METHODS

Human pancreatic samples. Human pancreatic samples were obtained after informed consent from their families and approval by the Hospital ethics committee. UTIP healthy series: Human non-pathogenic pancreases for immunohistochemistry were obtained from 7 human cadaveric organ donors from the Unit of Islet Transplantation (UTIP)/Tissue Bank Resource at the University Hospital of Bellvitge (Barcelona, Spain). Clinic adenocarcinoma series: Samples from 14 pancreatic adenocarcinomas were obtained from the Department of Pathology at the Hospital Clinic. Fragmented pancreases were fixed overnight in formaldehyde 4% (Sigma, St Louis, MO, USA) at 4ºC, dehydrated and embedded in paraffin, prior to sectioning. PAC481 array: A tissue array (US Biomax, Rockville, MD, USA) containing 3 cores from non-pathogenic pancreases and 20 from pancreatic adenocarcinomas was purchased. SEER array: Another tissue array was generously obtained from the National Cancer Institute and the Surveillance Epidemiology and End Results program (Beteshda, MD, USA), which characteristics were described previously [1]. Briefly, it contains 15 cores from non-pathogenic pancreases, 24 cores from pancreatic adenocarninoma biopsies, 72 cores form pancreatic adenocarcinoma excisions and 5 cores from neuroendocrine pancreatic tumors. Islets: Healthy pancreases for islet isolation were obtained from 11 human cadaveric organ donors from the Transplant Services Foundation at the Hospital Clínic (Barcelona, Spain). Islets were isolated as previously described [2] and kept by Biobanc Clínic-IDIBAPS. Pancreatic donors characteristics are described below.

Main characteristics of the pancreatic donors

A.-For Real Time PCR

n: 8

Sex: 5 men and 3 women.

Age: mean 46.75 years (SEM 6.89 years)

Death cause: 5 cerebrovascular accident, 2 aneurysm rupture and 1 traumatic brain injury.

Other diseases: 1 hypertension and 1 epilepsy.

B.-For Western Blot

n: 3

Sex: 1 man and 2 women

Age: mean 43 years (SEM 8.69 years).

Death cause: 1 cerebrovascular accident, 1 traumatic brain injury and 1 aneurysm rupture.

C.-For Immunohistochemistry

UTIP healthy series

n: 7

Sex: 4 men and 3 women.

Age: mean 52.83 years (SEM 5.12 years).

Death cause: 5 cerebrovascular accident, 1 traumatic brain injury and 1 aneurysm rupture.

Other diseases: 1 prostate cancer and 1 benign prostatic hyperplasia.

Clinic adenocarcinoma series

n: 14

Sex: 10 men and 4 women.

Age: mean 65.29 years (SEM 3.63 years).

Grade: I: 3

II: 9

III: 2

PAC 481 array

Healthy cores

n: 3

Sex: 2 men and 1 women.

Age: mean 50.67 years (SEM 4.55 years).

Adenocarcinoma cores

n: 20

Sex: 6 men and 14 women.

Age: mean 52.75 years (SEM 2.88 years).

Grade: I: 5

II: 9

III: 6

More characteristics at the manufacturer's webpage:
http://www.biomax.us/tissue-arrays/Pancreas/PAC481

SEER array

Healthy cores

n: 15

Discarded cores: 1

Adenocarcinoma cores

n=96

Pancreatic biopsies: 24

Pancreatic excisions: 72

Sex: 51 men and 45 women.

Discarded cores: 5

Neuroendocrine pancreatic tumors cores

n=5

Pancreatic excisions: 5

Sex: 3 men and 2 women

More information under request at the Statistics, Epidemiology, and End Results (SEER) Residual Tissue Repository (RTR):
http://seer.cancer.gov/biospecimen/resources.html

Animals. Principles of laboratory animal care were followed (European and local government guidelines). C57B/6 adult mice were obtained from Charles River (Wilmington, MA, USA). Mice were sacrificed by cervical dislocation and pancreases were removed, fixed in neutral buffered 10% formalin solution (Sigma) at 4ºC, dehydrated, and embedded in paraffin prior to sectioning. For islet studies, islets were isolated by collagenase digestion, as previously described [2]**.** Cdk4 knockout mice pancreas lysate and tissue sections were kindly provided by Dr. S. Ortega (National Center for Oncological Studies (CNIO), Madrid, Spain) [3].

Immunohistochemical studies. Sections of pancreas (4μm) were mounted, deparaffinated and rehydrated. Antigen retrieval was performed with citrate buffer REAL Target Retrieval Solution (Dako, Glostrup, Denmark) using a microwave oven or a pressure-cooker (see detailed protocol below). After permeabilization and blocking, sections were incubated overnight with guinea pig anti-insulin (1:500) (Dako, A0564) and rabbit anti-CDK4 (C-22) (1:100) (Santa Cruz Biotechnology, sc-260), rabbit anti-CDK4 (H-22) (1:500) (Santa Cruz Biotechnology, sc-601) or rabbit anti-CDK6 (C-21) (1:50) (Santa Cruz Biotechnology, sc-601). Samples were next incubated for 3 hours at room temperature with the secondary antibodies, donkey anti-rabbit Cy3 (1:500) (Jackson Immunoresearch, Cambridgeshire, UK, 711-165-152) and donkey anti-guinea pig Cy2 (1:500) (Jackson Immunoresearch, 706-225-148). Then nuclear staining was performed with Hoechst (Sigma, 1 μg/ml in PBS), for 10 min at room temperature. Finally, slides were mounted with Mowiol 4-88 (Calbiochem, La Jolla, CA, USA).

Epifluorescence images were captured with a Leica DM R microscope (Leica Microsystems Gmbh, Wetzler, Germany).

RNA isolation. Islet RNA was isolated with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). RNA integrity was analyzed using a Lab-on-a-chip in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Real-time PCR. Total RNA was retrotranscribed with Superscript III (Invitrogen, Carlsbad, CA, USA). Real-time PCR was carried out in a 7900HT Real Time System (Applied Biosystems, Foster City, CA, USA) using SYBR Green fluorophore. A standard curve for each primer set was generated from serial dilutions of cDNA. The PCR products were verified by dissociation curve analysis using SDS software (Applied Biosystems). Expression levels obtained were normalized to the housekeeping gene *TBP* (TATA box binding protein). Amylase alpha 2a levels were determined to ensure high islet purity.

Primer sequences.

CDK4 (NM_000075)
Forward: CGACCAGTTGGGCAAAATCT
Reverse: GATACATCTCGAGGCCAGTCATC
Forward: GCCCGAAACGCCGAATAT
Reverse: CGTGGCTCTCTTATCCTCATGA
AMY2A (amylase, alpha 2a) (NM_000699)
Forward: CAATGACTGGGTCTGTGAACATC
Reverse: AGGCTGGCCATCCACTACAT

Western Blot. 25 μg of human islets, human pancreas, wild type and Cdk4 knockout mice pancreas, wild type mice islets lysate and HeLa cell line protein lysates were resolved using 10% SDS-PAGE, transferred to PolyScreen PVDF Membrane (Perkin Elmer, Waltham, MA, USA). Western Blot analysis was performed by using a specific rabbit antibody against rabbit anti-CDK4 (C-22) (1:500), mouse anti-CDK4 (DCS-31) (1:1000) (Sigma, C8218), rabbit anti-CDK4 (H-22) (1:1000), mouse anti-CDK4 (DCS-

156) (1:5000) (Becton Dickinson, Franklin Lakes, NJ, USA, ref.559677) or rabbit anti-β-actin (1:500) (Sigma, A2066).

Competition studies. 2 or 0.4 ug / ml (for immunophistochemistry or immunoblotting respectively) of rabbit anti-CDK4 (C-22) was pre-incubated with or without 20 or 4 ug/ml (for immunohistochemistry or immunoblotting respectively) of competition peptide (corresponding to the C-terminus fraction of CDK4) (Santa Cruz Biotechnology, sc-260-P) overnight at 4ºC.

Antibodies.

Recognized protein	Clone	Commercialized by	Reference number
CDK4	C-22	Santa Cruz Biotechnology	sc-260
CDK4	DCS-156	Becton Dickinson	559677
CDK4	DCS-31	Sigma	C8218
CDK4	H-22	Santa Cruz Biotechnology	sc-601
CDK6	C-21	Santa Cruz Biotechnology	sc-177

Recognized protein	Clone	Epitope
CDK4	C-22	22 aas length between the region 253-303 aas (C-terminus)
CDK4	DCS-156	20 aas length between the region 270-290 aas (C-terminus)
CDK4	DCS-31	Full length
CDK4	H-22	22 aas length between the region 253-303 aas (C-terminus)
CDK6	C-21	15-25 aas length between the region 276-326 aas (C-terminus)

Recognized protein	Clone	Origin of the epitope	Cross reactivity	Host
CDK4	C-22	Mouse	Mouse, human, rat	rabbit
CDK4	DCS-156	Human	Mouse, human	mouse
CDK4	DCS-31	Human	Mouse, human, rat	mouse
CDK4	H-22	Human	Mouse, human, rat, pig	rabbit
CDK6	C-21	Human	Mouse, human, rat	rabbit

			Applications	
Recognized protein	Clone	Isotype	WB	IHC
CDK4	C-22	IgG	+	+
CDK4	DCS-156	IgG1	+	-
CDK4	DCS-31	IgG2a	+	-
CDK4	H-22	IgG	+	+
CDK6	C-21	IgG	+	+

[1] Takikita M, Altekruse S, Lynch CF, et al. (2009) Associations between selected biomarkers and prognosis in a population-based pancreatic cancer tissue microarray. Cancer Res 69: 2950-2955

[2] Marzo N, Mora C, Fabregat ME, et al. (2004) Pancreatic islets from cyclindependent kinase 4/R24C (Cdk4) knockin mice have significantly increased beta cell mass and are physiologically functional, indicating that Cdk4 is a potential target for pancreatic beta cell mass regeneration in Type 1 diabetes. Diabetologia 47: 686-694

[3] Martin J, Hunt SL, Dubus P, et al. (2003) Genetic rescue of Cdk4 null mice restores pancreatic beta-cell proliferation but not homeostatic cell number. Oncogene 22: 5261-5269

DETAILED IMMUNOHISTOCHEMISTRY PROTOCOL

This protocol works with paraffined samples fixed with formaldehyde 4% or formalin 10%. Other fixatives have not been tested.

Frozen tissues have not been result successfully.

All processes are at room temperature except that other temperature is specified.

Fixation

Immerse a piece (1 cm^3 approximately) of pancreas in buffered neutral formalin 10% overnight (~13 hours) at 4ºC.

Dehydratation

Immerse the sample in:

20 min--------- EtOH 50%

20 min--------- EtOH 50%

20 min--------- EtOH 50%

20 min--------- EtOH 70%

20 min--------- EtOH 70%

20 min--------- EtOH 70%

20 min--------- EtOH 95%

20 min--------- EtOH 95%

20 min--------- EtOH 95%

20 min--------- EtOH 100%

20 min--------- EtOH 100%

20 min--------- EtOH 100%

20 min--------- Xylol 100%

20 min--------- Xylol 100%

20 min--------- Xylol 100%

Overnight------Xylol:paraffin (1:1)

1 hour----------Paraffin at 65ºC

1 hour----------Paraffin at 65ºC

1 hour----------Paraffin at 65ºC

Embed in paraffin.

Cutting

Cut the sample with a microtome at 4 μm width and put the sections in polilysine treated slides.

Overnight at 37ºC.

Deparaffinization

Immerse the slide in:

10 min -------- Bucket with Xylol

10 min -------- Bucket with Xylol

10 min -------- Bucket with EtOH 100%

10 sec shaking--Bucket with EtOH 100%

10 sec shaking--Bucket with EtOH 100%

10 sec shaking--Bucket with EtOH 96%

10 sec shaking--Bucket with EtOH 70%

10 sec shaking--Bucket with EtOH 40%

10 sec shaking--Bucket with EtOH 20%

10 sec shaking--Bucket with H2O distillated

Unmasking

Pressure cooker

Immerse the samples in citrate solution inside a pressure cooker.

Heat the pressure cooker with a plate until it arrives to its maximum pressure, remaining heating for 5 min.

Separate the pressure cooker of the heating plate for 15 min, permitting that the samples arrive to room temperature.

Washing

5 min----------Tris 100mM, pH 7.4

Permeabilization

30 min ---------Tris 100mM, pH 7.4, BSA 3% and Triton® X-100 1%.

Washing

5 min----------Tris 100mM, pH 7.4.

Blocking

1 hour----------Tris 100mM, pH 7.4, BSA 3%, Triton® X-100 0.1% and FC Block 1:10.

Washing

5 min----------Tris 100mM, pH 7.4

Primary Antibody

Anti-CDK4 C-22 [rabbit] 1:100

Or

Anti-CDK4 H-22 [rabbit] 1:500 (more concentrated gives unspecific signal)

Or

Anti-CDK6 C-21 [rabbit] 1:50

And

Anti-insulin [guinea-pig] 1:500

In Tris 100mM, pH 7.4, BSA 3% and Triton® X-100 0.1%.

Overnight at 4ºC in a humidified chamber

Washing

5 min----------Tris 100mM, pH 7.4

5 min----------Tris 100mM, pH 7.4

5 min----------Tris 100mM, pH 7.4

Secondary Antibody

anti-Rabbit Cy3 [donkey] 1:500

And

anti-Guinea Pig Cy2 [donkey] 1:500

In Tris 100mM, pH 7.4, BSA 3% and Triton® X-100 0.1%.

3 hours at room temperature in a humidified chamber.

Washing

5 min----------Tris 100mM, pH 7.4

5 min----------Tris 100mM, pH 7.4

5 min----------Tris 100mM, pH 7.4

Nuclear staining

Hoechst 33342 1/1000 in PBS

10 min at room temperature in a humidified chamber

Washing

5 min----------Tris 100mM, pH 7.4

Mounting

Mowiol

Reagents:

Accustain® formalin solution, 10% neutral buffered (Sigma, HT501320-9.5L) (Sigma, St Louis, MO, USA)

Formaldehyde 36,5-38% (Sigma, F-8775)

EtOH absolute (Panreac, 141086,1214) (Panreac, Castellar del Valles, Spain)

Xylol (Panreac, 211769,1714)

Paraffin (Cymit, 22-0587-1Kg) (Cymit, Barcelona, Spain)

Poly-L-lysine solution 0.1% in water (Sigma, P 8920)

Triton® X-100 (Sigma, X100-500ML)

BSA (Albumin, Bovine, 96,99%) (Sigma, A3311-100G)

Tris (Tris(hydroxymethyl)aminomethane, research grade) (SERVA, 37190) (SERVA, Heidelberg, Germany)

Citrate solution, pH6.0 (Dako REAL Target Retrieval Solution, S2031) (Dako, Glostrup, Denmark)

Hoechst 33342 (Sigma, B2261) diluted in deionised water at 1mg/mL

Mowiol 4-88 (Calbiochem, 475904) (Calbiochem, La Jolla, CA, USA)

Antibodies:

Fc block, purified anti-mouse CD16/CD32 (Fcγ III/II receptor) clone 2.4G2 (BD Pharmingen, 553142) (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA)

Anti-Cdk4 (C-22) rabbit (Santa Cruz Biotechnology, sc-260)

Anti-Cdk4 (H-22) rabbit (Santa Cruz Biotechnology, sc-601)

Anti-insulin guinea pig (Dako, A0564)

Anti-rabbit Cy3 donkey (Jackson Immunoresearch, 711-165-152) (Jackson Immunoresearch, Cambridgeshire, UK)

Anti-Guinea Pig Cy2 donkey (Jackson Immunoresearch, 706-225-148)