatic neuroendocrine tumors (Pan-NETs). Conversely, in humans with this disease, mutations in genes of the retinoblastoma pathway have rarely been detected, even in genome-wide sequencing studies. In this study, we took a closer look at the role of the retinoblastoma pathway in human Pan-NETs.

Experimental Design: Pan-NET tumors from 92 patients were subjected to immunohistochemical staining for markers of the retinoblastoma pathway. To search for amplifications of retinoblastoma pathway genes, genomic DNAs from 26 tumors were subjected to copy number analysis. Finally, a small-molecule activator of the retinoblastoma pathway was tested for effects on the growth of two Pan-NET cell lines.

Results: A majority of tumors expressed high amounts of Cdk4 or its partner protein cyclin D1. High amounts of phosphorylated Rb1 were present in tumors that expressed high levels of Cdk4 or cyclin D1. The copy numbers of Cdk4 or the analogous kinase gene Cdk6 were increased in 19% of the tumors. Growth of the human Pan-NET cell line QGP1 was inhibited in a xenograft mouse model by the Cdk4/6 inhibitor, PD 0332991, which reactivates the retinoblastoma pathway.

Conclusions: Inactivation of the retinoblastoma pathway was indicated for most Pan-NETs. Gene amplification and overexpression of Cdk4 and Cdk6 suggests that patients with Pan-NETs may respond strongly to Cdk4/6 inhibitors that are entering clinical trials. Clin Cancer Res; 18(17): 4612–20. ©2012 AACR.

Introduction

Pancreatic neuroendocrine tumors (Pan-NETs) represent the second most common epithelial neoplasm in the pancreas, accounting for 1% to 2% of pancreatic tumors (1). Originally considered to be a benign group of insulin-producing tumors (insulinoma), it has now become apparent that 50% or more Pan-NETs are nonfunctional and help to comprise a heterogeneous group of tumors with often unpredictable and varying degrees of malignancy (2). Pan-NETs pose significant challenges in clinical management. Up to 50% of patients have liver metastases at the time of initial diagnosis, and median survival for patients with liver metastasis is 24 months (3). Currently, there are no reliable biomarkers to predict the recurrence and progression of the disease.

Although Pan-NETs may respond to streptozotocin or temozolomide, the majority of low- and intermediate-grade tumors are insensitive to standard cytotoxic chemotherapy. This has led to efforts to explore pathways affected by oncogenes and tumor suppressors in these tumors in hopes of predicting better treatment strategies. Mutations associated with pancreatic neuroendocrine tumorigenesis occur in genes involved in chromatin remodeling, such as MEN1, ATRX, and DAXX (4, 5), but these associations have not yet led to treatments. On the other hand, patients with tuberous sclerosis occasionally present with Pan-NETs (6–8), and the fact that mutations causing tuberous sclerosis activate the mTOR protein led to the idea that patients with Pan-NETs would benefit from treatment with the mTOR inhibitor rapamycin. Indeed, the rapamycin analogue everolimus was recently approved as a treatment for this disease (3); moreover, mutations in mTOR pathway genes TSC2 and PTEN have recently been found in 15% of Pan-NETs. At the level of tumor anatomy, Pan-NETs are remarkably vascularized, and the angiogenesis inhibitor sunitinib has also been approved for treatment of patients with Pan-NETs (9).

A critical step in tumorigenesis is entry into S-phase of cell cycle in the absence of growth signals. The Rb1 tumor suppressor plays an important role in regulating this step by preventing the activity of E2F transcription factors that are critical for expression of genes involved in S-phase metabolism (Fig. 1; ref. 10). Loss of Rb1 function results...
in unchecked transcriptional activation by elongation factor 2 (E2F) and can occur either by loss of Rb1 protein itself via Rb1 gene mutations or by aberrations in other regulatory elements of the Rb1 pathway that increase phosphorylation of the Rb1 protein (Fig. 1). In fact, 80% of cancers maintain an intact Rb1 protein but display genetic alterations of other components of the Rb1 pathway (11). Rb1 is negatively regulated via phosphorylation by cyclin-dependent kinases Cdk4 and Cdk6, which are activated by cyclin D1 and negatively regulated by a number of cyclin-dependent kinase inhibitors. Tumors with an intact Rb1 gene may show chromosomal rearrangement or amplification of Cdk4 or D-cyclin genes, as well as loss or repression of p16\textsuperscript{ink4a}, which encodes an inhibitor of Cdk4/6 (12–14).

In this study, we investigated abnormalities in the Rb1 pathway in Pan-NETs and identified significant genetic aberrations which culminated in attenuated Rb1 function, and which could be restored by a small molecule, PD 0332991.

Materials and Methods

Patients and clinical data

Cases of well-differentiated Pan-NETs, including both functioning and nonfunctioning tumors, and corresponding clinical data were collected from the institution databases from 1996–2008. The study was approved by the Internal Review Board of the Memorial Sloan-Kettering Cancer Center. Disease-related factors included the date and location of recurrence and survival, as measured to the time of last follow-up or death. Patient status at last follow-up was documented as no evidence of disease, alive with disease, dead of disease, and dead of other causes. Clinopathologic variables were assessed for their association with recurrence and survival. Recurrence-free survival (RFS) and disease-specific survival (DSS) were calculated using the Kaplan–Meier method, and comparisons were made using the log-rank test. Significance was defined as P < 0.05.

Tissue samples

Formalin-fixed and paraffin-embedded (FFPE) tissue blocks (N = 92) were used for tissue microarray (TMA) construction for immunohistochemical staining and also for FISH (see below). Fresh-frozen tissue was available for gene expression assay by quantitative real-time PCR (qRT-PCR) on 41 cases, 36 of which were also within the FFPE TMA.

Cell lines

The Pan-NET cell line QGP1 was purchased from the Japan Health Sciences Foundation. BON1 was a gift from the lab of Kjell Oberg. MCF7 and SAOS2 were purchased from American Type Cell Culture. Cell lines were grown at 37°C under 5% CO\textsubscript{2}. All lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS except QGP1, which was grown in RPMI supplemented with 10% FBS. DMEM and RPMI were purchased from Invitrogen. FBS was purchased from Sigma-Aldrich.

Construction of TMAs

Hematoxylin- and eosin-stained slides of the pancreatic resection specimens were reviewed by one pathologist, and slides containing tumor were marked and matched with corresponding paraffin blocks. Tissue cores of 0.6 mm were then punched out in triplicate from locations randomly selected within the marked tumor areas and mounted in blank recipient blocks using an automated tissue microarrayer (Beecher Instruments, Inc.).
**Immunohistochemistry**

Immunohistochemical staining for Cdk4, phospho-Rb1, and cyclin D1 were conducted on TMA from representative paraffin blocks with tumor tissues in triplicate cores for each case. The sections were deparaffinized in xylene, rehydrated with ethanol, and then steamed for 30 minutes with citrate buffer (0.01 mol/L citric acid, pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked using 95 mL of methanol plus 5 mL of 3% hydrogen peroxide solution. Non-specific protein binding was blocked with 1% bovine serum albumin (BSA) for 60 minutes. Rabbit anti-phospho-Rb1 antibody (Ser807/811), rabbit monoclonal cyclin D1, and mouse monoclonal Cdk4 were from Cell Signaling Technology, Neo Markers, and Invitrogen, respectively. Positive controls were included with each antibody and negative controls were obtained by omitting the primary antibodies. Correlation between protein expression was determined using Pearson product-moment coefficient (R) with P values.

**DNA copy number assays**

Genomic DNA was isolated from either human tumors or cell lines using a Wizard kit from Promega. Copy number assays for Cdk4, Cdk6, Cdk2, or Gli1 were conducted by RT-PCR using an Applied Biosystems Prism 7500. TERT and RNaseP were used to normalize the quantitation, and only tumors that showed amplification relative to both TERT and RNaseP were called amplified. All copy number assays were purchased from Applied Biosystems.

**Quantitative real-time PCR**

RNA was extracted from fresh-frozen tissue using Qiagen RNAeasy Mini Kit (Qiagen), then reversely transcribed into cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems). Cdk4 and Cdk6 transcription was evaluated using TaqMan Real Time PCR Gene Expression Assays (Applied Biosystems). The reaction was carried out on the Applied Biosystems 7500 Fast Real Time PCR System using TaqMan Real Time PCR Gene Expression Assays. To determine steady-state expression of Cdk4, NET cell lines were plated for 12 hours before addition of nocodazole, washed in PBS, and fixed in 70% ethanol. Propidium iodide was then added and cell-cycle analysis was conducted using a FACScan® digital cell cycle analysis (BD Biosciences). The Bliss additivity value was computed as the sum of the percentage G1 arrest caused by either drug alone, minus the product of both of the percentage G2 arrest values.

**Immunoblot**

Antisera raised against Cdk4, Rb1, or Rb1 phosphorylated at serine 780 were purchased from Novus Biologicals. β-Actin antisera was purchased from Sigma Aldrich. To determine steady-state expression of Cdk4, NET cell lines QGP1, BON1, or H727 were plated for 36 hours and then protein was extracted using radioimmunoprecipitation assay buffer. To measure the effect of PD 0332991 on Rb1 phosphorylation, BON1 or QGP1 cells were plated for 24 hours and drug was then added along with 100 ng/mL nocodazole (Sigma-Aldrich) for 16 hours. Cells were harvested by trypsinization 16 hours after addition of nocodazole, washed in PBS, and fixed in 70% ethanol. Propidium iodide was then added and cell-cycle analysis was conducted using a FACScan® digital cell cycle analysis (BD Biosciences). The Bliss additivity value was computed as the sum of the percentage G1 arrest caused by either drug alone, minus the product of both of the percentage G2 arrest values.
either exclusively in the nucleus, or else in both nucleus and cytoplasm. There was a statistically significant correlation between phospho-Rb1 and Cdk4 protein expression ($r = 0.55; P = 0.01$). Another kinase of Rb1, Cdk6, was not tested due to poor antisera, but we did look at cyclin D1 expression. Cyclin D1 staining occurred in 68% of Pan-NETs but not in normal islets. Like Cdk4, cyclin D1 expression correlated well with Rb1 phosphorylation ($r = 0.51; P = 0.03$). High expression of cyclin D1 has previously been reported in Pan-NETs (18, 19), but high amounts of Cdk4, phospho-Rb1, and correlation analyses are novel results. We found no significant correlation of either Cdk4 or high phospho-Rb1 with either recurrence or disease-free survival with a mean clinical follow-up of 55 months ($P > 0.05$).

qRT-PCR was conducted using mRNA from fresh-frozen samples of well-differentiated Pan-NETs. When compared with normal pancreas tissue, the Cdk4 transcript was markedly increased ranging from 1.2- to 97-fold (mean $12.5 \pm 2.5$) (Fig. 3A). In contrast, increased Cdk6 transcript was less common with mean transcription levels of $0.8 \pm 0.1$ when compared with control normal tissue (Fig. 3B). Interestingly, Cdk4 transcripts were significantly higher in nonfunctional Pan-NETs (mean $14.6 \pm 3.0$) when compared with functional Pan-NETs (mean $3.8 \pm 0.5$), which were represented by clinically symptomatic insulinoma, glucagonoma, and vasoactive intestinal peptide (VIP) producing tumor (VIPoma; Fig. 3C).

Although recent exomic sequencing of Pan-NETs did not reveal point mutations in retinoblastoma pathway genes (4), our observations of high expression of Cdk4 RNA and protein, along with high Rb1 phosphorylation, indicated the possibility of amplification of the Cdk4 gene in these tumors. Indeed, RT-PCR analysis revealed Cdk4 or Cdk6 to be amplified in a subset of tumors (Fig. 4A and B). Out of 26 tumors tested, 3 had a higher dosage of the Cdk4 gene, and 2 had a higher dosage of the Cdk6 gene. Consistent with the overlapping functions of the 2 genes, tumors with amplified Cdk4 did not have amplified Cdk6,

Figure 2. Immunohistochemistry of proteins involved in Rb1 phosphorylation. Representative tumors expressing Cdk4, phosphorylated Rb1, and cyclin D1 are shown. Out of 92 cases, immunoreactivity of Cdk4, phosphorylated Rb1, and cyclin D1 was detected in 58%, 68%, and 68% of Pan-NETs, respectively.

Results

Pan-NETs express high levels of Cdk4 and its product phospho-Rb1

Immunohistochemistry was carried out on a TMA constructed from 92 cases of well-differentiated Pan-NETs. Clinical characteristics of this database were previously published (16, 17). Representative cases are shown in Fig. 2. Although neither Cdk4 nor phospho-Rb1 could be detected in normal islets (data not shown), Cdk4 staining was detected in 58% of Pan-NETs, and phospho-Rb1 was detected in 68% of tumors. Phospho-Rb1 was found to be detected in normal islets (data not shown), Cdk4 staining was detected in 58% of Pan-NETs, and phospho-Rb1 was detected in 68% of tumors.
although this observation was not statistically significant due to the small sample size. Altogether, 19 percent of tumors showed amplification of either Cdk4 or Cdk6. The level of amplification was small, in the range of 1 to 2 additional copies (Fig. 4A and B). We also used RT-PCR as a method to test for amplification of genes proximal to Cdk4, and saw no examples within this 26 patient data set of increased copy number of the Gli1 or Cdk2 genes, which reside within 300 and 1,800 kilobases, respectively, on either side of the Cdk4 gene on chromosome 12 (data not shown). Therefore, amplification of Cdk4 appears to be specific to this gene locus. Cyclin D1, which resides on chromosome 11, was not amplified within this data set (data not shown).

We further investigated Cdk4 gene status by FISH of the 92 cases within our TMA and observed tumors in which Cdk4 was present in 3 or 4 copies but in which there were only 2 copies of the centromere of chromosome 12 (Fig. 4C). These tumors therefore appear to have extra copies of Cdk4 due to amplification, and not due to G2 arrest or polysomy.

**Neuroendocrine cell lines and Cdk4/6**

We examined pancreatic neuroendocrine cell lines BON1 and QGP1 and pulmonary neuroendocrine cell line H727 in hopes of finding an in vitro model system for Cdk4 amplification-expression in human NETs. While BON1 did not show amplification of Cdk4, the QGP1 and H727 cell lines had 6 and 3 copies of the Cdk4 gene, respectively (Fig. 4A). Gene copy number correlated with Cdk4 mRNA and protein expression levels in these 3 cell lines as assessed by qRT-PCR and Western blot, respectively (Fig. 5A). There was an extra copy of Cdk6 in QGP1, but this gene was not amplified in H727 or BON1 (Fig. 4B).

The pharmaceutical compound PD 0332991 selectively inhibits the activity of both Cdk4 and Cdk6 (20). Growth of the 2 pancreatic neuroendocrine cell lines, QGP1 and BON1, responded in a dose-dependent fashion to treatment with PD 0332991 (Fig. 5B). Interestingly, QGP1, the cell line with amplified Cdk4, was particularly sensitive to PD 0332991, with an IC50 of only 36 nmol/L. BON1, which expresses less Cdk4 and is not amplified for the gene, had a higher IC50 of 135 nmol/L. As shown in Fig. 5C, treatment
of QGP1 and BON1 cells with PD 0332991 for only 7 hours significantly decreased phosphate-Rb1 levels without changing the total amount of Rb1 protein. The human breast cell line MCF7 was previously reported to be a strong responder to PD 0332991 (21), and we measured an IC50 value of 125 nmol/L for this line (data not shown), which compares favorably with the IC50 of QGP1 and BON1. We also measured a very high IC50 (>1,000 nmol/L; data not shown) for the osteosarcoma cell line SAOS2. This is consistent with a known deletion of the Rb1 gene in SAOS2, which renders its growth less dependent on Cdk4 activity.

If PD 0332991 inhibits cell line growth by targeting Cdk4/6, then it should arrest growth at the G1-phase of the cell cycle. Indeed, a very large percentage of BON1 and QGP1 cells remained in G1 phase upon PD 0332991 treatment (Fig. 5D). In this experiment, growth arrest of cells treated with nocodazole alone, which blocks in G2, were compared with cells pretreated with PD 0332991 for 3 hours, then cotreated with PD 0332991 and nocodazole. Pretreatment with PD 0332991 resulted in a 10-fold and 6-fold increase in G1 arrest for QGP1 and BON1, respectively. These data show that low concentrations of PD 0332991 can block Cdk4 activity in 2 Pan-NET cell lines, reactivate Rb1, and halt cell growth at G1.

Next, we tested the effect of PD 0332991 on growth of the QGP1 cell line in vivo. QGP1 cells were injected subcutaneously into the flanks of nude mice to establish xenografted tumors. After 2 weeks, the mice were treated either with phosphate-buffered saline or with 150 mg/kg of PD 0332991. As shown in Fig. 6A, the tumors within the PD 0332991-treated group did not grow. The difference in tumor volume between the 2 groups was statistically significant by day 10, and remained statistically significant at days 12 and 14.

Finally, we investigated the effect of rapamycin upon Cdk4 expression and upon the activity of PD 0332991. A previous study showed that rapamycin treatment can decrease expression of Cdk4 by other cell lines (22), and in Figs. 6B and C we show that this is also true for BON1 and QGP1. The dose of rapamycin required to lower Cdk4 is much less than the IC50 of either cell line, which we determined to be 1 and 3 nmol/L for BON1 and QGP1, respectively (data not shown). We wondered whether rapamycin and PD 0332991 might be able to function in combination to block the cell cycle: rapamycin, by lowering Cdk4 expression and PD 0332991, by inhibiting the Cdk4 that remained. As shown in Fig. 6D, a sub-IC50 dose of rapamycin alone, or PD 0332991 alone, resulted in very small increases in the population of cells in G1. However, combined low doses of the 2 drugs increased G1 arrest. The observed G1 arrest caused by the combination of drugs is
much higher than the Bliss additivity value, which estimates the effect of 2 drugs if they act independently. Therefore in either cell line, rapamycin decreases Cdk4 expression and acts synergistically with PD 0332991.

Discussion

In this study, we have shown that high expression of Cdk4 coincides with Rb1 phosphorylation in a majority of Pan-NETs. High Cdk4 expression is due at least in part to increased transcription, as we find higher levels of Cdk4 mRNA in Pan-NETs when compared with normal pancreatic tissue. One mechanism for increased transcription may be amplification of the Cdk4 gene. In 12 percent of the samples tested, the copy number of Cdk4 increased. But amplification alone does not account for all of the tumors in which we observed high Cdk4 expression (12% amplification vs. 58% overexpression). In 2 pancreatic neuroendocrine cell lines, we show that Cdk4 expression decreases upon inhibiting mTOR with rapamycin; thus, an alternative mechanism for increased Cdk4 expression may be through activation of the mTOR pathway, which occurs by mutation of PTEN and TSC2 in patients with Pan-NETs.

There are other ways to inactivate the retinoblastoma pathway in Pan-NETs. For instance, hypermethylation of the promoter for the cyclin-dependent kinase inhibitor gene p16\(^{INK4a}\) has been reported to occur in Pan-NETs (23). Also, we observe increased copy number of Cdk6 in 8% of the samples. Thus, about 1 in 5 Pan-NETs have higher dosage of Cdk4 or Cdk6. In all cases of amplification of both Cdk4 and Cdk6, the increased gene dosage was a modest 1 to 2 copies. Amplification of the Cdk4 gene has previously been reported for a number of tumor types, including breast, melanoma, lymphoma, sarcoma, and glioma (24). The involvement of the Cdk4/6-cyclin D1 complex in tumorigenesis of mantle cell lymphoma is particularly well-established due to the presence of a t(11;14)(q13;q32) translocation that puts Cyclin D1 under control of an immunoglobulin enhancer in a high majority of these patients (25). Interestingly, low copy number (<5) increases in dosage of the Cdk4 gene, without coamplification of a nearby gene on chromosome 12q13, have been found in the more aggressive blastic form of these tumors (26). As is true for mantle cell lymphoma, we have also found high expression of cyclin D1 as well as low copy number, locus-specific increases in the dosage of the Cdk4 gene. Given this

Figure 6. Effect of PD 0332991 in vivo, or in combination with rapamycin. A, tumor xenografts composed by human QGP1 pancreatic neuroendocrine cells were established for 2 weeks in nude mice, which were then treated either with PBS or with the Cdk4/6 inhibitor PD 0332991 at a dose of 150 mg/kg. The upper line shows the control group and the lower line shows the drug-treated group. The difference in tumor volume between the control and drug-treated group was statistically significant (P < 0.03 by 2-tailed t test) on days 10, 12, and 14. BON1 (B) or QGP1 (C) cells were treated for 14 hours with the indicated dose of rapamycin, and then protein extracts were prepared and separated by SDS-PAGE. The expression of Cdk4 and β-actin was detected by Western blot. D, BON1 and QGP1 cell lines were treated with 0.2 nmol/L rapamycin alone (RAP), 20 nmol/L PD 0332991 alone (PDO), or both 0.2 nmol/L rapamycin and 20 nmol/L PD0332991 (R + P). After 7 hours, nocodazole was added and cells were grown for another 16 hours to enhance detection of G1 arrest. G1 arrest was measured by flow cytometry. Also shown is the Bliss additivity value, which estimates the level of G1 arrest that would occur by combining the 2 drugs if they acted independently. For both QGP1 and BON1, the difference between the Bliss value and the actual value of the drugs in combination was statistically significant (P < 0.01 by 2 tailed t test).
sensitivity to PD 0332991. The RPI1-Tag2 mouse develops insulinomas due to insulin promoter–driven expression of the SV40 T antigen, which inactivates both the retinoblastoma and p53 families of tumor suppressor genes (28). Mice doubly knocked out for the p18$^{INK4C}$ and p27$^{RPI1}$ cyclin-dependent kinase inhibitor genes develop islet cell hyperplasia (29). Cdk4(R24C) mice express an allele of Cdk4 that cannot be downregulated by p16$^{INK4A}$, and these mice produce a large variety of tumors, including Pan-NETs (30, 31). Indeed, Cdk4 has been called a “driver” of retinoblastoma pathway–dependent tumorigenesis due to the high penetrance of tumor formation in Cdk4(R24C) mice as compared to much weaker cancer phenotypes for mice with mutations in other genes of the retinoblastoma pathway (32). In a complementary experiment, mice lacking Cdk4 were shown to be relatively healthy with the notable exception of diabetes, due to depletion of pancreatic beta cells (30). More recently it was shown that Cdk4 acts at a very early stage in islet cell development by stimulating the replication of neuroendocrine stem cells (33).

The present study may be of significant clinical value. PD 0332991 is currently in phase II trials for other human malignancies, and another Cdk4/6 inhibitor is in a phase I trial (http://clinicaltrials.gov/ct2/show/NCT01237236; http://clinicaltrials.gov/ct2/show/NCT01037790). Tumors in which Cdk4 or Cdk6 are overexpressed may respond particularly well to treatment with PD 0332991 as does the QGP1 cell line, which has a Cdk4 amplification and high sensitivity to PD 0332991.

Importantly, PD 0332991 shows synergy with rapamycin in vitro. Rapamycin treatment can result in side-effects including infections due to immunosuppression, and it is possible that combined treatment with PD 0332991 may lower the required dose of rapamycin analogues and thereby reduce its side-effects. Interestingly, the BON1 cell line, which has lower expression of Cdk4 than QGP1 but high sensitivity to rapamycin, was particularly sensitive to combination treatment with PD 0332991 and rapamycin. Thus, the efficacy of Cdk4/6 inhibition may not be strictly dependent on Cdk4 amplification.

Disclosure of Potential Conflicts of Interest
D.S. Klimstra is a consultant/advisory board member for Novartis. No potential conflicts of interest were disclosed by the other authors.

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