Prkar1a gene knockout in the pancreas leads to neuroendocrine tumorigenesis

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Abstract

Carney complex (CNC) is a rare disease associated with multiple neoplasias, including a predisposition to pancreatic tumors; it is caused most frequently by the inactivation of the PRKAR1A gene, a regulator of the cyclic AMP (cAMP)-dependent kinase (PKA). The method used was to create null alleles of prkar1a in mouse cells expressing pdx1 (Δ-Prkar1a). We found that these mice developed endocrine or mixed endocrine/acinar cell carcinomas with 100% penetrance by the age of 4–5 months. Malignant behavior of the tumors was seen as evidenced by stromal invasion and metastasis to locoregional lymph nodes. Histologically, most tumors exhibited an organoid pattern as seen in the islet-cell tumors. Biochemically, the lesions exhibited high PKA activity, as one would expect from deleting prkar1a. The primary neuroendocrine nature of these tumor cells was confirmed by immunohistochemical staining and electron microscopy, the latter revealing the characteristic granules. Although the Δ-Prkar1a mice developed hypoglycemia after overnight fasting, insulin and glucagon levels in the plasma were normal. Negative immunohistochemical staining for the most commonly produced peptides (insulin, c-peptide, glucagon, gastrin and somatostatin) suggested that these tumors were non-functioning. We hypothesize that the recently identified multipotent pdx1+/insulin− cell in adult pancreas, gives rise to endocrine or mixed endocrine/acinar pancreatic malignancies with complete prkar1a deficiency. In conclusion, this mouse model supports the role of prkar1a as a tumor suppressor gene in the pancreas and points to the PKA pathway as a possible therapeutic target for these lesions.

Introduction

Carney complex (CNC; Online Mendelian Inheritance in Man 160987) is a multiple endocrine neoplasia syndrome characterized by endocrine tumors, schwannomas, cardiac and extracardiac myxomatosis and spotty skin pigmentation (Stratakis et al. 2001). Specific examples of the endocrine tumors include multiple hyper-secretory...
adrenal nodules (described pathologically as primary pigmented nodular adrenocortical disease), thyroid and gonadal neoplasms and growth hormone-secreting pituitary tumors. Several hundreds of patients with CNC have been reported so far worldwide, and a genetic defect has been identified for the majority of them (Bertherat et al. 2009, Rothenbuhler & Stratakis 2010). Inactivating mutations in the PRKAR1A, which codes for the regulatory subunit type 1α (Ra) of the cAMP-dependent protein kinase A (PKA) and is located on chromosome 17q23–24 have been detected in approximately 70% of the kindreds with known CNC (Kirschner et al. 2000, Bertherat et al. 2009, Horvath et al. 2010, Salpea et al. 2014). Inactivating mutations of the PRKAR1A gene lead to aberrant function of PKA and increased phosphorylation of the downstream targets implicated in cycle progression and apoptosis, as well as cell transcription (Bossis & Stratakis 2004). Thus, in patients with CNC, PRKAR1A seemingly functions as a tumor suppressor gene (Boikos & Stratakis 2006, 2007).

An unexpectedly high prevalence of rare pancreatic neoplasms has been detected among CNC patients (Gauloux et al. 2011). Nine patients (2.5%) with CNC and pancreatic neoplasms within an international cohort of 354 CNC patients were identified. Loss of heterozygosity (LOH) and immunohistochemistry studies suggested that PRKAR1A might function as a tumor suppressor gene in pancreatic tissue, at least in the context of CNC.

Previous studies from our group have shown that Prkar1a heterozygous mice (Prkar1a+/−) develop various tumors, which include schwannomas, thyroid neoplasms and tail bone lesions, in a spectrum that overlaps with that observed in CNC patients (Kirschner et al. 2005). Genetic analysis indicated that allelic loss occurred in a subset of tumor cells. Some pancreatic tumors at a low frequency (5%) also formed in the aged heterozygote subset of tumor cells. Some pancreatic tumors at a low frequency (5%) also formed in the aged heterozygote subset of tumor cells.

To better understand the role of PRKAR1A in the pancreas, we knocked out Prkar1a in pdx1-expressing cells. Interestingly, these mice developed with high-penetrance malignant neuroendocrine pancreatic neoplasms with an acinar component. This finding may support the role of PKA as a potential druggable target in the neuroendocrine tumors of the pancreatic gland.

Materials and methods

Animal studies

All mice were housed three to four per cage with same-sex littermates, with ad libitum access to food and water and maintained on a 12 h light: 12 h darkness schedule (lights on at 06:00h). All animal procedures were conducted in accordance with the standards approved by the NIH Guide for the Care and Use of Laboratory Animals. All animal protocols received prior approval at the NIH.

Prkar1a+/− and Pdx1-CRE mice were previously reported (Lammert et al. 2001, Kirschner et al. 2005) and were kept in a mixed genetic background (C57Bl/6×FVB). Both Prkar1a alleles were conditionally deleted during development by generating the Prkar1a+/−; Pdx1-Cre+ mice (Δ-Prkar1a).

Histology and immunostaining

Normal pancreatic tissues and tumors were collected from mice at predetermined time periods or when their condition deteriorated and they had to be killed. Tissues were fixed in 4% neutral-buffered formaldehyde for at least 24 h, followed by dehydration and paraffin embedding. For all immunostaining experiments, appropriate positive and negative controls were run concurrently for all the applied antisera on the adjacent pancreas sections. Histopathological analysis was carried out on 3-μm sections stained with Mayer’s H&E. Immunostaining was performed on serial sections essentially, as previously described (Kirschner et al. 2005, Szarek et al. 2008) using primary antibodies against PRKAR1A (ab38936, 1:100; Abcam), Chromogranin A (ab15160, Abcam), Chymotrypsin (ab118845, Abcam), PDX1 (ab47383, Abcam) and Insulin (ab7842, Abcam). Antibody solutions 1:200 in blocking buffer (PBS, 0.01% azide, 10% normal donkey serum; Sigma-Aldrich) were used.

Transmission electron microscopy (EM)

EM was performed on mouse tissues fixed overnight at 4°C with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and washed with cacodylate buffer three times. The tissues were fixed with 2% OsO4 for 2 h, washed again with 0.1 M cacodylate buffer three times, washed with water and placed in 1% uranyl acetate for 1 h. The tissues were subsequently serially dehydrated in ethanol and propylene oxide and embedded in EMBed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections, ~80 nm thick, were obtained by using the Leica ultracut-UCT ultramicrotome (Leica) and placed onto 300-mesh copper grids and stained with saturated uranyl acetate in 50% methanol and then with lead citrate. The grids were viewed in the JEM-1200EXII electron microscope (JEOL) at 80 kV, and images were recorded on the XR611M, mid-mounted, 10.5 million pixel, CCD camera (Advanced Microscopy Techniques, Woburn, MA, USA).
Isolation of mouse pancreatic islets and PCR analysis

Mice were killed by CO₂ inhalation. Next, 10 mL of a 0.25 mg/mL solution of Liberase RI (Roche) in DMEM was injected into the common bile duct. Each pancreas was removed, placed in a 50 mL tube and incubated at 37°C for 25 min. After the incubation, 40 mL of cold DMEM + 10% FBS was added. Tubes were shaken vigorously for 5–10 s by hand to break up the tissue. The rest of the isolation was done at room temperature. Tubes were centrifuged at 250 × g for 1 min, the supernatant was poured off, 35 mL of DMEM (without serum) was added and vortexed gently. Centrifugation was repeated, and the supernatant was discarded. The tissue was resuspended in 10 mL of DMEM and filtered through a wire mesh with 1.5 mm holes to remove the remaining undigested tissue, fat and lymph. An additional 5 mL of DMEM was added to the original tube to wash out any remaining islets, and the wash was also filtered through the wire mesh. This filtrate was then filtered through a wire mesh with 0.8 mm holes and centrifuged at 350 × g for 90 s. The pellet was resuspended in 20 mL of Ficoll and overlain with 10 mL of DMEM. The sample was spun for 15 min at 900 × g. The topmost layer of media was aspirated. The islet layer was then collected from the interface with a 10 mL pipette and placed in a new 50 mL tube. The islets were washed several times with DMEM and resuspended in 10 mL of DMEM. Islets were hand-picked for later procedures.

DNA was extracted and amplified using the following three primers: (5’-AGCTAGCGTGGGAGGCTTA-3’, 5’-AAGCAGGGAGCTATTAGTTTAT-3’ and 5’-CATCCATCTCCATCCCTTT-3’). Each PCR reaction was carried out in a 25-μL reaction mixture containing 1 μL of template DNA, 1 μM of each primer, 1 mM of each deoxynucleoside triphosphate and 1 unit of Tag polymerase. The reaction mixture was denatured for 5 min at 94°C and incubated for 35 cycles (denaturing at 94°C for 30 s, annealing at 58°C for 45 s and extending at 72°C for 10 s).

Quantification of blood glucose and serum insulin, c-peptide and glucagon levels

All measurements were carried out on animals of 2–6 months of age. Mice were fasted 18 h before blood and serum collection. Blood samples were collected from the tail vein, and glucose concentration was determined using a Glucotrend 2 kit (Roche). Serum was obtained after clotting, and separation was obtained by centrifugation. Quantification was performed with a solid-phase, two-site ELISA immunoassay specific for mouse insulin (Ultrasensitive mouse insulin ELISA, Mercodia, Cat No 10-1247-01), c-peptide (c-peptide ELISA, Mercodia, Cat No 10-1172-01) and glucagon (Glucagon ELISA, Mercodia Cat No 10-1271-01). Assays for each serum were performed in duplicate.

PKA activity

PKA enzymatic activity was measured by the method described previously (Rohlff et al. 1993).

Results

Loss of Prkar1a in pancreatic progenitor cells does not affect normal pancreas development but leads to endocrine pancreas tumorigenesis

We evaluated the loss of Prkar1a in both exocrine and endocrine pancreatic cells. Transgenic mice expressing the Cre recombinase under the control of the Pdx1 (pancreatic and duodenal homeobox 1) promoter allowed target gene deletion in all pancreatic endocrine and exocrine cells due to Pdx1-Cre expression in pancreatic progenitors (Gu et al. 2002). The Pdx1-Cre transgenic mice bred with mice whose Prkar1a alleles were flanked by loxP sequence sites in exon 2 (Prkar1afl/fl) (Kirschner et al. 2005) and gave expected Mendelian frequencies for all genotypes. Prkar1afl/fl, Pdx1-CRE− (wild type (WT)) served as control animals. It has already been shown that Prkar1a

Figure 1

(A) The Prkar1afl/fl; Pdx1-CRE+ mice at the age of 4–6 months developed distended abdomen due to abundant accumulation of hemorrhagic ascites. (B) There were large masses present in the upper abdomen 2–3 cm in diameter, with adhesions to the stomach, spleen, mesentery and intestines. The lungs were atelectatic due to pleural effusions, whereas the lymph nodes appeared normal both in size and number. (C) A macroscopic view of the pancreatic tumor. Note the fibrotic appearance of the tumor. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0443.
is more than 90% reduced in the islets from Δ-prkar1a mice compared to controls with consequent increase in PKA activity (Song et al. 2011).

To study the consequences of Prkar1a deletion in tumor development, we monitored the Δ-Prkar1a (Prkar1af/f; Pdx1-Cre+) mice and their wild-type littermates until the development of signs that mandated to kill them according to the NIH animal committee guidelines or at the age of 18 months (whatever happened first). All Δ-Prkar1a mice developed a syndrome of distended abdomen and respiratory distress with complete penetrance by the age of six months (Fig. 1A). The distention was due to the accumulation of a red-tinged fluid into the peritoneal cavity, whereas the respiratory distress was caused by the accumulation of thoracic effusion that caused lungs atelectasis. There was a tissue mass in the region of the pancreas that was primarily solid with a few cystic components, and with adhesions to the liver, stomach, mesenteric tissues, spleen and intestinal tract (Fig. 1B and C). The lymph nodes appeared normal in size and number, whereas the spleen was diffusely modestly enlarged. WT mice from the same background and corresponding age and gender were also phenotyped, and no obvious abdominal abnormalities were found.

Cre-Lox recombination resulted in loss of Prkar1a expression and increased PKA activity

Genomic DNA isolated from pancreatic tumor cells showed the expected deletion of Prkar1a allele in Prkar1aΔ/+; Pdx1-Cre+ (Fig. 2A). Furthermore, using total tumor lysate and isolated islets from the control mice, we confirmed a significant increase of the PKA activity in tumors from the experimental animals (Fig. 2B). These results, in addition to the loss of Prkar1a expression in the tumors compared to the exocrine and endocrine pancreas (Fig. 2C and D) showed that Pdx1-Cre-mediated loss of Prkar1a had an oncogenic effect in the pancreas.

Figure 2
Recombination resulted in loss of prkar1a expression and increased PKA activity.
(A) Deletion of the Prkar1a allele in tumors from Δ-Prkar1a mice. Top bar: flox 350 bp. Bottom bar: the presence of deleted Prkar1a allele 185 bp.
(B) Increased total PKA activity in Δ-Prkar1a mice pancreatic tumors compared to islets isolated from controls. (C and D) Immunohistochemical staining of pancreas sections with antibodies against Prkar1a shows total loss of Prkar1a expression in tumors compared to normal exocrine and endocrine pancreas. Magnification x4 (C) and x20 (D). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0443.
Histopathologic analysis revealed pancreatic tumors of neuroendocrine origin

The striking feature of the Δ-Prkar1a mice was the early development of pancreatic tumors. These lesions first appeared at the age of 2 months (Fig. 3A) and became progressively enlarged (Fig. 3B and C). All (100%) the mice were affected by the age of 6 months. The tumors were well demarcated with significant fibrotic reaction of the surrounding tissue similarly to the human pancreatic tumors. Histologically, most lesions exhibited an organoid pattern as seen in the islet-cell tumors (Fig. 3D). Higher magnification (Fig. 3D, square) showed that some tumor cells are small round with scant cytoplasm and nuclei with fine chromatin and others are larger polygonal and exhibit ample eosinophilic cytoplasm and occasionally hyperchromatic nuclei or prominent nucleoli (Fig. 3E). In addition, some tumors exhibited foci suggestive of acinar cell differentiation (Fig. 3E). The exocrine pancreas remained unaltered.

Microscopically, there were signs of local invasion, neoplastic emboli to the lymphatic vessels and occasionally metastases to the loco-regional lymph nodes (Fig. 4A and B). These findings, but most importantly the decreased survival of the Δ-Prkar1a mice compared to their WT littermates (median survival: 200 days vs not reached; \( P < 0.001 \)) (Fig. 4C), were strong indications of the malignant behavior of these tumors.

To study further the initial interpretation of the neuroendocrine origin for the pancreatic tumors, we applied electron microscopy (EM) that is considered one of the most helpful tools in the diagnosis of such
lesions. The ultrastructural hallmark of neuroendocrine neoplasms in pancreas (and elsewhere) is the presence of cytoplasmic neurosecretory granules; indeed, all mouse lesions studied had this feature (Fig. 5).

**Mixed cell population in the pancreatic tumor of the Δ-Prkar1a mice**

To study the cell origin of the tumors in Δ-Prkar1a mice, single immunofluorescent staining for multiple pancreatic cell markers was applied. The stainings were done in sections from pancreatic tumor and lymph node metastasis from a 5-month-old female Δ-Prkar1a mouse. To characterize the identity of the tumor cells the following markers were studied: chromogranin A (Ehrhart et al. 1986, Yantiss et al. 2002, Taupenot et al. 2003), chymotrypsin (Yantiss et al. 2002), PDX1 (pancreatic and duodenal homeobox 1) (Park et al. 2011) and insulin (Okabayashi et al. 2013). Indeed, the pancreatic tumors from Δ-Prkar1a mice appeared to have a mixed population
of cells (Fig. 6). Some of the cells had neuroendocrine features giving positive staining for chromogranin A but not for insulin. Interestingly, within the same tumor, there were acinar-like cells, which were negative for neuroendocrine markers but positive for chymotrypsin.

**Inactivation of the Prkar1a in the endocrine pancreas did not lead to an obvious hormonal syndrome**

We measured blood glucose as well as serum insulin and c-peptide levels in Δ-Prkar1a mice after a fast of 18 h. Although blood glucose levels remained normal until the age of 2 months, it decreased with age thereafter compared to that of the control animals (Prkar1afl/fl-Pdx1Cre-) (Fig. 7A) as the animals were getting sicker. However, serum insulin levels were not different compared to age-matched controls (Fig. 7B). It is important to note that despite the low blood glucose, endogenous insulin concentration remained into the range of basal levels (<1 ng/mL), a finding that practically rules out the diagnosis of insulinomas. Accordingly, c-peptide levels were also similar among the studied groups of mice (Fig. 7C); all tumors also stained consistently negative for insulin (Fig. 7D). Immunohistochemistry was also negative for peptides produced by the alpha (gastrin; Fig. 7E), gamma (pancreatic peptide; 7F) and delta islet cells (somatostatin; 7G).

**Discussion**

In the present study, we aimed to elucidate the role of prkar1a as a tumor suppressor in the adult pancreas. We used a mouse model wherein Prkar1a was deleted in the progenitor cells that give rise to both exocrine and endocrine pancreas during development. We showed that pancreatic progenitor cells expressing pdx1 and lacking Prkar1a are able to differentiate into normal endocrine and exocrine pancreas. However, adult cells lacking Prkar1a developed tumors with a mixed cell population from both the exocrine and the endocrine pancreas.
We established, therefore, that loss of Prkar1a is sufficient to cause endocrine (as well as exocrine) tumorigenesis in the pancreas.

We have shown previously that Prkar1a haploinsufficiency in a mouse model led to the development of tumors arising in cAMP-responsive tissues, such as the bone, Schwann and thyroid follicular cells (Kirschner et al. 2005). Although the spectrum of tumors overlapped with what is seen in CNC patients, this mouse model did not present some of the most common CNC tumors, such as heart myxomas and pituitary adenomas, whereas pancreatic neoplasms were rarely found. A different mouse model that led to significantly higher Prkar1a downregulation and consequently augmented CAMP signaling produced a more severe phenotype with a significant decrease in the overall lifespan (Griffin et al. 2004a,b). This observation raised the hypothesis that decreased Prkar1a expression, more than simple haploinsufficiency, could generate a more aggressive phenotype that was closer to CNC in various tissues. We have also investigated the Prkar1a haploinsufficiency in the background of Tp53 or Rb1 haploinsufficiency (Almeida et al. 2010). Tp53+/− mice developed bone sarcomas, a tissue frequently affected by Prkar1a haploinsufficiency (Kirschner et al. 2005). Doubly heterozygous mice (Prkar1a+/−/Tp53+/−) mice exhibited significantly decreased survival and developed significantly more sarcomas than the Tp53−/− ones. Similarly, Rb1+/− Prkar1a+/− mice experienced reduced overall survival when compared with Rb1−/− and Prkar1a+/− mice; Prkar1a+/−/Rb1+/− mice developed more pituitary tumors and medullary thyroid carcinomas than Rb1−/− mice. Therefore, Prkar1a haploinsufficiency acts as a generic but relatively weak tumorigenic signal that is enhanced by the deficiency of other tumor suppressor or tissue-specific factors to induce tumors. Likewise, an expanded skeletal tumor distribution was observed when Prkar1a was conditionally deleted in early osteoblastic progenitors (Molyneux et al. 2010). Tumors arose across the skeleton, including both long and flat bones, and were not restricted to the tail as is seen in Prkar1a−/− mice. Similar to the bone, a thyroid-specific knockout mouse model of Prkar1a led to hyperthyroidism and thyroid cancer (Pringle et al. 2012). The resulting mice developed follicular thyroid neoplasm by 12 months of age, including follicular thyroid cancer in over 40% of animals. However, in none of the mouse models of Prkar1a deficiency reported so far, the oncogenic effect was as powerful as that in our study. Deletion of both Prkar1a alleles in early pancreatic progenitors caused full penetrance of pancreatic tumors with malignant behavior and significantly decreased survival; no mouse survived longer than six months. The oncogenic potency of Prkar1a in the Pdx1+ pancreatic progenitors compares favorably with the effect of Kras activation that leads to intraductal neoplasia development and additional inactivation of other tumor suppressor genes is required for the progression to advanced dysplasia or carcinoma (Pérez-Mancera et al. 2012). These data indicate that pancreatic tissue is highly sensitive to PKA alteration due to Prkar1a inactivation.

It has been shown that tissues are extraordinarily sensitive to modest changes in the type of PKA signaling (Skalhegg & Tasken 2000, Bossis & Stratakis 2004). When PKA abnormalities were enhanced in the bone of a mouse with double heterozygosity for Prkar1a and the type A catalytic subunit (Ca) of PKA (Prkar1a+/−/Prkaca1+/−) a particular population of adult bone stromal cells that are responsive to cAMP signaling and alternate PKA catalytic subunits was identified (Tsang et al. 2010). These cells were recruited from the pool of bone marrow stem cells and under the abnormal PKA signaling were unable to follow the regular process of maturation to hypertrophic chondrocytes or mature osteoblasts, rather forming tumors with irregular and undermineralized matrix. We hypothesized that a similar population exists in the adult pancreas. Actually, the recently described population of multipotent Pdx1+/Insulin− cell might be the cell of origin that gave rise to the tumors in our mouse model (Fig. 2). This cell type has been suggested to be the source of the endocrine cells within foci of ductal metaplasia observed in the tamoxifen-treated Pdx1CreERtm; LSL-KrasG12D) mice and the endocrine cells arising from the ductal lining after pancreatic duct ligation (Xu et al. 2008). It is possible that this cell type is activated only in certain settings such as injury and neoplasia and may be particularly susceptible to PKA-induced proliferation and differentiation. It might also be the source of the ductal structures located in the islets, if it resides both in the ductal lining as well as the islets or if it migrates to the islets under certain conditions (Xu et al. 2008).

Interestingly, the Δ-Prkar1a mice did not exhibit any physiological abnormality suggestive of hormonal dysregulation. At a relatively young age (6–8 weeks), Δ-Prkar1a mice did not have fasting hypoglycemia, but exhibited augmented insulin secretion as glucose levels rose due to increased PKA signaling in potentiating beta-cell glucose-stimulated insulin secretion (GSIS) (Song et al. 2011). We noted that the decreased blood glucose levels
apparently paralleled the development of tumors, but the serum insulin levels remained suppressed, a finding that practically ruled out the diagnosis of insulinomas. Additionally, we did not find any evidence of other common peptide production by immunostaining or serum analysis. We concluded that these tumors were non-functional, similarly to what has been shown in the most recent clinical series of human pancreatic neuroendocrine tumors (Halfdanarson et al. 2008, Turaga & Kvols 2011).

These data indicate that Prkar1a is a tumor suppressor in the pancreas and that loss of this gene leads to non-functional neuroendocrine tumors with an acinar component. These data are in support of the human data showing that CNC patients have an increased predisposition to pancreatic tumors, although histologically the human and mouse lesions were not identical. We speculated that PKA may be a valuable new target for molecularly targeted therapy in pancreatic neuroendocrine and possibly exocrine neoplasms.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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