Universidad de Alcalá
COMISIÓN DE ESTUDIOS OFICIALES
DE POSGRADO Y DOCTORADO

ACTA DE EVALUACIÓN DE LA TESIS DOCTORAL

(FOR EVALUATION OF THE ACT DOCTORAL THESIS)

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DOCTORANDO *(candidate PHD)*: **MAZARICO GALLEGO, JOSE MARÍA**
D.N.I./ PASAPORTE (*Id.Passport*): ******2307H**
PROGRAMA DE DOCTORADO *(Academic Committee of the Programme)*: **D420-CIENCIAS DE LA SALUD**
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En el día de hoy 28/11/16, reunido el tribunal de evaluación, constituido por los miembros que suscriben el presente Acta, el aspirante defendió su Tesis Doctoral **con Mención Internacional** (*In today assessment met the court, consisting of the members who signed this Act, the candidate defended his doctoral thesis with mention as International Doctorate*), elaborada bajo la dirección de (*prepared under the direction of*) FRANCISCO XAVIER REAL ARRIBAS // VÍCTOR JAVIER SÁNCHEZ AREVALO LOBO.

Sobre el siguiente tema *(Title of the doctoral thesis)*: *DEREGULATION OF CHOLINE KINASE IN PANCREATIC CANCER AND ITS INHIBITION AS A POTENTIAL THERAPEUTIC STRATEGY*

Finalizada la defensa y discusión de la tesis, el tribunal acordó otorgar la CALIFICACIÓN GLOBAL[1] de (**no apto, aprobado, notable** y **sobresaliente)** *(After the defense and defense of the thesis, the court agreed to grant the GLOBAL RATING (fail, pass, good and excellent)*: Sobresaliente (Excelente)

Alcalá de Henares, a 28 de noviembre de 2016

Fdo. *(Signed)*: Atanasio Pandiella

Fdo. *(Signed)*: José Palacios Calvo

Fdo. *(Signed)*: M.ª Teresa García Pérez

Fdo. *(Signed)*: Bruno Saiz Ladera

Fdo. *(Signed)*: Sophie Vasseur

FIRMA DEL ALUMNO (candidate's signature),

Fdo. *(Signed)*:

Con fecha 21 de diciembre de 20[illegible]
Delegada de la Comisión de Estudios Oficiales de Posgrado,
a la vista de los votos emitidos de manera anónima por el tribunal que ha juzgado la tesis, resuelve:

☐ Conceder la Mención de "Cum Laude"
☒ No conceder la Mención de "Cum Laude"

La Secretaria de la Comisión Delegada

[1] La calificación podrá ser "**no apto**" "**aprobado**" "**notable**" y "**sobresaliente**". El tribunal podrá otorgar la mención de "cum laude" si la calificación global es de sobresaliente y se emite en tal sentido el voto secreto positivo por unanimidad. (*The grade may be "**fail**" "**pass**" "**good**" or "**excellent**". The panel may confer the distinction of "cum laude" if the overall grade is "Excellent" and has been awarded unanimously as such after secret voting.).*

INCIDENCIAS / OBSERVACIONES:
(Incidents / Comments)

Por ausencia justificada del Dr. David Olmos se incorpora como miembro del Tribunal la Dra. Laura García Bermejo.

Universidad de Alcalá

COMISIÓN DE ESTUDIOS OFICIALES DE POSGRADO Y DOCTORADO

En aplicación del art. 14.7 del RD. 99/2011 y el art. 14 del Reglamento de Elaboración, Autorización y Defensa de la Tesis Doctoral, la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado y Doctorado, en sesión pública de fecha 21 de diciembre, procedió al escrutinio de los votos emitidos por los miembros del tribunal de la tesis defendida por *MAZARICO GALLEGO, JOSE MARÍA*, el día 28 de noviembre de 2016, titulada *DEREGULATION OF CHOLINE KINASE IN PANCREATIC CANCER AND ITS INHIBITION AS A POTENTIAL THERAPEUTIC STRATEGY*, para determinar si a la misma se le concede la mención "cum laude", arrojando como resultado, 4 votos a favor y 1 en contra.

Por lo tanto, la Comisión de Estudios Oficiales de Posgrado **resuelve no otorgar la Mención de "cum laude"** a dicha Tesis.

Alcalá de Henares, 21 de diciembre de 2016
EL PRESIDENTE DE LA COMISIÓN DE ESTUDIOS OFICIALES DE POSGRADO Y DOCTORADO

UNIVERSIDAD DE ALCALÁ - COMISIÓN DE ESTUDIOS OFICIALES DE POSGRADO Y DOCTORADO

Juan Ramón Velasco Pérez

Copia por e-mail a:
Doctorando: MAZARICO GALLEGO, JOSE MARÍA
Secretario del Tribunal: JOSÉ PALACIOS CALVO.
Directores de Tesis: FRANCISCO XAVIER REAL ARRIBAS//VÍCTOR JAVIER SÁNCHEZ AREVALO LOBO

FACULTAD DE MEDICINA Y CIENCIAS DE LA SALUD
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DOCTORAL THESIS:

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José María Mazarico Gallego.

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Tesis doctoral realizada por José María Mazarico Gallego bajo la dirección del Dr. Francisco Xavier Real Arribas y el Dr. Víctor Javier Sánchez-Arévalo Lobo.

Alcalá de Henares, Julio de 2016

This thesis work has been accomplished in the Epithelial Carcinogenesis Group, Spanish National Center for Cancer Research (CNIO) under the supervision of F.X Real and V.J. Sánchez Arévalo.

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Table of contents

Abbreviations

5-FU	5-fluorouracil.
AA	Arachidonic acid.
ABCB-1	ATP-binding cassette transporter-1.
ABCB-4	ATP-binding cassette transporter-4.
ACC	Acetyl-CoA carboxylase.
ACLY	ATP citrate lyase.
ACSS2	Acyl-CoA Synthetase Short-Chain Family Member 2.
ATP	Adenosine Triphosphate.
BCL-XL	B-cell lymphoma-extra large.
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A.
CEA	Carcinoembryonic antigen.
CHK	Choline Kinase.
CHKA	Choline Kinase alpha.
CHKB	Choline Kinase beta.
CHKI	Choline Kinase inhibitor.
CHO	Choline.
c-MYC	Avian myelocytomatosis viral oncogene.
DAG	Diacylglycerol.
ECOG	Eastern Cooperative Oncology Group.
ELOVL	Elongation of long chain fatty acids.
FA	Fatty acid.
FADH2	Flavin adenine dinucleotide.
FASN	Fatty Acid Synthase.
FGF	Fibroblast growth factor.
Gem	Gemcitabine.
GPC	Glycerophosphocholine.
GLUT-1	Glucose Transporter 1.
HIF	Hypoxia-Inducible Factor.

IHC	Inmunohistochemistry.
KRAS	Kirsten Rat Sarcoma Viral Oncogene.
MAPK	Mitogen-Activated Protein Kinases.
MDR	Multidrug resistant.
NADH	Nicotinamide adenine dinucleotide.
NADPH	Nicotinamide adenine dinucleotide phosphate.
NFK-β	Nuclear Factor Kappa Beta.
OS	Overall survival.
OX	Oxaliplatin.
PA	Phosphatidic acid.
PanINs	Pancreatic Intraepithelial Neoplasm.
PC	Phosphatidylcholine.
PCHO	Phosphocholine.
PDAC	Pancreatic ductal adenocarcinoma.
PDGF	Platelet derived growth factor.
PE	Phosphatidylethanolamine.
PET	Positron Emission Tomography.
PFS	Progression-free survival.
Pgp	Permeability Glycoprotein.
PI	Phosphatidylinositol.
PI3K	Phosphoinositol-3-Kinase.
PKB	Protein Kinase B.
PS	Phosphatidylserine.
qPCR	Quantitative polymerase chain reaction.
REDD1	Regulated in Development and DNA damage responses.
ROCK	Rho-Associated Protein Kinase.
SCD-1	Stearoyl-CoA desaturase 1.
Sh-CHKA	Choline kinase-α short-hairpin RNA.

SHH	Sonic Hedgehog.
Sh-Nt	Non-targeting short-hairpin RNA.
ShRNA	Short-hairpin RNA.
SMO	Smoothened.
SREBP	Sterol regulatory element-binding protein.
TCA	Tricarboxylic Acid Cycle.
tCHO	Total choline.
TGF-β	Transforming growth factor beta.
Vinc	Vinculin.
WB	Western blotting.

Summary

In this dissertation I describe the role of Choline Kinase Alpha (CHKA) in pancreatic ductal adenocarcinoma (PDAC) and its proposal as a therapeutic target.

I show that PDAC cell lines and human PDAC overexpress CHKA. Immunohistochemistry (IHC) of human PDAC samples reveals an intense CHKA staining that is absent from normal pancreatic tissue and chronic pancreatitis. There is lack of correlation between cytoplasmic staining and survival but a positive correlation between nuclear staining and survival in well and moderately-differentiated tumors.

In PDAC cells CHKA is important for cell proliferation; genetic approaches and pharmacological inhibition support this hypothesis. PDAC cell lines express high levels of CHKA compared with non-malignant pancreatic cell lines (HPDE/HPNE) and similar to CHKA overexpressing tumors. Genetic CHKA down-regulation, using a specific shRNA, decreases growth rate by more than 50% in all cell lines without morphological changes. I assessed the therapeutic potential of CHKA inhibitor, MN58b. This drug induces apoptosis on several PDAC cells lines depending on their CHKA levels suggesting that it is a predictive marker. MN58b is also effective against Pancreatic Stellate Cells (PSC) one of the main cell types of PDAC stroma that contributes to its chemorresistance.

MN58b also synergizes with different chemotherapeutic agents (Gemcitabine, Oxaliplatin and 5-Fluorouracil) commonly used in PDAC treatment. Genetic CHKA down-regulation sensitizes PDAC cell lines to these drugs. Moreover gemcitabine-resistant PDAC cells show enhanced sensitivity to MN58b.

Resistance to chemotherapy is a general problem in Oncology. For that reason I went beyond the therapeutic potential of MN58b and generated a MN58b resistant cellular model. Resistant cells show less choline (CHO) uptake, slower proliferation and reduced migration capacities than its counterpart. I describe ABCB-1 and ABCB-4 up-regulation as the mechanism of resistance. These two proteins belong to the multidrug resistance proteins family (MDR), and extrude drugs from inside of the cell. Consistently with this mechanism, blockade of these trans-membrane proteins restores the original MN58b-sensitivity.

In summary this data suggest that CHKA support PDAC development and its pharmacological inhibition could be a new therapeutic approach.

Resumen

En el presente trabajo describo el papel de la Colina Quinasa Alfa (CHKA) en el adenocarcinoma de páncreas (PDAC) y su posible papel como diana terapéutica contra el mismo.

Demuestro que, tanto las líneas celulares como las muestras tumorales de PDAC sobreexpresan CHKA. El análisis inmunohistoquímico de muestras de tumores humanos de PDAC revela un intenso patrón de tinción de CHKA, negativo para muestras de tejido pancreático normal y de pancreatitis crónica. No se aprecia correlación entre tinción citoplasmática y supervivencia pero sí, en cambio, entre tinción nuclear y supervivencia en tumores bien y moderadamente diferenciados.

Experimentos genéticos y de inhibición farmacológica muestran que CHKA es importante para la proliferación celular de PDAC. Las líneas celulares de PDAC expresan altos niveles de CHKA en comparación con líneas celulares de cáncer de páncreas no transformadas (HPDE/ HPNE) y niveles similares a los de tumores con sobreexpresión de CHKA. El descenso de los niveles de CHKA mediante un shRNA específico, disminuye la tasa de proliferación de las líneas celulares más de un 50% sin que se observen cambios en su morfología. He testado el potencial terapéutico de un inhibidor de CHKA, el MN58b. Dicho fármaco induce apoptosis en varias líneas celulares de PDAC en función de sus niveles de CHKA lo que sugiere que CHKA podría ser un factor predictivo de respuesta al MN58b. MN58b también posee efecto citotóxico contra las Pancreatic Stellate Cells (PSC), uno de los subtipos celulares principales del estroma de PDAC y responsables, en gran medida, de su quimioresistencia.

MN58b tiene un efecto sinérgico con varios quimioterápicos (Gemcitabina, Oxaliplatino y 5-Fluorouracilo) usados habitualmente para el tratamiento de PDAC. El descenso genético de los niveles de CHKA aumenta la eficacia de dichos fármacos en líneas celulares de PDAC. Además, las células de PDAC resistentes a la gemcitabina muestran una mayor sensibilidad a MN58b que las no resistentes.

La adquisición de resistencia a la quimioterapia constituye uno de los mayores problemas en Oncología. Para profundizar en los mecanismos que la producen, se ha generado una línea celular de PDAC resistente a MN58b que mostraba, respecto a las basales, un descenso en la captación de colina, una menor tasa de proliferación y una reducción en sus capacidades migratorias. Describo la sobreexpresión de ABCB-1 y ABCB-4 como el mecanismo responsable de la adquisición de resistencia. Estas dos proteínas pertenecen a la familia de proteína de resistencia de múltiples fármacos que expulsan los fármacos del interior de las células.

De acuerdo con lo previo, el bloqueo de dichas proteínas de transmembrana restituyó la sensibilidad originaria al MN58b.

En conclusión, los resultados obtenidos en el presente trabajo sugieren que CHKA puede ser relevante en el desarrollo de PDAC así como una novedosa e interesante diana terapéutica.

Introduction

1. The pancreas.

The pancreas is a secretory organ located in the retroperitoneum. Its name derives from the Greek words "pan"- meaning "all" - and "creas" - meaning "flesh" - reflecting its unusual composition without any cartilage, bone or hence. It is localized in the abdomen between the spleen, stomach and small intestine and it is anatomically divided into four parts: head, neck, body and tail (Fig I1A).

Functionally, we distinguish two compartments:

- *Endocrine pancreas,* responsible for the production and secretion of metabolic hormones. It contains compact structures called islets of Langerhans which account for approximately 5% of the pancreas and include 5 different cell types, each of them producing different hormones: glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells), ghrelin (ε-cells) and pancreatic polypeptide (PP-cells) (Fig I1. B and C) (1).

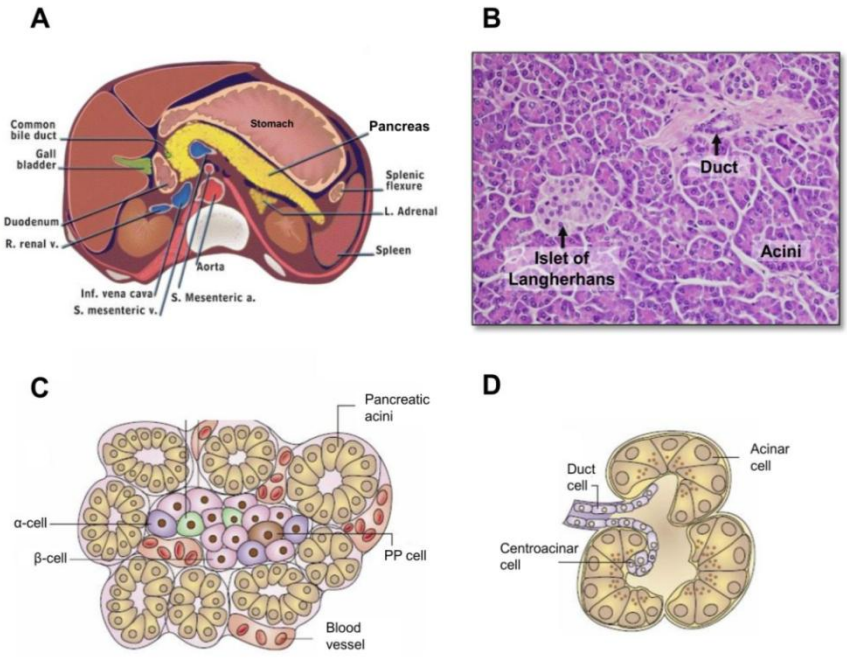


Figure I1. Anatomy and histology of the pancreas. (A) Cross-sectional diagram showing the anatomy of the pancreas. *Adapted from Pandol, 2010*. (B) Hematoxylin-eosin staining of a pancreatic section showing endocrine (Islet of Langerhans) and exocrine (Duct and Acini) compartments. (C) Schematic representation of islet of Langerhans embedded in the exocrine parenchyma. (D) Schematic representation of an acinus connected to a ductule. *Adapted from Bardeesy and Depinho, 2002.*

- *Exocrine pancreas,* composed by three main cell types: acinar (constituting 85% of the organ), centroacinar (CAC) and ductal cells (2). The exocrine functional unit is composed by acinar cells - forming a structure resembling a cluster of grapes named acinus- and a duct. Acinar cells produce digestive enzymes that are secreted into the lumen of the acinus and go through the ductal system to end in the duodenum, where they perform their hydrolytic action (Fig I1. D).

2. Molecular features of Pancreatic Ductal Adenocarcinoma (PDAC).

Tumors of the pancreas are relatively uncommon representing 3% of all solid tumors (3); the majority of them arise from the exocrine pancreas. Pancreatic ductal adenocarcinoma (PDAC) is the most frequent of them and it accounts for approximately 85% of all pancreatic tumors. The rest of this introduction, unless otherwise specified, refers to PDAC.

Less frequent pancreatic neoplasms include:

- *Intraductal papillary mucinous neoplasms (IPMNs)* (1-3%). Formed by columnar mucin-producing cells that grow inside the ductal system, can evolve from adenoma to invasive carcinoma, and are often associated with PDAC (4). They belong to the category of pancreatic cystic tumors that are receiving increasing attention due to their high prevalence in the healthy aging population and their ability to become malignant (4).
- *Acinar cell carcinomas (ACCs).* They are solid and unifocal differentiated tumors that secrete acinar digestive enzymes. They often present with genetic alterations in Wnt/β- catenin signaling pathway (5).
- *Pancreatoblastoma.* It is the most frequent pancreatic tumor of childhood and it also frequently shows Wnt/β- catenin alterations (6).
- *Pseudopapillary neoplasms (SPNs).* These tumors lack features of differentiated pancreatic cells and their cell origin is unknown. They tend to metastasize to liver or peritoneum (7).

2. 1. Etiology.

Several factors are associated with an increased risk of PDAC development (Table I1).

A. Lifestyle/environmental exposures:

- *Tobacco smoking* increases PDAC risk by 2.5 to 3.6 times (8), and it is estimated that it contributes to 20-30% of cases of PDAC (9).
- *Chemical occupational exposure* to several agents such as beta-naphthylamine and benzidine (10).
- *Body mass index and low physical activity*. Walking 1.5 hours or more per week is associated with 50% reduction of the risk of PDAC (11).
- *Allergies*. Recent studies show that atopic people (asthmatic or with nasal/ skin allergies) have decreased risk of developing PDAC suggesting the connection between inflammatory processes and pancreas carcinogenesis (12, 13).
- *25-hydroxyvitamin plasma levels*. This correlation is still under debate; while some authors find correlation between low levels of plasma 25-hydroxyvitamin and increased risk of developing pancreatic cancer (14), other results do not support this hypothesis (15).

B. Pancreas disorders:

- *Chronic pancreatitis (CP)* increases the risk by 7.2-fold (16, 17). This association is even stronger (up to 70-fold) when we consider only hereditary chronic pancreatitis (18).
- *Diabetes mellitus (DM).* The association between PDAC and DM is complex, as the development of PDAC - by destroying pancreatic parenchyma- can cause DM. The relative risk of PDAC in persons with new-onset non-insulin-dependent DM is double the risk of persons without DM. It is estimated that 1% of patients diagnosed of DM at age 50 years or younger will develop PDAC (19-22). Furthermore, DM is a factor of poor prognosis among patients with PDAC (23). The association between DM treatments and PDAC is also being studied; insulin and sulfonylurea use is associated with an increased risk of PDAC (24) while metformin intake is associated

with reduced incidence and improved survival in patients with non-metastatic PDAC (25).

C. Genetic predisposition. Familial aggregation of pancreatic cancer (two or more first-degree relatives with PDAC without criteria for inherited cancer syndromes) is found in approximately 5% of patients with PDAC (26). Some of the patients/families display features compatible with familial cancer syndromes but, for most of the remaining cases, the genetic alterations responsible for the aggregation remain unkown. The risk of developing PDAC is 2, 6 or 30 times higher in patients with 1, 2 or 3 relative affected respectively (27). The genes in which germline mutations have been reported to be associated with increased PDAC risk are summarized in Table 1 (28-32).

Variable	Times
Risk factor	
• Smoking	2.5-3.6
• Diabetes mellitus	2
• Sporadic chronic pancreatitis	2-6
• Obesity	2
• Non-O blood group	1-2
• Atopy	Unkown
• Low 25-hydroxyvitamin levels	Unkown
• Exposition to chemical agents	Unkown
Genetic syndrome and associated genes	
• Hereditary pancreatitis (*PRSS1*)	50-70
• Familial atypical multiple mole and melanoma syndrome (*p16/INK4A*)	10-20
• Hereditary breast and ovarian cancer syndrome (*BRCA1*, *BRCA2*, *PALB2*)	2-3
• Peutz-Jeghers syndrome (*STK11*)	30-40
• Lynch syndrome (*MLH1*, *MSH2*, *MSH6*)	4
• Ataxia-telangiectasia (*ATM*)	Unkown
• Li-Fraumeni syndrome (*TP53*)	Unkown

Table I1. Risk factors and inherited cancer syndromes associated with increased risk of developing pancreatic cancer.

Genome wide association studies have revealed that low penetrance genetic variations can contribute to PDAC (33). Among the most striking variants are those responsible for the ABO blood group: subjects of A or B blood groups have an increased risk of developing PDAC (34). Nevertheless, this information cannot be used in the clinical setting for risk stratification.

2.2. Biology.

2.2.1. PDAC initiation and progression.

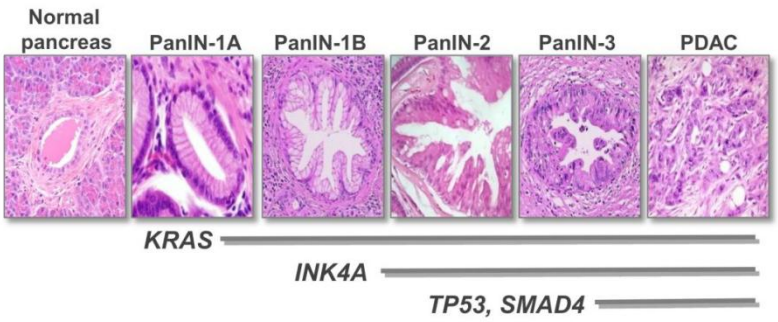
The pancreas of patients with PDAC commonly contains hyperplastic or dysplastic lesions that are considered precursors of the malignant tumor. These lesions have been designated as "Pancreatic Intraepithelial Neoplasms" (PanINs). Following a stepwise model, it is proposed that normal cells (low cuboidal cells in a single layer epithelium) evolve to PanIN-1A (hyperplastic epithelium without dysplasia; elongated cells with mucin production) that progresses to PanIN-1B (papillary hyperplasia) then to PanIN-2 (mild hyperplasia with nuclear abnormalities) and finally to PanIN-3 (severe dysplasia with nuclear atypia) which are carcinomas *in situ* and the direct precursor of invasive adenocarcinomas (Fig. I2A and 2B). PanIN-1 lesions are frequent in old people without evidence of PDAC whereas PanIN-3 lesions are almost exclusively detected in PDAC patients, suggesting than PanIN-2 is the turning point in the progression to carcinoma (35).

This stepwise malignant progression is paralleled by the successive accumulation of genetic alterations (Fig. I2A); the initial event is a *KRAS* activating mutation that is found in up to 90% of PanIN-1 lesions. This model posits that subsequently there are mutations in tumor-suppressor genes such as *CDKN2A* (which encodes the inhibitor of cyclin-dependent kinase 4, *INK4A/CDN2A*) and *TP53* which is found in 50-70% of PanIN-3 with severe dysplasia (36). A late event, present in PanIN-3 and invasive lesions, is *SMAD4* inactivation (37). This model is supported by experiments in genetically engineered mice in which targeted activation of *Kras* and inactivation of *Tp53* or *Cdkn2A/Ink4A* lead to the development of a pancreatic cancer recapitulating the human disease (38).

The cell of origin of PDAC is still under debate. Historically, ductal cells were proposed as the origin of PDAC based on their morphological resemblance. However, the use of genetic mouse models aimed at recapitulating the human disease, has shown that acinar cells can give rise to PDAC due to its plasticity (38). These cells can trans-differentiate into other cell types, i.e. adipocytes and ductal cells. The trans-differentiation to ductal cells, a process known as acinar-ductal metaplasia (ADM), can occur upon severe damage (i.e. chronic pancreatitis) (38). Intriguingly, it has proven very difficult to initiate PDAC in ductal cells using genetic mouse models. Also it is discussed whether mouse models are a reliable model of human disease; the initiation of PDAC in mouse models depend on the expression of oncogenes and loss of tumor suppressors in pan-pancreatic progenitor cells

during embryonic development, while in the human disease genetic alterations occur in adult life (39).

A



B

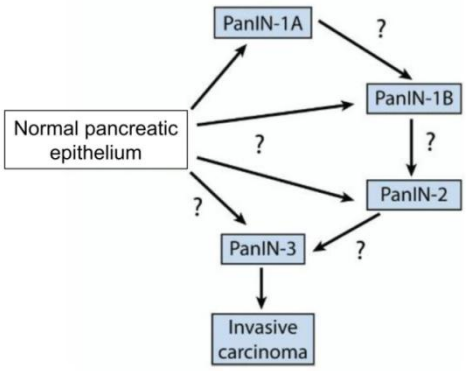


Figure I2. PDAC progression. (A). Pancreatic Ductal Adenocarcinoma (PDAC) linear progression model according to which ductal cells that acquire *KRAS* activating mutations proliferate and form PanIN-1A/B lesions which - due to mutations in tumor suppressors - gradually progress into lesions of higher grade (PanIN-2 and PanIN-3) to finally yield ductal adenocarcinoma. (B) An alternative model of PDAC progression suggesting that different types of PanINs lesions might arise directly from normal pancreatic epithelium and that low grade lesions might arise through mechanisms independent from those leading to carcinoma formation. *Adapted from Real, 2003 (40)*.

2.2.2. Genetic alterations in PDAC.

PDAC is characterized by a successive accumulation of genetic alterations (mutations, deletions or amplifications). Nearly 100% of PDAC present activating mutations in *KRAS* codon 12 or 13 and inactivation of *P16/CDKN2A* (a regulator of the G1-S transition of the cell cycle). Less frequent, but highly prevalent (60-80% of tumors), are the inactivation of *TP53* (which allows cells to evade apoptotic stimuli and contributes to genomic instability) and deletion of *SMAD4/DPC4* (resulting in an aberrant signaling by TGF-β).

Other relevant genetic events found in 35% of PDAC is c-MYC overexpression; activation of *KRAS* and loss of *SMAD4* contribute to the increased levels of c-Myc (41). PDAC also

shows constitutive activation of NF-Kβ that induces chemoresistance through the up-regulation of BCL-2 and BCL-XL (42). Finally, members of the Sonic Hedgehog pathway (SHH) such as SHH, SMO and PTC1 are overexpressed in PDAC and are in part responsible for the stromal formation (43).

Recent whole exome/genome sequencing and CNV analyses support the enormous genetic heterogeneity of PDAC. The first genomic analysis of 24 PDAC, using standard PCR and Sanger sequencing, pointed to an average of 63 mutations per tumor that could be grouped in 12 functional cancer-relevant pathways i.e. apoptosis, G1/S phase transition and KRAS signaling among others (44). Subsequent studies, using massive parallel sequencing, revealed different sub-clones in the primary tumor arising from a common cell and a considerable heterogeneity between primary and metastatic samples (45). Further analyses have confirmed chromosomal rearrangements leading to disruption of genes (*TP53, SMAD4, CDKN2A*) known to be important for PDAC development and have confirmed significant intratumoral heterogeneity in PDAC (45, 46). The existence of PDAC subgroups based on both genome wide transcriptomic analyses (47) and genome rearrangements (46) suggests that a PDAC molecular taxonomy is emerging.

2.2.3. Role of the stroma in PDAC.

PDAC is characterized by the occurrence of a dense stroma surrounding tumoral cells (Fig. I3A and B). This desmoplastic reaction can account for up to 90% of tumor volume and constitutes a physical barrier contributing to the reduced flood flow and high interstitial pressure that impairs drug delivery and promotes intra-tumoral hypoxia (48). Mouse models have shown that the stroma impairs gemcitabine penetration in PDAC, and its depletion using a SHH inhibitor increases intratumoral gemcitabine concentration (49). The stroma is also a dynamic compartment which is crucial for tumor initiation, progression and metastatic capacity (50) through the expression of proteins such as cyclooxygenase-2 or PDGF receptor, among others. The molecular composition of the PDAC associated stroma is undergoing extensive characterization. The stroma has two main components:

- A cellular component that includes fibroblasts, Pancreatic Stellate Cells (PSC), adipocytes, endothelial cells and immune and inflammatory cells. PSC, also called myofibroblasts, are essential for the formation and turnover of the stroma; upon activation by growth factors (PDGF, FGF, TGFβ1), they secrete collagen, metalloproteases and other components of the extracellular matrix (51) reducing tumor vascularization (52).

- An extracellular matrix composed by collagen I and III, laminin, fibronectin, matrix metalloproteinases (MMP), metallopeptidase inhibitor 1 (TIMP1), secreted protein acid and rich in cysteine (SPARC), and a wide range of cytokines including connective tissue growth factor (CTGF).

Cytokines recruit immunosuppressive regulatory T-cells and infiltrating B-lymphocytes (53). Feig et al showed that by targeting CXCL12- a chemokine ligand secreted in the stroma- it is possible to restore cytotoxic T-cell recruitment, leading to tumor regression (54) which points at the importance of therapeutically targeting the stroma.

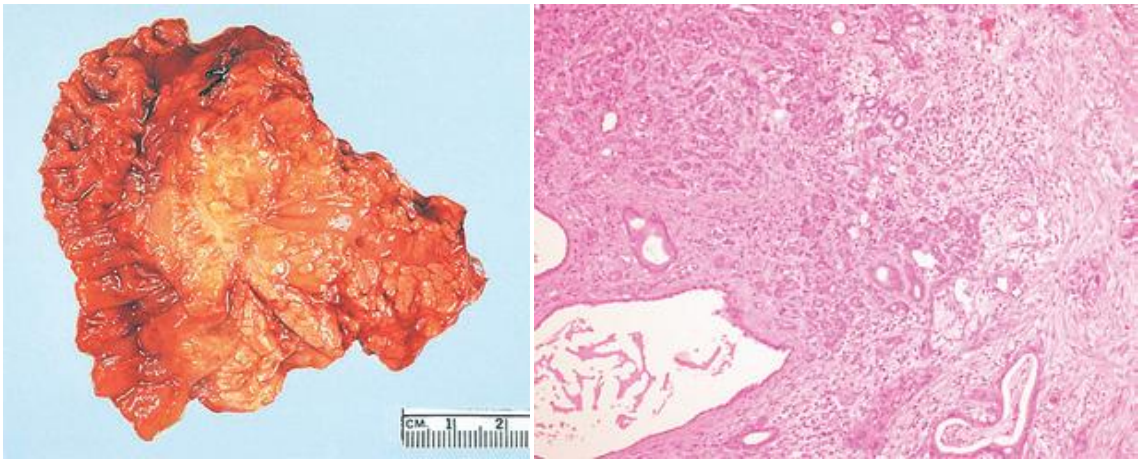


Figure I3. Pancreatic ductal adenocarcinoma (PDAC): macroscopic and microscopic aspect. (A) Resection specimen of an undifferentiated pancreatic ductal adenocarcinoma showing cystic features with hemorrhage. (B) Hematoxylin-eosin staining of human PDAC with very abundant fibrotic tissue. *Adapted from Hidalgo et al. (55)*

2.2.4. Metabolic alterations in PDAC.

Metabolic adaptation is crucial for tumor initiation and development. PDAC cells adapt their metabolism to stress, including that originated by the desmoplastic reaction that surrounds the tumor. This hypoxic environment, with reduced nutrient availability, forces the tumor to modify its metabolism in different ways (56):

- Increasing glycolysis to metabolize glucose through the tricarboxylic acid cycle (TCA) cycle to provide intermediates for biosysnthetic reactions.
- Increasing glutamine uptake that is diverted to the TCA to generate NADPH and anti-oxidants.
- Increasing macropinocytosis and autophagy to scavenge lipids and provide intermediary metabolites.

- Reducing redox state by the expression of genes coding for proteins with anti-oxidant function.

3. Metabolism and cancer.

Metabolism is the set of biochemical and physicochemical reactions required to transform nutrients into energy and structural components to fulfill the requirements of dividing cells. Cellular metabolism deregulation is considered an emerging crucial hallmark of cancer (57) (Fig. I4A). Key oncogenic signaling pathways converge to adapt tumor metabolism to support growth, survival, and cell division. Upon oncogenic activation, tumor cells reprogram glycolysis and glutaminolysis to facilitate carbon assimilation into different macromolecules (58, 59) (Fig. I4B).

Otto Warburg first described in 1930s the importance of metabolism in malignant transformation and described that neoplastic cells, even in the presence of normal oxygen concentrations, reprogram their glucose metabolism and undergo aerobic glycolysis instead of mitochondrial oxidative phosphorylation (60). Although there is a clear energetic loss (36 molecules of ATP less per molecule of glucose are produced through anaerobic glycolysis), the main advantage of the Warburg effect is the synthesis of structural molecules, such as nucleotides and amino acids, which sustain rapid cell growth (61). Cancer cells compensate the deficient ATP production by enhancing glucose uptake through the up-regulation of the main glucose transporter (GLUT1) (59). This property of neoplastic cells has allowed the development of a radiolabeled analog of glucose (18-F-Deoxyglucose) as a reporter in positron emission tomography (PET) - a non-invasive technique of cancer diagnosis that is routinely used by clinicians.

c-MYC, a major human oncogene, enhances glycolysis through the transcriptional activation of lactate deshydrogenase A (LDHA) and it also increases mitochondrial biogenesis and glutamine catabolism, contributing to the Warburg effect (62). The reliance on glycolysis is accentuated under hypoxic conditions, a common situation in rapidly growing tumors; the hypoxia response acts pleiotropically to up-regulate glucose transporters and multiple enzymes of the glycolytic pathway (58, 63). For example, KRAS activation in hypoxic conditions increases the levels of the transcription factors HIF1a and HIF2a that up-regulate glycolysis (64).

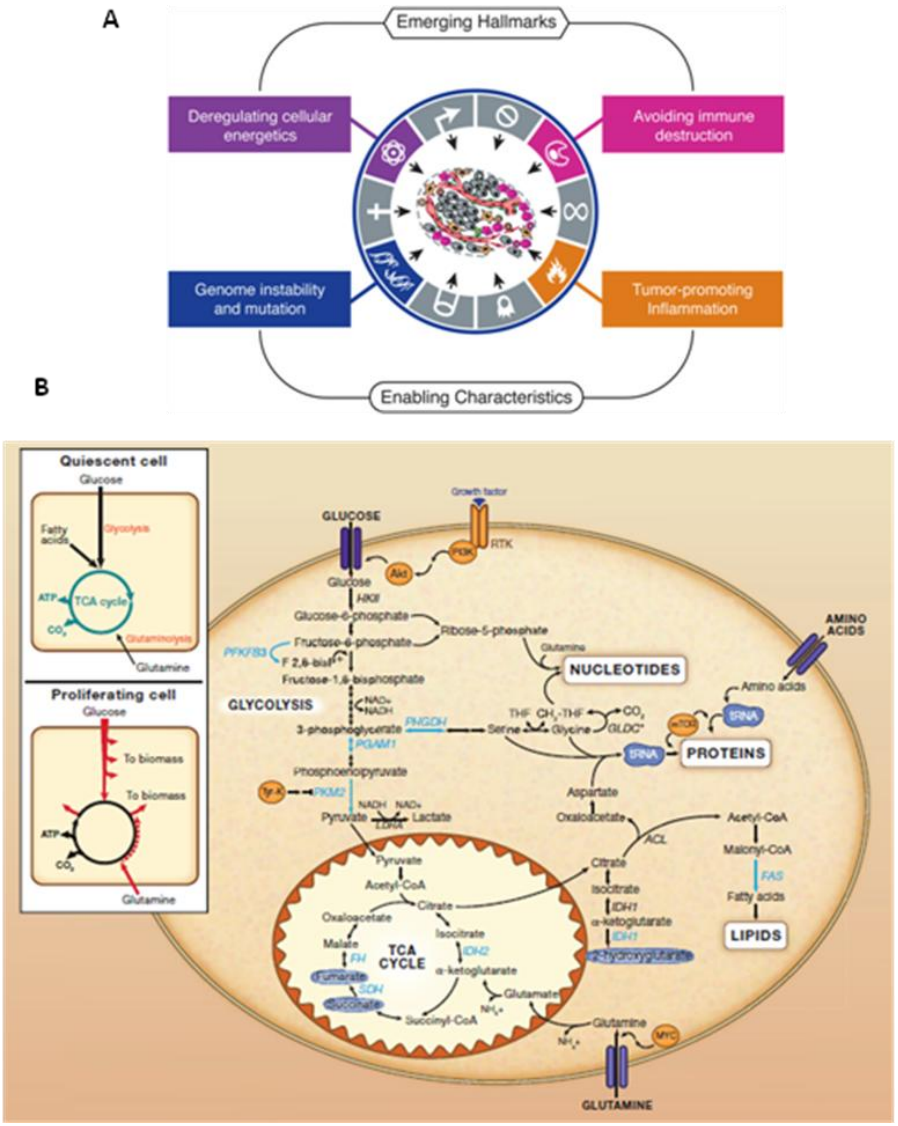


Figure I4. Metabolic changes in neoplastic transformation. (A) Deregulation of cellular energetics is one of the new features of cancer cells described in the last decade. (B) Schematic illustrating prominent metabolic pathways in malignant cells. Glycolysis is the 10-step metabolic pathway that converts glucose into pyruvate. The energy released in this process is used to form the high-energy compounds such as ATP and NADH. The pentose phosphate pathway generates NADPH and 5-carbon sugars as alternatives to glycolysis. Nucleotide synthesis provides molecules that make up the individual structural units of RNA and DNA. In addition, nucleotides participate in cell signaling (cGMP and cAMP) and are cofactors of enzymatic reactions, and nucleoside triphosphates are sources of chemical energy. Fatty acid synthesis from acetyl-CoA and malonyl-CoA precursors occurs via fatty acid synthases. *Adapted from Hanahan et al.*

Glutamine is a key amino acid for cancer cells; it provides two N- atoms to synthesize hexosamines, nucleotides and other amino acids required to sustain cell growth. Glutamine is transformed into the cell in glutamate, with different fates: i) it is transformed to glutathione - one of the most abundant antioxidants in mammalian cells,

responsible of maintaining redox status; ii) Glutathione can be converted into alpha-ketoglutarate that enters the TCA cycle to produce other amino acids and fatty acids; iii) Finally, glutamine can also contribute to the TCA cycle and produce NADPH (Fig. I4B).

3.1. Lipid metabolism and cancer.

Lipids, which include triacylglicerides, phosphoglicerides, sterols and sphingolipids (Fig. I5), contribute to energy production (triacylglicerides) and are a source of structural components of cells (phosphoglycerides and sphingolipids). Lipids are grouped as follows:

Non-saponifiable lipids			**Saponifiable lipids**	
Terpenoids:	*Steroids:*	*Eicosanoids:*	*Simple:*	*Complex:*
- Vitamin A - Vitamin K - Vitamin E	- Vitamin D - Cholesterol - Bile salts - Sexual hormones	- Linoleic acid - Arachidonic acid	- Waxes -Acylglycerols	- Phospholipids - Glycolipids

The connection between lipid metabolism and cancer has been known for several decades (65) and, nowadays, obesity is described as a major risk factor for many types of cancers such as those arising in the breast, endometrium and esophagus.

Tumors activate *de novo* lipogenesis, to provide intermediates for membranes synthesis during unrestrained growth and second messengers for cell signaling (66).

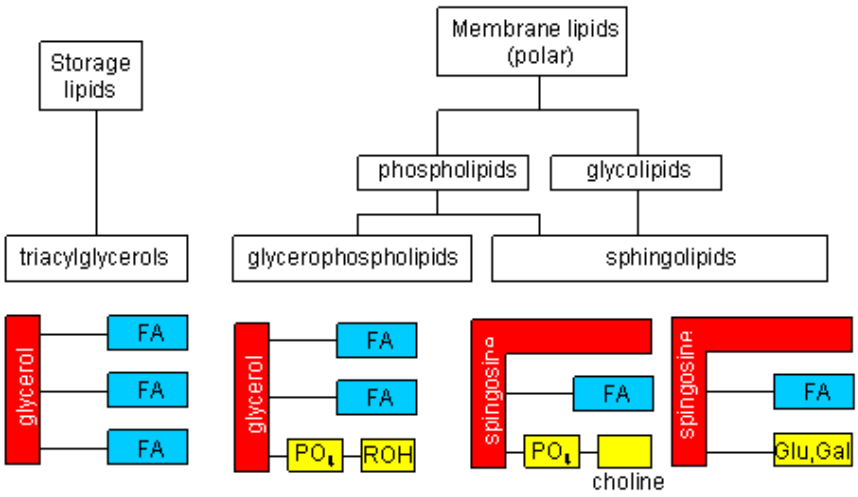


Figure I5. Lipid classification. According to their biological role we can distinguish between energy lipids (triacylglycerols) and storage lipids (phospholipids and glycolipids). *Adapted from Lehninger et al.*

3.1.1 Fatty acids.

3.1.1.1. Fatty acid synthesis and oxidation.

Fatty acids (FA) can be obtained directly from the diet or synthesized *de novo* from glucose through the tricarboxylic acid cycle (TCA); glucose is converted to citrate, then to acetyl-CoA, and finally to malonyl-CoA which is the direct precursor of FA (Fig. I6). Sterol regulatory element-binding protein (SREBP), which has three isoforms (1a, 1c and 2), is the master regulator of most of the enzymes implicated in lipid synthesis (67) and its expression is controlled by the intracellular concentrations of sterols and phospholipids (68). For example, depletion of phosphatidylcholine (PC) in mammalian cells leads to an increased nuclear accumulation of SREBP-1. Also, the PI3K/AKT/PKB pathway has an important role in SREBP regulation through mTOR activity (69).

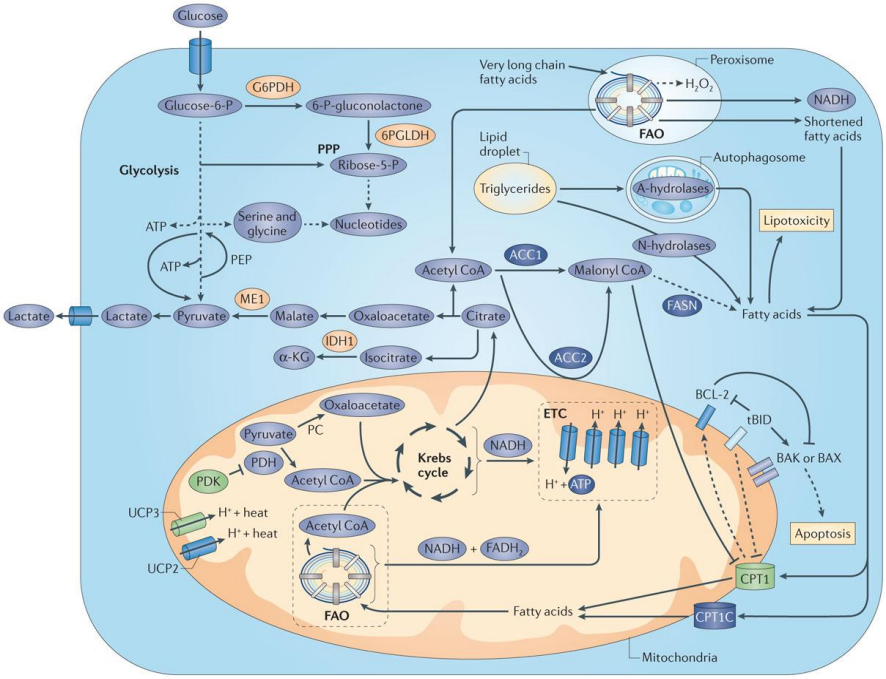


Figure I6. Fatty acid synthesis and oxidation. Citrate is converted to acetyl-coenzyme-A (acetyl-Co-A), then to malonyl-Co-A, and finally to saturated fatty-acids through three steps that are catalyzed by ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty-acid synthase (FASN), respectively. Different saturases (SCD-1) and elongases (ELOVL1-7) generate FA of variable length and saturation. Unsaturated fatty acids are generated by stearoyl-CoA desaturases (SCD), mainly SCD-1. This enzyme introduces a double bond in the D9 position of palmitic and stearic acids to produce mono-unsaturated fatty acids that are essential for the synthesis of phosphoglycerides, the major components of cell membrane. Fatty acids used for FAO can either be of extracellular origin or be obtained through the metabolism of triglycerides from lipid droplets. The fate of FAO products is summarized: NADH and $FADH_2$ are oxidized in the ETC for ATP production and acetyl-CoA enters the Krebbs cycle to produce citrate, which can be exported to the cytoplasm to engage NADPH-producing reactions. *Adapted from Pandolfi et al.*

Fatty acid oxidation (FAO) is the catabolic process by which FA molecules are broken down in the mitochondria to generate acetyl-CoA, NADH and FADH2. FA are an important source of energy for the cell. Mitochondrial FAO can produce more than twice ATP per mole of FA than glucose or amino acid oxidation.

3.1.1.2 Fatty acids and cancer.

Under stress conditions (low oxygen or nutrient deprivation), scavenging extracellular lipids has emerged as an important mechanism to maintain tumor growth independently of *de novo* synthesis. Scavenging, rather than synthesis, liberates tumor cells from the need to supply carbon and NADPH for this demanding pathway. Hypoxia and oncogenic RAS signaling stimulate lysophospholipid uptake conferring resistance to SCD1 inhibition. In fact, some neoplasias - such as B-cell lymphomas - prefer FAO to glucose or glutamine under normal oxygen and nutrient conditions (70).

Several oncogenes and tumor suppressor genes regulate lipid metabolism. Fatty-acid synthase (FASN), one of the master regulators of lipid metabolism and a prognostic marker in breast cancer, is up-regulated in several KRAS-dependent tumors (71); in PDAC its enhanced expression partially relies on the EGFR/ERK signaling pathway (72). FASN inhibition allows caspase-2 dimerization through REDD1 and induces apoptosis in ovarian cancer cells, suggesting that FASN may be a therapeutic target (73). ATP-citrate lyase (ACLY), whose expression is upregulated by KRAS and c-MYC, participates in malignant transformation and is overexpressed in ovarian cancer (74). c-MYC induces the expression of acetyl-CoA synthase (ACSS) to produce acetyl-CoA and contributes to FA biosynthesis during the cell cycle. However, the effect of c-MYC on lipid metabolism is tissue-specific (75). c-MYC overexpressing lymphomas show a different lipid profile compared to non-transformed B-lymphocytes, with increased phospholipids (76). Moreover, pharmacological inhibition of c-MYC leads to lipid accumulation and down-regulation of enzymes involved in FA synthesis (77). Tp53 increases lipid synthesis via SREBP1 and promotes metastasis in ovarian cancer (78).

In the case of PDAC, de novo synthesis of lipids is elevated due to the deregulation of some lipid (79) enzymes such as FASN, which indeed has been proposed as prognostic marker (80) or citrate synthase and ACC (81-83).

3.1.2. Phospholipids and other structural lipids.

Besides their role as an energy source, lipids have a fundamental structural role, for example as components of membranes in eukaryotic ells. As we described previously, most phospholipids contain a diglyceride, a phosphate group and a simple organic molecule (Fig. I7). Among phospholipids we find sphingolipids containing a 18 carbon amino-alcohol backbone called sphingosine.

The most important glycerophospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). PC, also called lecitin and discovered in the 19th century in the egg yolk, is the most abundant phospholipid in biological membranes and, together with other phospholipids (PE and neutral lipids), forms the characteristic bilayer structure of cell membranes. Besides its structural function, PC has a crucial role in cell signaling. Products of its biosynthesis and hydrolysis, such as diacylglicerol (DAG) or arachidonic acid (AA), function as second messengers in several signaling pathways (RAS-RAF-MAPK cascade and protein kinase C pathway) (84). Diacylglicerol regulates cell cycle progression from G1 to S through up-regulation of cyclin D1 and cyclin D3 expression (85). Together with inositol phospholipid metabolism, choline metabolism provides a sustained activation of mitogenic signal transduction via a positive feedback loop (86).

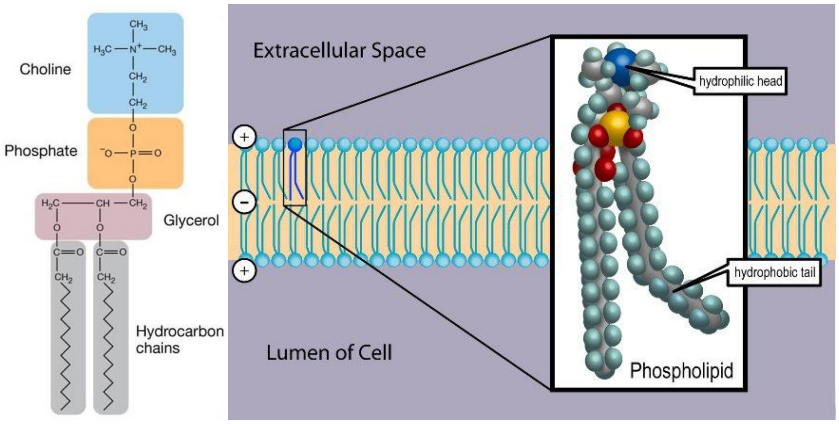


Figure I7. Phosphatidylcholine (PC) structure. Phospholipids form the characteristic lipid bilayer of eukaryotic cells and are composed by a positively charge hydrophilic head, in contact with the extracellular space, and a hydrophobic negatively charged tail. Their basic structure contains a phosphate and glycerol group, two hydrocarbon chains and an organic molecule that varies according to the phospholipid. *Adapted from Lehninger.*

3.2. Choline metabolism.

Choline is an important nutrient that belongs to the vitamin B group. It can be obtained either directly from the diet or from PE that is converted to PC and then hydrolyzed to choline. Physiologically, choline and its metabolites participate in several processes: structural integrity of cell membrane, cell signaling, cholinergic neurotransmission, and DNA methylation (66) (Fig I8).

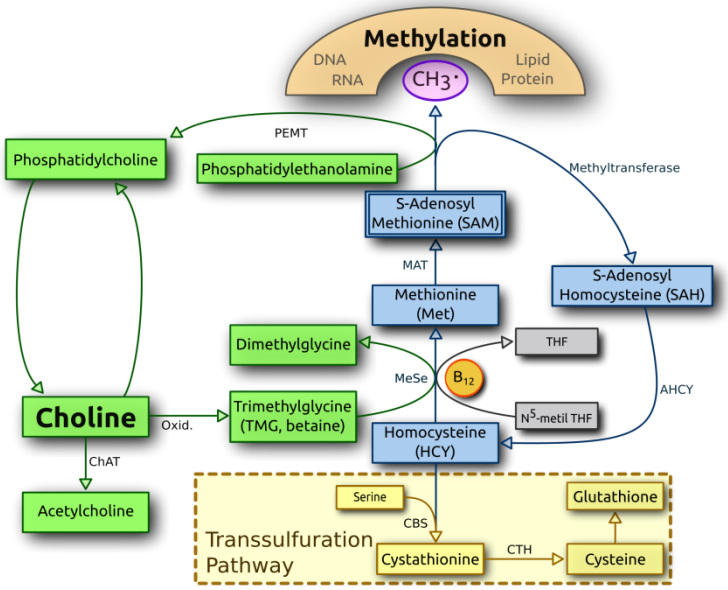


Figure I8. Physiological roles of Choline. Choline is the source of PC, the most abundant phospholipid in eukaryotic cell membranes. It is also the direct precursor of acetylcholine, the main neurotransmisor, and - finally - the source of methyl groups used in epigenetic DNA control.

3.2.1.Phosphatidylcholine synthesis: The Kennedy pathway.

The intestine absorbs dietary choline as lysophosphatidylcholine and converts it to PC through two pathways: the PE methylation pathway, which is only significant in hepatocytes, and the Kennedy pathway.

Through the Kennedy pathway, described in 1956 by Weis and Kennedy (87), choline is transformed first to phosphocholine, then to CDP-Choline and finally to PC (Fig.9). Choline is incorporated into the cell by choline transporters. There are four families of transporters: high-affinity choline transporters (CHTs), choline transporter-like proteins (CTLs), organic cation transporters (OCTs), and organic cation/carnitine transporters (OCTNs). Inside the cell, choline kinase (CHK), phosphocholine cytidyltransferase (CCT), and diacylglicerol cholinephosphotransferase 1 (CHPT1) synthesize PC which is then incorporated to the membrane. Finally, phosphocholine-specific phospholipase D (PC-PLD

1 and 2) and phosphocholine-specific phospholipase C (PC-PLC) hydrolyze PC to produce phosphatidic acid (PA) and DAG, respectively, which are intracellular second messengers

crucial for cell proliferation, survival signaling, and tumor progression (Fig. I9).

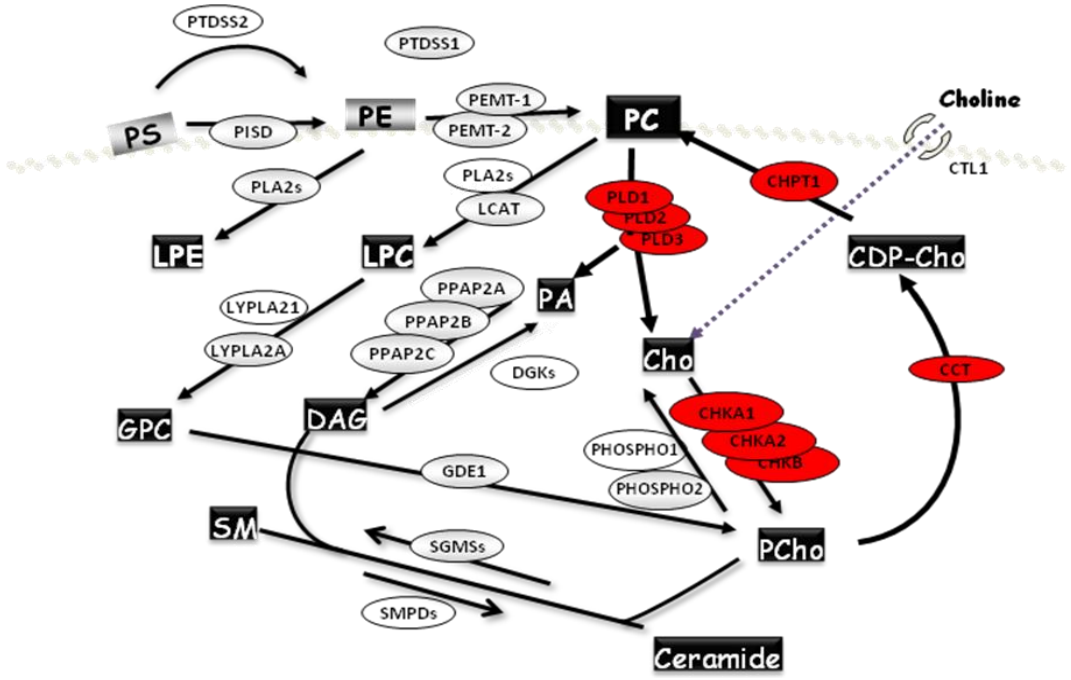


Figure I9. The Kennedy Pathway: **synthesis and regulation of PC.** CHO is incorporated into the cytoplasm and converted to PC through several enzymes that include Choline Kinase (CHK), Phosphocholine cytidyltransferase (CCT) and Diacylglicerol Cholinephosphotransferase 1 (CHPT1). PC is hydrolyzed by phospholipases (PLD) and it yields intracellular messengers, such as phosphatidic acid (PA) and diacylglicerol (DAG), and molecules such as sphyngomielin (SM), ceramide and glycerophosphocholine (GPC).

3.2.2. Choline Kinase (CHK)

Wittenberg and Kornberg discovered Choline Kinase in 1953 (88). Hosaka cloned the α-isoform (CHKA) in 1992 (89) and, in 1998, Aoyama reported the cloning of a new isoform of CHK (CHKB) from a rat kidney cDNA library. This cDNA encoded a protein of 45 KDa and 394 residues with a 60% similarity with CHKA (90).

CHK localizes in the cytoplasm and catalyzes the phosphorylation of free choline to phosphocholine (PCHO) using ATP as a phosphate donor. There are three isoforms of CHK in mammalian cells, encoded by two genes*: CHKA* located on 11q13 and *CHKB* on 22q13. *CHKA* generates, by alternative splicing, two isoforms, CHKA1 (435 aa) and CHKA2 (453 aa) whose functional difference is currently unkown. *CHKB* generates one isoform, CHKB (90). Both, *CHKA* and *CHKB* genes contain 11 exons. Northern and western blotting analyses using mouse tissues show that CHKA and CHKB are ubiquitously and

concurrently expressed in all tissues, with a predominance of CHKA in liver and testis and CHKB in heart and liver. Functionally CHK acts as a homodimer or a heterodimer and the proportion of each is tissue-specific. In mouse liver, 40% of CHK corresponds to α or β homodimers, while the remaining 60% is composed by α/β heterodimers. CHKA1 and 2 homodimers display a dual choline and ethanolamine activity, CHKB homodimer has an ethanolamine activity, and the CHKA/β heterodimer possesses intermediate substrate specificity (91).

While CHKB knockout mice are viable, CHKA loss is embryonically lethal and mice die within the first 3 postnatal days, suggesting that CHKA is essential and that it can compensate for CHKB loss (92).

3.2.2.1. CHKA and the regulation of choline metabolism.

Different transcription factors control CHKA expression. In the liver, c-Jun drives CHKA transcription through its binding to the distal activator protein 1 (AP-1) binding site in the promoter of CHKA. This activity has been linked with proliferation and transformation in liver cancer (93). MYC regulates CHKA levels; Myc +/+ rat fibroblasts show elevated CHK and PCHO levels compared with Myc -/- cells (75). Hypoxia increases CHKA levels and choline metabolism in a model of prostate cancer, presumably through HIF1 (94). A putative hypoxia response element has been described in the *CHKA* promoter.

Different signaling pathways control CHKA activity (Fig. I10). Serum-stimulated KRAS-transformed NIH3T3 fibroblasts show activation of CHK and elevated PCHO levels and several studies indicate that CHK activation downstream of oncogenic KRAS occurs via Ral-GDS and ROCK kinases (95, 96). There is also a positive feedback between the PI3K-AKT pathway and CHKA activity: inhibition of the PI3K-AKT pathway blocks choline uptake in lung adenocarcinoma cell lines (97) and knockdown of CHKA attenuates MAPK and PI3K-AKT pathway (86, 98).

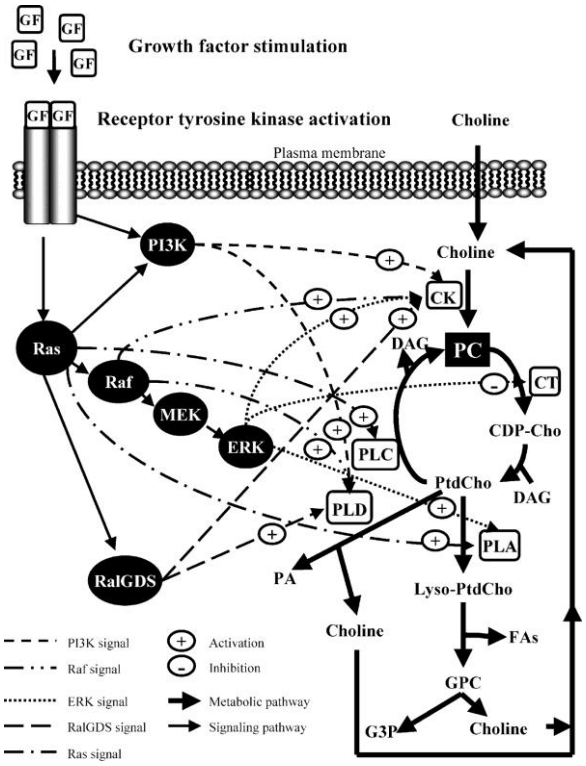


Figure I10. Kennedy pathway regulation. Some of the most relevant survival and proliferative signaling pathways, such as PI3K and MAPK, regulate the Kennedy pathway by directly activating (mostly) or inhibiting some of its key enzymes, including CHK. *Adapted from Yalcin et al.*

3.2.3. Choline metabolism and CHKA in malignant transformation.

Introduction of magnetic resonance spectroscopy (MRS) in the 80s revealed that neoplastic cells show an active choline metabolism characterized by an increase of PCHO and total choline (99). Levels of PCHO and tCho are high in brain, breast, and endometrial cancer, among others, and these tumors show enhanced CHKA expression (100-107).

Overexpression of CHKA and abundance of choline metabolites have been proposed as prognostic markers in different tumor types (108-113). In prostate cancer, the increase of tCHO metabolites measured by MRS permits to distinguish malignant and normal prostatic tissue and its expression correlates with Gleason score and aggressiveness (114).

Choline metabolism has also been proposed to be involved in metastatic dissemination. Many tumors (breast, prostate, and ovarian) show a reduction of the ratio of glycerophosphocholine (GPC) to choline. Endometrial differential 3 (EID3) is the enzyme responsible of the conversion from GPC to choline; its silencing increases the intracellular GPC/PC ratio and inhibits cell migration via disruption of the PKCα signaling pathway (115). In addition, CHKA inhibition - either using shRNA or small molecules - has strong

antiproliferative effects in different tumor types, supporting its role as a therapeutic target (116, 117).

3.2.4. Clinical applications of altered choline metabolism in cancer.

3.2.4.1. Cancer diagnosis.

During malignant transformation choline uptake increase can be measured by MRS, due in part to the overexpression of choline transporters such as CTL1 and OCT3 (118).

In ^{1}HMR spectra, the signal of total choline compounds (CHO, PCHO and GPC) in tumors is detected as a single peak observed between 3.2 ppm and 3.3 ppm (Fig. I11).

Recently, ^{11}C- and ^{18}F-labelled choline has been evaluated for the non-invasive diagnosis of tumor recurrence using PET. ^{11}C Choline PET-CT has shown a much higher sensitivity for the detection of lymph nodes in prostate cancer compared to MRI (81.5% vs. 51.9%) and is considered a promising imaging strategy (110).

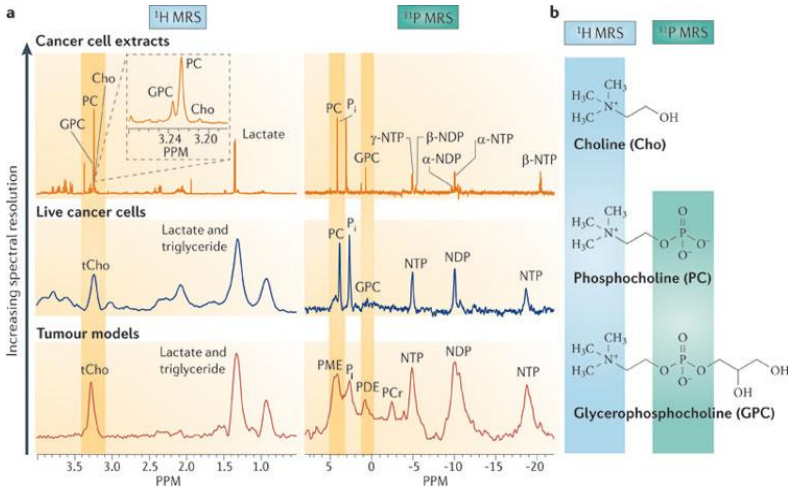


Figure I11. Detection of deregulated cancer CHO metabolism via MRS. In ^{1} H MR spectra obtained *in vivo*, the total choline-containing compounds (tCHO) signal in tumors is detected as a single peak observed between 3.2 and 3.3 ppm. This peak consists of CHO, PCHO and GPC. *Adapted from Glunde et al.*

3.2.4.2. CHKA as a therapeutic target.

A wide variety of cancers overexpress CHKA rendering it an attractive therapeutic target. Genetic down-regulation of CHKA reduced intracellular levels of tCho and PCHO and impaired cell proliferation from different tumor types *in vitro*. For example, CHKA down-regulation in two metastatic breast cancer cell lines (MCF-7 and MDA-MB-231), reduced cell proliferation and induced differentiation (119). The combination of CHKA silencing

and 5-fluorouracil treatment showed synergistic effect on cultured breast cancer cells (120) and its combination with cisplatin and doxorubicin synergized in ovarian cancer (121).

The first compound with antiproliferative activity due to CHKA inhibition was Hemicholinium-3 (HC-3), a low-affinity sodium-independent choline transport and CHK inhibitor (122) that drastically reduces the entry of cells in S phase after growth factor stimulation. In these experiments, addition of exogenous PCHO to culture medium restored G1-S transition (123, 124). However, HC-3 could not be introduced in the clinic due to its lack of specificity for CHKA (125) and respiratory paralyzant toxicity (126). JC Lacal and colleagues developed further compounds based on the structure of HC-3, with up to 1000-fold increased activity compared with HC-3. These compounds showed significant anti-proliferative activity and induction of apoptosis. Some of these first HC-3-derived compounds were JCR89C, JCR1043B, JCR795B, JCR947A, JCR791B, and JCR987B with an *in vitro* IC50 of 4 μM showing a dramatic increase in antiproliferative activity compared to HC-3, which, under the same experimental conditions, had an IC50 of 2500 μM (127).

More compounds were developed from HC-3, reducing the IC50 to the range of 2 μM: MN288b, MN276b, MN352b, MN284b, MN308b, MN356b, MN94b, MN336b, MN58b, MN280b, MN82b, MN90b, MN304b, MN168b, MN332b and FK19. All these compounds reduced intracellular levels of PCHO by 75% and, more importantly, were well tolerated in mice when injected at doses of 5-10 mg/kg for 5 consecutive days. Of all, the compound which demonstrated optimal *in vitro* and *in vivo* antiproliferative activity with better toxicity profile was MN58b (1,4-[4-40-Bis-{[4-(dimethylamine) pyridinium-1-yl] methyl} diphenyl] butane dibromide) (117).

Second generation CHKI were developed from HC-3 showing better *in vivo* and *in vitro* antiproliferative results than MN58b. The best characterized is RSM932A (also named TCD-717), nowadays in Phase I clinical trial for the treatment of solid tumors (http://clinicaltrials.gov/ct2/show/NCT01215864).

Another compound with anti-proliferative effect due to its activity through the Kennedy pathway is Hexadecylphosphocholine (HePC, also known as miltefosine), which is a synthetic alkylphosphocholine that inhibits phosphocholine cytidyltransferase activity by preventing its translocation to the membrane (128).

CHKI induces cytotoxicity by apoptosis through two mechanisms: 1) endoplasmatic reticulum stress that induces the pro-apoptotic transcription factor CHOP (127, 129, 130), and 2) an increase of intracellular levels of ceramide, a pro-apoptotic molecule (127, 129, 130).

4. Pancreatic Ductal Adenocarcinoma (PDAC).

4.1. Epidemiology.

PDAC is the 10th most frequent neoplasm in western countries and the fourth cause of cancer death among men (after lung, prostate and colorectal cancer) and women (after breast, lung and colorectal cancer). During 2014 there were 46,420 new PDAC cases in the United States, with 39,590 deaths (131), and 39,084 PDAC cases with 38,885 deaths in the UE (132). In Spain, it is estimated that 2,400 men and 2,000 women die of PDAC every year (Spanish Society of Clinical Oncology, www.seom.org).

The median age at diagnosis is between 65 and 70 years and the incidence in males is 30% higher than in women (3). PDAC incidence has increased during the 1999-2008 period (133) and its mortality rates have remained largely unchanged for the last five decades (131, 134), despite the recent progress in our understanding of the biology of this cancer. Estimations predict that, by 2030, pancreas cancer will be the second cancer-related cause of death in USA after lung cancer (135) if no major progress in diagnosis and/or therapy occurs.

4.2. Diagnosis.

Unfortunately, we are currently unable to detect PDAC tumors in asymptomatic premalignant or early malignant stages and approximately 80% of patients with PDAC present with locally advanced or metastatic cancer hence, only 20% of patients are candidates for surgery with radical intention (136).

Symptoms depend on tumor localization and stage but generally include abdominal pain, fatigue, anorexia, nausea, and weight loss. Jaundice may occur in patients with tumors located in the head of the pancreas and it reflects the obstruction of the intra-pancreatic portion of the bile duct (3). Based on the known risk factors, a suspicion of PDAC should arise in patients older than 50 in whom a sudden onset of type 2 DM ensues or in diabetic patients who develop unusually severe deterioration of health status.

Once a diagnosis of PDAC has been made, the most important clinical issue is to assess its resectability. Multi-detector row helicoidal CT scan and magnetic resonance (MRI) with intravenous contrast are the optimal techniques for PDAC work-up. They permit to assess the involvement of contiguous structures (superior mesenteric artery, celiac axis, superior mesenteric vein, and portal vein) and distant metastases to establish resectability with 80-90% accuracy (137). Endoscopic ultrasound (EUS) helps to assess lymph nodes and blood vessels and is useful in patients without a mass identifiable on CT. It also helps to characterize proximal and cystic lesions (138) and it is the preferred technique to obtain a biopsy for tissue diagnosis. Although the use of PET-CT scan is not common to diagnose PDAC, in combination with standard CT it improves the sensitivity to detect metastatic disease (139). Endoscopic retrograde cholangiopancreatography (ERCP) shows the pancreatic and bile duct anatomy and can be used to obtain tissue and place a stent in patients with jaundice.

Pathologic diagnosis, using endoscopic Fine Needle Aspiration (FNA) is necessary prior to administration of neoadjuvant therapy and for patients with locally advanced unresectable PDAC or metastatic disease (3).

Macroscopically, PDAC is generally a firm, sclerotic mass with poorly defined boundaries that sometimes contains cystic features. Microscopically, it contains infiltrating ductal glands with - and around - an intense desmoplastic reaction (140). The pathological differential diagnosis includes chronic pancreatitis, tumors of the lower biliary tract, and ampullary tumors. Due to the lack of established markers to identify PDAC, the immunohistochemical analysis of keratins, mucins and CEA can help (141).

The most clinically useful serum biomarker in PDAC is CA 19.9, which has potential uses in diagnosis, staging, determining resectability, as a prognostic marker after resection, and as a predictive marker of response to chemotherapy (142). In symptomatic patients it has a sensitivity of 80% and a specificity of 90% (143). Furthermore, pre-operative CA 19.9 levels correlate with AJCC staging and resectability (144, 145). Finally, it is a prognostic marker after resection and it predicts response to chemotherapy (142, 146, 147) except for patients who are non-secretors (10% of the population) whose tumors do not synthesize CA19.9.

4.3. Staging.

According to the American Joint Comittee on Cancer (AJCC), the staging of PDAC and the stage-specific survival rates (148) are summarized in Table I2.

Depending on the probability of achieving a negative margin after resection (R0), we classify PDAC into resectable, borderline resectable, and unresectable (either locally advanced or metastatic) (Fig. I12). Resectable disease includes stages I and II, we define stage III as locally advanced disease, and stage IV corresponds to metastatic disease.

STAGE	T	N	M	OS (m)
IA	1 (Tumor limited to the pancreas <2cm)	0	0	24.1
IB	2 (Tumor limited to the pancreas > 2cm)	0	0	20.6
IIA	3 (Tumor extends beyond the pancreas without involvement of the celiac axis or the superior mesenteric artery)	0	0	15.4
IIB	1, 2 or 3	1	0	12.7
III	4 (Tumor invades celiac axis or superior mesenteric artery)	0/1	0	10.6
IV	1, 2, 3 or 4.	0/1	1	4.5

Table I2. Clinical staging of PDAC according to AJCC and specific survival rates.

4.4. Treatment.

To define the best treatment, a multi-disciplinary team including surgeons, medical oncologists, and radiologists should evaluate the patient (149). When possible, patients should be referred to high-volume centers.

Current therapeutic options for PDAC patients include surgery, radiotherapy, and chemotherapy. Optimal treatment or combinations depend on tumor stage and patient performance status. Symptomatic care should be always considered specially in unresectable patients.

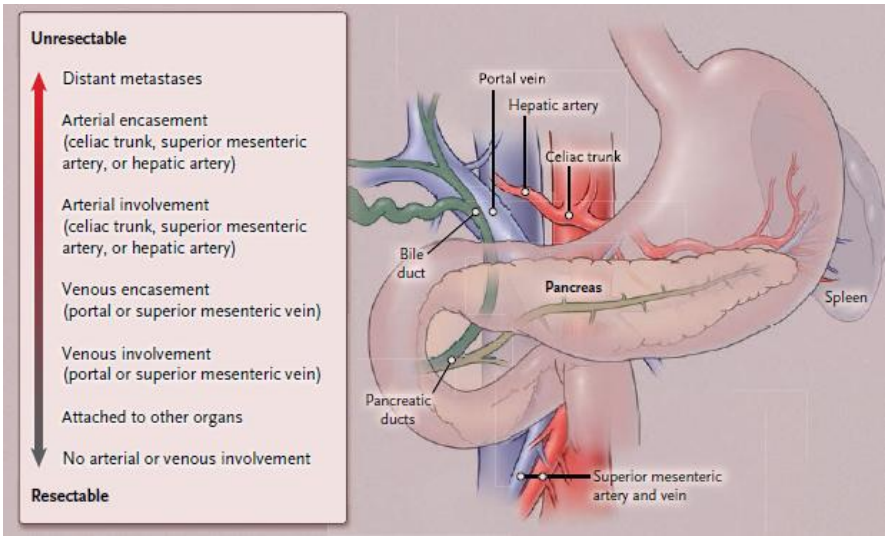


Figure I12. General criteria for PDAC resectability. The pancreas is a retroperitoneal organ in close relation with other organs and vascular structures. Its surgical management depends on the involvement of the surrounding structures. *Adapted from Hidalgo et al.*

4.4.1. Resectable and borderline disease.

For patients with resectable and borderline disease, surgery is the main treatment since it is the only option for long-term survival. Surgery can be accompanied by chemo and/or radiotherapy, either pre- (neoadjuvant) or post-surgery (adjuvant).

- **Surgery:** There is not a single definition for resectable disease and physicians/institutions differ in their consideration of loco-regional involvement. The likelihood of achieving negative surgical margins (R0) is the standard criterion accepted for resectability (150, 151). Tumor is defined as "resectable" when there is a clear fat plane around the celiac axis, hepatic artery, and superior mesenteric artery and no radiologic evidence of superior mesenteric vein or portal vein distortion. To decide whether a patient is candidate for resection, other factors to be considered are: age, co-morbidities, performance status, and frailty. The type of resection depends on tumor location. Distal pancreatectomy and splenectomy is the treatment of choice for tail tumors. When the tumor locates in the body of the pancreas or it involves the pancreas diffusely, guidelines recommend total pancreatectomy. In the case of head tumors, duodenopancreatectomy (Whipple procedure), either open or laparoscopic, should be performed. The goal of surgery is to achieve negative margins (R0). Outcome of patients with vein (portal vein/mesenteric vein) resection and vascular reconstruction are the same as patients without vein invasion (152).

Lymph node involvement constitutes a negative prognostic factor; therefore, a lymphadenectomy including >12-15 nodes should be performed in all patients undergoing surgery. Classical lymph node resection includes nodes at the duodenum and pancreas, the right side of the hepatoduodenal ligament, the right side of the superior mesenteric artery (SMA), and the anterior and posterior pancreatoduodenal lymph nodes (153). In patients with N0, the number of examined lymph nodes is a prognostic factor whereas, in N1 patients, the lymph node ratio (positive nodes/total nodes) is also a prognostic factor (154). There is no clear association between extended lymphadenectomy (including retroperitoneal lymph nodes) and overall survival (155).

Prognostic factors after surgery for PDAC patients include: margins involvement (R0/R1) and distance to margin (</>1 mm), tumor size, tumor grade, nodal involvement, tumor DNA content and pre and post-operative CA 19.9 levels (156-161).

- **Adjuvant therapy** (Table I3): The first evidence of benefit from adjuvant therapy came in 1985; postoperative chemoradiation with 5-FU improved survival at 2 years (42% vs. 15% p< 0.05) (162). Also adjuvant treatment with gemcitabine improved progression-free survival (PFS) (13.4 vs. 6.9 months), overall survival (OS) (22.8 vs. 20.2 months), and 5-year survival (21% and 9%). Gemcitabine and 5-FU yield similar OS rates of 23 and 23.6 months, respectively (163). No single regime has been firmly established as being superior to others. The current options include gemcitabine or fluoropyrimidine-based chemoradiation with additional gemcitabine, continuous infusion 5-FU or 5-FU/leucovorin or chemotherapy alone with gemcitabine and continuous infusion of 5-FU or 5-FU/leucovorin. If the option of only chemotherapy is chosen, guidelines recommend gemcitabine over 5-FU/leucovorin because of its favorable toxic profile. Finally, capecitabine - an oral fluropyrimidine - should be considered when other options are unsuitable because of patient´s performance status (NCCN guidelines, 2016).

  The role of adjuvant radiotherapy is highly controversial. It is commonly used in the US but less frequently in Europe. Some meta-analyses found the same disease-free survival (DSF) for adjuvant chemotherapy and radiation (164). A recent trial pointed at the harmful effect of adding radiation to chemotherapy after surgery (165).

Study	Patients	Treatment	Survival	P
GITSG	43	• Observation • 5-FU+ RT	10% at 2 yr 20% at 2 yr	0.007
EORTC	218	• Observation • 5-FU+ RT	26% at 2 yr 34% at 2 yr	0.1
ESPAC-1	289	• Observation • Chemoradiotherapy • 5-FU • Chemoradiotherapy+ 5FU	16.9 m 13.9 m 21.6 m 19.9 m	
CONKO-01	368	• Observation • Gemcitabine	10.4% at 5 yr 20.7% at 5 yr	0.001
ESPAC 3	1088	• 5-FU • Gemcitabine	23 m 23.6 m	0.39
RTOG 9704	451	• 5-FU+ RT • Gemcitabine+ RT	22% at 5 yr 18% at 5 yr	0.12
JASPAC-01	378	• S-1(oral fluoropyrimidne) • Gemcitabine	70% at 2 yr 53% at 2 yr	<0.001

Table I3. Main clinical trials of adjuvant chemotherapy in PDAC published.

- **Neoadjuvant therapy:** Preoperative chemotherapy or chemoradiotherapy is currently under investigation to improve overall survival in patients with borderline tumors (166). The aim of neoadjuvant therapy is to reduce tumor size and allow an R0 resection. It may also help to "test" the responsiveness of the tumor and contribute to reduce/eliminate potential micro-metastatic burden. Histologic confirmation is required before administering neoadjuvant treatment and FNA through EUS is the preferred method.

  Both, chemoradiation or chemotherapy alone are valid options. The regimens used are the same applied to patients with locally advanced disease: continuous 5-FU infusion, capecitabine or gemcitabine in combination with radiotherapy, or induction 5-FU or gemcitabine followed by chemoradiation (167, 168). For resectable lesions, neoadjuvant treatment may be considered, although it is only recommended in clinical trials or in patients with initial adverse features (NCCN guidelines, 2016). Upon restaging after therapy, 25% of patients show disease progression and - therefore - are excluded from surgery. The median survival for patients operated after neoadjuvant treatment is 21 months and, at 14 months, 32% of patients were alive (169).

4.4.2. Recurrent disease.

Unfortunately, approximately 80% of patients operated with curative will intent relapse. The treatment decision depends on performance status: for fit patients (ECOG 0-2), we should consider the enrollment in clinical trials as first option after a second biopsy. In case of local recurrence, chemoradiation can be considered - if not administered earlier - and in case of distant recurrence the time to relapse (interval from treatment ending to recurrence diagnose) is important: if it is shorter than 6 months, the previous regimen can re-administrated and, if longer, a new chemotherapeutic agent should be chosen. In general, gemcitabine-based regimens are preferred after the administration of a fluoropyrimidine (CapeOx or 5-FU/leucovorin/oxaliplatin) (170, 171). Unfit patients (ECOG 3-4) should be offered best supportive care (BSC).

4.4.3. Locally advanced and metastatic disease.

Palliation and improving survival are the main goals. Systemic therapy is the treatment of choice in these patients but PS is a critical factor to decide whether to give or not chemotherapy and to select the most appropriate regimen.

For fit patients, possible chemotherapy regimens are:

- *Gemcitabine alone*. It is the preferred first-line treatment for metastatic disease in patients with bad PS (172).
- Gemcitabine combinations:
    - *Gemcitabine plus nab-paclitaxel*. The addition of nab-paclitaxel - an albumin-bound nanoparticle form of paclitaxel - to gemcitabine as first-line treatment for metastatic PDAC increases overall survival by 1.8 months (8.5 vs. 6.7 months, $p<0.001$) (173). It also improves significantly response rate, PFS, and 1- and 2-year survival. The most common adverse effects attributable to nab-paclitaxel are fatigue, neuropathy, and neutropenia.
    - *Gemcitabine plus erlotinib.* The addition of erlotinib, an EGFR antibody, to gemcitabine only slightly increases overall survival and 1-year survival (6.24 months and 23% vs. 5.91 months and 17%, respectively; $p< 0.05$) (174). This combination is not used extensively because of its very limited improvement in therapeutic activity.

- *Gemcitabine plus cisplatin.* Although cisplatin treatment does not benefit the general PDAC population, there is a subgroup of patients - those who have a tumor with a defect in homologous recombination repair - who may benefit from addition of platinum drugs to gemcitabine. In this context the survival appears to be significantly increased (22.9 months vs. 6.3 months, p<0.001). (Oliver GR, ASCO abstracts, 2010).

- *Gemcitabine plus capecitabine.* It has shown improved response rates and PFS compared to gemcitabine alone and it constitutes a reasonable option for good performance patients (175).

- *Gemcitabine plus docetaxel and capecitabine (GTX).* In good performance patients, this combination offers a response rate of 29% with 31% of patients showing stable disease; the OS is 11.2 months (176).

- *5-fluorouracil + irinotecan + oxaliplatin (FOLFIRINOX)*. This combination is the most active reported in PDAC, with a major improvement in OS (11.1 months vs. 6.8 months, p<0.001) and PFS (6.4 vs. 3.3 months, p< 0.001) compared to gemcitabine (177). However, FOLFIRINOX has a remarkably high rate of grade 3 and 4 adverse events (45.7% neutropenia). FOLFIRINOX, together with gemcitabine-nab-paclitaxel, is the treatment of choice for fit patients (ECOG 0-1).

TRIAL	PATIENTS	TREATMENT	OS (m)	P
Burris et al	126	• 5-FU	4.4	0.002
		• Gemcitabine	5.6	
NCIC	569	• Gemcitabine	5.9	0.04
		• Gemcitabine+erlotinib	6.2	
Ueno et al	834	• Gemcitabine	8.8	<0.001
		• S-1	9.7	
Conroy et al	342	• Gemcitabine	6.8	<0.001
		• FOLFIRINOX	11.1	
Von Hoff et al	861	• Gemcitabine	6.7	<0.001
		• Gemcitabine+nab-paclitaxel	8.5	

Table I4. Key clinical trials in metastatic pancreatic cancer.

5. Mechanisms of resistance to chemotherapy: the ABC family.

5.1 Resistance and cancer.

Resistance to chemotherapy is one of the major limitations in cancer therapy and is an important cause of tumor progression and poor clinical outcome. There are two types of resistance; intrinsic/primary and extrinsic/acquired. Intrinsic resistance occurs because of the presence of resistance-mediated factors prior to the treatment, whereas extrinsic/acquired resistance arises during treatment because of secondary events. In general, the mechanisms involved in primary resistance are less well- established. There are at least seven well-defined mechanisms of acquired resistance in cancer: deregulation of specific drug target, enhanced drug metabolism, enhanced cellular repair mechanisms, reduced drug uptake, enhanced drug efflux, drug compartmentalization, and activation of alternative pathways or inactivation of downstream cell death signaling pathways (Fig. I13)(178). For targeted therapies, a wider variety of mechanisms of resistance have been recently identified including the activation of feedback signaling pathways and selection of mutant, drug-resistant clones.

Up-regulation of drug efflux ATP-binding cassette (ABC) transporters is one of the most common and best- studied mechanisms of drug resistance. ABC transporters efflux drugs outside the cell and reduce intracellular concentrations.

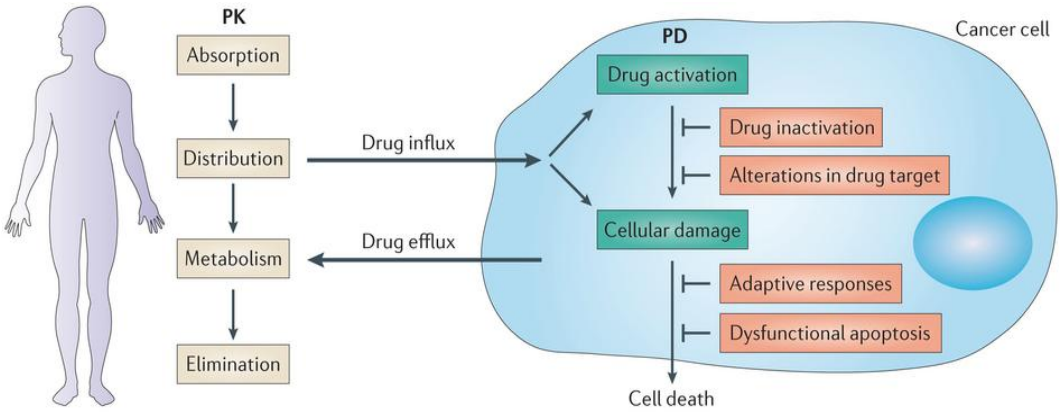


Figure I13. General principles of drug resistance. Phamacokinetic (PK) factors such as absorption, distribution, metabolism or elimination determine the amount of drug that reaches the cell, while pharmacodynamic (PD) factors are responsible of the effects of the drug once in the cell. *Adapted from Holohan et al.*

5. 2. The ABC family: function and structure.

Initially reported in 1973 because of its role in daunomycin-resistance, the ABC superfamily includes 49 proteins, subdivided in 7 families (A-G) according to their sequence homology and domain organization (179). The ABC proteins bind ATP and use its energy to transport a wide variety of molecules across plasma membranes as well as intracellular membranes of the peroxisome, mitochondria and endoplasmatic reticulum (ER). Structurally, they are composed by two ATP-binding domains, also known as nucleotide binding folds (NBF), and two sets of transmembrane (TM) domains typically containing six membrane-spanning -helices (180).

ABC proteins are constitutively expressed at low levels in almost all tissues but they are found at much higher levels on the surface of epithelial cells with excretory roles, such as those lining the kidney, colon, small intestine and pancreatic ducts, among others (181). Their function and role depend on their localization (Table I5).

FAMILY	MEMBERS	LOCALIZATION	FUNCTION
ABCA	12	Brain, lung, rod receptors, muscle, heart, liver, testis, spleen, thymus, ovary, stomach	* Regulation of cholesterol and phospholipids homeostasis. * N-retinylidiene-PE efflux. * Drug resistance.
ABCB	11	Endoplasmatic reticulum and mitochondria of cells. Tissues with barrier function.	* Drug resistance * Transport of peptides, PC, iron and bile salts
ABCC	13	Lung, testes, liver, intestine, prostate, kidney, pancreas, heart, muscle.	* Drug resistance. * Organic anion efflux. * Nucleoside transport
ABCD	4	Peroxisomes.	* Peroxisomal import of FA and Acyl-CoA.
ABCE	1	Ovary, testes, spleen	* Protein synthesis and inflammatory processes.
ABCF	3	Ubiquitous	* Unkown
ABCG	5	Liver, intestine, placenta	* Cholesterol and sterols efflux. * Drug resistance

Table I5. The ABC family; proteins, localization and function.

Among all ABC proteins, three have been related with multidrug resistance (MDR) in cancer: MDR protein 1 (MDR1), also known as P-Glycoprotein (Pgp) or ABCB1; MDR-associated protein 1 (MRP1) or ABCC1, and breast cancer resistance protein (BCRP) or ABCG2.

5.2.1. ABCB-1.

ABCB-1, also known as multidrug resistance protein 1 (MDR1) or permeability glycoprotein 1 (Pgp1) was discovered as a protein overexpressed in drug-resistant cell lines. *ABCB-1* is located on 7q21 and encodes for a transmembrane glycoprotein of 1280 Aa that is expressed mainly in tissues with barrier functions such as the blood-brain barrier, liver, kidney, placenta and colon (180).

The protein contains two regions, each one with 6 transmembrane domains followed by a large cytoplasmic domain with an ATP-binding site (Fig. I14). The transmembrane regions bind neutral or positively charged hydrophobic drug substrates that are presented to the transporter directly from the lipid bilayer. Substrate binding to the transmembrane regions stimulates the ATPase activity - two ATP hydrolysis events occur - causing a conformational change that leads to the efflux of the substrate to the extracellular space (182). ABCB-1 has a broad substrate specificity and transports across the membrane several types of compounds such as lipids, steroids, xenobiotics, peptides or billirrubin, and diverse drugs such as digoxin, chemotherapeutics (etoposide, doxorrubicin, vinblastine), colchicine, tacrolimus, glucocorticoids or HIV antiretroviral therapy among others (180).

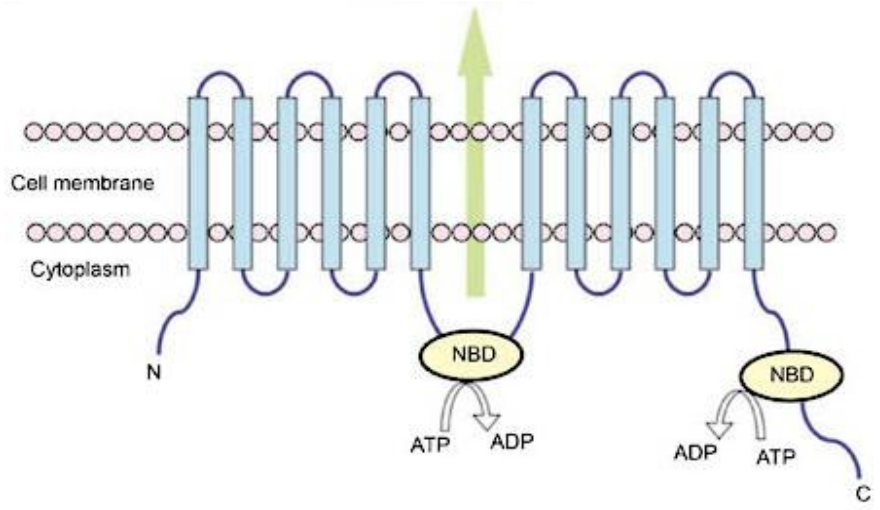


Figure I14. PGP-1 structure. ABCB1 is a full-length transporter of two identical halves as shown; with two nucleotide binding domains (NBD) that contain conserved sequences of the ABC. *Adapted from Dean et al.*

5.2.1.1. ABCB-1 and cancer.

ABCB-1 participates both in intrinsic and extrinsic drug resistance. Many tumors with intrinsic drug resistance overexpress ABCB-1; cancer stem cells (CSC) -a cell-subpopulation in the tumor with self-renewal properties- show high levels of MDR

proteins, which are responsible for their chemoresistance, and correlates with poor survival (183, 184). ABCB-1 expression can also be induced/selected by chemotherapy resulting in the acquired development of the MDR phenotype, which is responsible for tumor progression in many cancers, including kidney, colon and liver cancers (185). Recent reports indicate that ABCB-1 is also a substrate of targeted therapies such as erlotinib, imatinib and sunitinib (186).

It has been reported that PDAC tumors with poor prognosis (7.5 vs. 14.1 month) overexpress ABCB-1 due to its intrinsic chemoresistance (187). Also ABCB-1 overexpression is, in part, responsible for the acquired resistance to gemcitabine and etoposide in some PDAC cell lines (188).

5.2.1.2. ABCB-1 inhibitors.

Once the mechanisms involved in MDR were discovered, the identification of drugs that could inhibit the transporters became a priority. Many drugs have since been shown to inhibit ABCB. There are two generations of ABCB-1 inhibitors. First-generation are non-specific drugs that were originally developed for other therapeutic indications and include calcium channel blockers such as verapamil, the immunosuppressive agent cyclosporin, analogues of the antihypertensive drugs reserpine and yohimbine, the neuroleptic trifluoperazine, and antiestrogens such as tamoxifen and toremifene. (189). These drugs cannot be used *in vivo* as their lack of specificity alters the pharmacokinetic profiles and clearance of co-administered chemotherapeutic agents (189).

Second-generation inhibitors possess a higher affinity for Pgp-1 and, among them, are Zosuquidar (also named LY335979), Tariquidar (also named XR9576) and Laniquidar. The first experiments with Zosuquidar, a substituted dibenzosuberane molecule, showed that it blocks ABCB-1-mediated drug resistance at concentrations of 50-100 nM and it restores sensitivity to vinblastine, doxorubicin, etoposide and taxol in leukemia cell lines (190). It also enhanced antitumor efficacy of taxol in a MDR human non-small cell lung carcinoma nude mouse xenograft model (191). Moreover, the administration of Zosuquidar with chemotherapeutic agents (doxorubicin, paclitaxel or etoposide) did not change their pharmacokinetic profile (192). Tariquidar, an anthranilic acid derivative, was also able to reverse *in vitro* the resistance to doxorrubicin, paclitaxel, vincristine and etoposide at doses between 25-80 nM in a panel of human MDR cell lines and it was also effective in mouse xenografts. Again, it had no major effect on phamacokinetics (193, 194).

On the basis of these promising results, clinical trials with specific Pgp1 inhibitors were initiated. Phase I clinical trials in patients with advanced malignancy showed that Zosuquidar could be given safely in combination with doxorubicin with little effect on doxorubicin toxicity and phamacokinetics (195, 196). The same profile was observed when Tariquidar was given in combination with vinorelbine in patients with advanced malignancies (197), or with docetaxel in lung, ovarian and cervical cancer (198).

However, phase II clinical trials using ABCB-1 inhibitors failed. A Phase II study in breast cancer found minimal effects on OS and PFS when zosuquidar was co-administered with docetaxel (199). Also, tariquidar has shown limited activity in combination with an anthracycline or taxanes in a small cohort of women with stage III–IV breast carcinoma (200).

Objectives

The main goal of this work is to describe the role of Choline Kinase Alpha (CHKA) in pancreatic adenocarcinoma (PDAC) and its pharmacological inhibition as therapeutic strategy.

The specific goals were the following:

1. Analyze the expression of CHKA in PDAC cell lines and tumor samples.

2. Describe, in vitro and in vivo, the effects of genetic an pharmacologic inhibition of CHKA.

3. Test the efficacy of MN58b, a selective inhibitor of CHKA, in PDAC cells, alone or in combination with other drugs used for PDAC treatment.

4. Understand the mechanisms leading to acquired resistance to MN58b and the possibilities to reverte it.

Objetivos

El objetivo principal del presente trabajo es describir el papel de la Colina Quinasa Alfa (CHKA) en el adenocarcinoma de páncreas (PDAC) así como el potencial terapéutico de su inhibición farmacológica.

Los objetivos específicos que se plantearon fueron los siguientes:

1. Analizar la expresión de CHKA en líneas celulares y muestras tumorales de PDAC.

2. Describir, in vitro e in vivo, los efectos de la inhibición genética y farmacológica de CHKA.

3. Comprobar la eficacia del inhibidor de CHKA, MN58b, en células de PDAC sólo y en combinación con otros fármacos utilizados en PDAC.

4. Comprender y profundizar en los mecanismos de resistencia al MN58b y en las posibilidades de revertir dicha resistencia.

Materials and methods

1.Cell experiments.

1.1. Cell culture. The following cell lines were used: Suit2 007, Suit2 028, SK-PC-1, T3M4, Bx-Pc-3, Patu-T, Panc-1, IMIM-PC-2, RWP-1, PK-9, SK-PC-3, MZ-PC-4, HPDE and HPNE (PDAC and ductal cell line respectively); J82, RT112 and HT1376 (bladder cancer); HT-29 (colon cancer); SK-BR-3, MDA-MB-231 (breast cancer), and 293FT**.** Cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 1% pyruvate and 1% penicillin/streptomycin under standard conditions**.** All cells were free of Mycoplasma.

1.2. Lentiviral infections. Control non-targeting or CHKA -targeting lentiviral particles were produced in 293FT cells using Sigma Mission plasmids following the manufacturer's instructions. The shRNA sequences were:
Sh-1:
GTACCGGGTGTTACTTGCAGGTACTTTGCTCGAGCAAAGTACCTGCAAGTAACACTTTTTTG.
Sh-2:
GTACCGGGCAGATGAGGTCCTGTAATAACTCGAGTTATTACAGGACCTCATCTGCTTTTTTG.
Sh-3: CCGGGCGATTAGATACTGAAGAATTCTCGAGAATTCTTCAGTATCTAATCGCTTTTT.
Sh-4: CCGGCCAAGAAACAACAGCTCCATTCTCGAGAATGGAGCTGTTGTTTCTTGGTTTTT.
Sh-5: CCGGGCCAAGATTTCATCTATTGAACTCGAGTTCAATAGATGAAATCTTGGCTTTTT.
pPAX2 and pCMV-VSVG packaging plasmids were used to produce the viral envelope. Cells were transfected with JetPrime, following manufacturer instructions. The plasmid ratio used was: 5μg of lentiviral plasmids 4 μg psPAX2 and 1.5 μg of pCMV-VSVG. Virus-containing supernatant was collected 36 h after transfection, 0.45 μm filtered and used to infect the corresponding cells in the presence of hexadimethrine bromide polybrene (5 mg/ml) (Sigma). Two rounds of infection were performed within 24 h, 12 h each; infected cells were selected for 48h in medium containing puromycin (2 mg/ml) (Sigma).

1.3. Generation of resistant cell lines: To generate IMIM-PC-2 MN58b-resistant cell line (IMIM-PC2-R), cells were seeded at 70% confluence in a 10 cm dish and treated with MN58b in standard DMEM supplemented medium (described previously) at an initial dose of 0.1 μM. In parallel IMIM-PC-2 cells without MN58b were grown. Medium was removed every 48 hours and dose of MN58b increased by approximately 20% weekly at each passage of the cells (split 1:3 when confluence was 90%). The final concentration achieved was 8 μM. To generate gemcitabine resistant pancreatic cancer cell lines an intermittent incremental dose approach was used. The starting concentration was 35 nM gemcitabine

(2´-deoxy-2´,2´-difluorocytidine monohydrochloride, Eli Lilly Ltd, Liverpool, UK) in RPMI media with 20% FCS. Media was changed with a constant concentration of gemcitabine every 24 hours. Drug dose was increased in increments of 1.5 - 2 times the previous dose at each passage of the cells (cells were passaged with a split of 1:5 at 80-90% confluence). The final concentration achieved was 250 nM gemcitabine. The resistant cells were authenticated as being the Suit2 cell line by STR profiling (Promega PowerPlex 16 HS).

1.4. Proliferation and viability assays. Cells were seeded in triplicate in 6-well plates (5x 10^4 per well), trypsinized at defined times, and counted in a Neubauer chamber. To determine viability, cells were seeded in 24-well plates (2x10^4 per well). After 24 h, medium was removed and MN58b was added at seven different concentrations (0.1, 0.2, 0.5, 1, 2, 5 and 10 μM). Each treatment was done in triplicate. After 72 h cells were fixed using formaldehyde at a final concentration of 3% during 1 h at room temperature, the medium was removed and wells washed twice with PBS1X. Cells were then incubated for an hour with 0.5% crystal violet in 25% methanol; crystal violet was eluted with 10% acetic acid, and absorbance was measured at OD590 nm (Eppendorf).

1.5. Migration assays. 5x 10^4 resistant/wild-type IMIM-PC-2 and shCHKA/shNt Suit2 028 cells were seeded in 500 μl serum-free media in a modified Boyden chamber assay. Cells were plated in the upper chamber of a noncoated Transwell insert (24-well insert; pore size, 8.0 μm; BD Biosciences) and media supplemented with serum was used as the chemoattractant in the lower chamber. At 24 and 48- hour cells in the upper chamber were removed with a cotton swab and cells which migrated through the pores were fixed and stained with Giemsa.

1.6. Colony-formation assays. Cells were seeded in 6-well plates (5x10^4 per well) and medium was replaced 24 h later with medium containing MN58b at 5 and 10 μM. After 72 h, cells were fixed, washed, and incubated with crystal violet as described before. Crystal violet was removed and plates dried during 24 hours. Representative photographs were taken.

2. Drug assays.

2.1. Apoptosis assays. Cells were seeded in 6-well plates (5x10^5 per well) in the presence of increasing concentrations of MN58b (1, 2, 5 and 10 μM). After 24 and 48 h, cells were washed with PBS 1X, re-suspended in Annexin V binding buffer, and incubated with 1

mg/ml APC-Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) in the dark for 15-20 min at four degrees. DAPI was added for 15 min and cell viability was assessed in a FACS Canto II flow cytometer (BD Biosciences). Results were quantified using FlowJo software (FlowJo version 7.6.1, TreeStar Inc., Ashland, OR, USA).

2.2. Synergy assays. Cells were seeded in 24-well plates ($2x10^4$ per well). After 24 h, medium was removed and drugs (MN58b, gemcitabine, oxaliplatin and 5-fluorouracil) were added for 72 hours, either alone or in combination, at a range of doses according to the previously estimated IC50 for each drug. Viability was determined and the Combination Index (CI) values for the different drug combinations calculated using the Chou and Talalay method with the Calcusyn software (Biosoft, Ferguson, MO, USA). A CI of 0.9-1.1 indicates an additive effect, a CI <0.9 indicates synergy, and a CI >1.1 indicates antagonism.

3. Biochemichal assays.

3.1. Calcein-AM assay. Cells were seeded in black 96-well plates ($20x10^4$ per well). After 24 h, Calcein-AM uptake and conversion to fluorescent calcein was determined using the Invitrogen Calcein-AM assay kit according to the manufacturer's instructions (Invitrogen, Paisley, UK). Fluorescence was determined at 490/520 nm using a luminometer (Pherastar).

3.2. Choline uptake. A pulse-chase *in cell* assay was used to determine uptake of choline. ^{3}H-Choline chloride was added to the culture media for 60 min; the media was then removed and cells washed 3 times with PBS 1X. Cells were lysed and cellular uptake of choline was determined as the amount of ^{3}H present in the lysates using a scintillation counter.

3.3. Choline Kinase activity. Free ^{3}H-Choline was added to the reaction mix (MgCl2 10mM, KCl 100 mM, ATP 500 µM, Tris pH 7.5 100 mM) and its phosphorylation to phosphocholine accounted to the amount of ^{3}H-Cho converted to ^{3}H-PCHO. The use of passive lysis buffer (PLB) ensured enzymatic activity in the lysates before ^{3}H-choline chloride was added. The reaction was stopped at 60 min with methanol/chloroform to effectively initiate the lipid extraction. Phase extraction, using tetraphenylborate, allowed Cho- isolation from the PCHO-containing fraction. The amount of ^{3}H in each was determined using a scintillation counter.

4. Proteins analysis.

4.1.Immunoblotting. Cells were lysed in NP-40 buffer (25 mM Tris-HCl ph 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) supplemented with the complete protease inhibitor cocktail (Roche). After sonication, 20 µg of protein was fractionated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with anti-CHKA mouse monoclonal antibody (Sigma-Aldrich, HPA624153), 1:1000 dilution in 5% skim milk TTBS. After washing, peroxidase-labeled anti-mouse Ig (DAKO) was added. Mouse anti-vinculin (Sigma) served as a loading control. Reactions were developed using enhanced chemiluminiscence (Amersham Biosciences).

5. RNA analysis.

5.1. RT-qPCR. Total cellular RNA was isolated using the GenElute Mammalian Total RNA kit following the manufacturer's instructions (Sigma). To eliminate genomic DNA contamination, samples were treated with DNaseI (Ambion DNA-free kit, Invitrogen). Retro transcription was performed using TaqMan reverse transcription reagents (Applied Biosystems). Quantitative PCR was performed using SYBR-green mastermix in a Prism 7900 HT instrument (Applied Biosystems). The following primers were used: CHKA AAAGAGGGATCCGAACAAGC (Forward) and AGTGACCTCTCTGCGAGAATG (reverse); ABCB-1 CTGTGAAGAGTAGAACATGAAG (Forward) TTGCACCTCTCTTTTATCTG (reverse); ABCB-4: GAGGTCAAAAACAGAGGATTG (Forward) CCTTTTCACTTTCAGTATCCAG (reverse). All reactions were performed in triplicate and expression levels were normalized to individual HPRT values using the ΔΔCt method.

5.2.RNA-seq and bioinformatics analysis. RNA from parental and MN58b-resistant cells was extracted and purified using Trizol. RNA integrity was assayed on an Agilent 2100 Bioanalyzer (range 8.4-10). PolyA+ fractions were purified and randomly fragmented, converted to double stranded cDNA and processed through subsequent enzymatic treatments of end-repair, dA-tailing, and ligation to adapters as in Illumina's "TruSeq Stranded mRNA Sample Preparation Part # 15031047 Rev. D". Adapter-ligated library was completed by 10 cycles of PCR with Illumina PE primers. The resulting purified cDNA library of template molecules was applied to an Illumina flow cell for cluster generation (TruSeq cluster generation kit v5) and sequenced on the Genome Analyzer IIx (GAIIx) with SBS TruSeq v5 reagents following manufacturer's instructions (SingleRead 1x40 bases). Image analysis and per-cycle base calling was performed with Illumina Real Time Analysis software (RTA1.13). Conversion to FASTQ was performed with CASAVA-1.8 (Illumina).

These files contain only reads that passed "chastity" filtering (flagged with a N, for *NOT filtered* in the sequence identifier line). "Chastity" parameter measures signal contamination in raw data and allows flagging unreliable reads. Quality check was done via fastqc (v0.9.4, Babraham Bioinformatics). The raw reads were aligned to the reference genome hg19/GRCh37 with tophat[1] (version 2.0.4) using the following parameters: --bowtie1, --max-multihits 5, --genome-read-mismatches 1, --segment-mismatches 1, --segment-length 19, --splice-mismatches 0, --library-type fr-firststrand. The gene expression levels (Fragments Per Kilobase of exon per Million fragments, FPKMs) were quantified with cufflinks[2] (version 2.0.2), as annotated in Ensembl version GRCh37.65, with the following parameters: -N, --library-type fr-firststrand, -u.

Sample	RIN	Barcode	Lane	Read	Clusters PF (x10^{6})	Reads mapped (x10^{6})	%Align PF
IMIM-PC-2 R-1	8.5	GCCAAT	5	1	16.1	13.9	86
IMIM-PC-2 R-2	8.6	CTTGTA	5	1	16.6	14.1	85
IMIM-PC-2 R-3	8.4	GTGAAA	5	1	16.8	14.8	88
IMIM-PC-2 WT-1	9.7	GCCAAT	4	1	13.2	11.3	85
IMIM-PC-2 WT-2	10.0	CTTGTA	4	1	13.4	11.3	84
IMIM-PC-2 WT-3	9.9	GTGAAA	4	1	25.1	21.8	87

Table 6. Results of analysis of RNA integrity.

5.3.Sample correlation, principal component analysis (PCA), and differential gene expression. The FPKM correlations and PCA clustering of samples were carried out with the R (version 2.14.1) functions cor and prcomp. The 3D plots were carried out with scatterplot3d library. The differential gene expression analysis was performed with the cuffdiff function included in cufflinks[2] (version 2.0.2) with parameters: -N, --library-type fr-firststrand, -u.

6. Immunohistochemistry.

Pancreatic Ductal Adenocarcinoma tissue microarrays (TMA) containing matched duplicate non-malignant and malignant cores from 96 patients treated at the Royal Liverpool University Hospital, Liverpool, UK were manufactured using Standard Operating Procedures conducted to Laboratory GCP. Cores were taken from tumor regions identified by an experienced pancreatic pathologist using haematoxylin and eosin (H&E) stained

sections. Tissue microarrays were prepared with two cores from each block. For all arrays control cores, comprising 3 cores each of colon, kidney, liver, normal pancreas and chronic pancreatitis, were arranged in a fence around the test samples. The tissue samples were collected under ethical committee approval for characterization of tumor markers for chemotherapy from the Liverpool (Adult) Research Ethics Committee (07/H1005/87).
After de-paraffinization and rehydration, endogenous peroxidase was blocked. Sections were incubated in boiling water for 10 min in citrate buffer pH 6. The slides were then incubated at 4 °C overnight with anti-CHK antibody (1:100) (Sigma, HPA024153). After rinsing in PBS 1x, the slides were incubated with a rabbit secondary antibody (DAKO) for 1 h at room temperature. Bound antibody was revealed with DAB and sections were counterstained with hematoxylin. The following variables were analyzed: intensity (scale 0-3) and proportion of cells (0-100%) displaying nuclear and cytoplasmic staining, and stromal staining. H-Score was calculated as the product of intensity and percentage of positive cells and classified as high (upper quartile), medium (two-mid quartiles) and low (lower quartile).

7. In vivo experiments.

7.1. ***In vivo*** **subcutaneous tumorigenic assay**. Suit028 CHKA-silenced (sh3 and sh5) and their non-target counterparts (shNt) cells were grown in 6-8 week old female BALB/c athymic Nu/Nu mice (Charles River or Harlan). Briefly, subcutaneous injections containing $2x10^6$ cells (100 ml in PBS) were performed at the height of the shoulder blades. Tumor growth was monitored over a three weeks period using an electronic calliper and tumor volumes calculated following the formula ($LxW^2x\ 0.5$). Mice were housed in IVC cages and all animal work was performed by licensed investigators in accordance with the United Kingdom Home Office Guidance on the Operation of the Animal (Scientific procedures) Act 1986 and Amended 2012 in line with the EU Directive 2010/63.

8. Statistical analysis.

8.1. CHKA expression in cancer cell lines. Expression values for gene CHKA were obtained from the Cancer Cell Line Encyclopedia (CCLE; http://www.broadinstitute.org/ccle/home). The cell lines were sorted by the expression of the probes included in the microarray (204266_s_at and 204233_s_at).
We adapted the method developed by Subramanian (201) and carried out a pre-ranked tissue enrichment analysis with the PDAC cell lines included in the CCLE. We used the pre-

ranked cell lines by CHKA expression to calculate an enrichment score for the PDAC cell lines. The score reflects how often PDAC cell lines appear at the top or bottom of the ranked data set as described in Subramanian A et al (201).

8.2. CHKA expression in PDAC patients. Expression values for gene CHKA were downloaded from the Gene Expression Omnibus serie GSE15471, probes 204266_s_at and 204233_s_at. Samples were classified in Classical-PDA, Exocrine-like-PDA and QM-PDA as defined previously by Badea L et al (202) . One way ANOVA was carried out to test differences in CHKA expression between PDAC classes and Tukey test was used to test pairwise comparisons. Statistical analysis and boxplots were done with the R (version 2.14.1) functions aov, TukeyHSD and boxplot, p-values < 0.05 were considered significant.

8.3. Survival analysis. The following clinical parameters were evaluated: survival, grade, stage, chemotherapy, gender, year of birth and date of surgery. The Kaplan–Meier method applying the log-rank test was used to estimate the differences in overall survival (OS). Multivariate analyses including age, gender, stage, grade, date of surgery and previous chemotherapy were performed.

Results

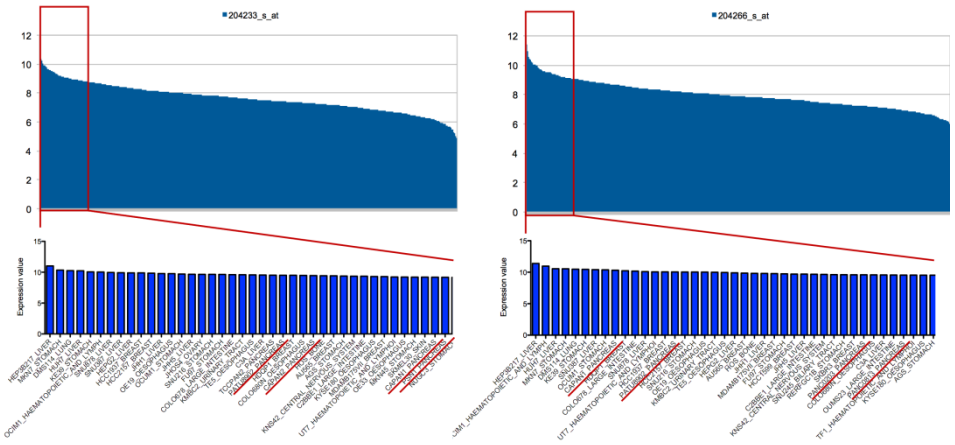
1. PDAC cells overexpress CHKA.

1.1. Identification of CHKA as a gene overexpressed in PDAC.

To obtain an overall view of the expression of CHKA in PDAC, I applied a similar strategy to GSEA (gene set enrichment analysis) with a pre-ranked gene list, but I replaced genes by cell lines obtained from the Cancer Cell Line Encyclopedia (CCLE). I designate this strategy tissue enrichment analysis (TEA). Cell lines derived from PDAC are significantly enriched among those displaying high level of expression of CHKA mRNA (Fig. 1A). This enrichment was significant for both CHKA probes for which information was available through CCLE.

In addition, I assessed whether CHKA expression associates with the three PDAC categories (quasi-mesenchymal (QM), classical and exocrine-like) recently defined by Collisson et al. As shown in Figure 1B, samples in the QM group express lower levels of CHKA mRNA than classical or exocrine-like samples, suggesting a relationship between CHKA expression and cell differentiation.

A



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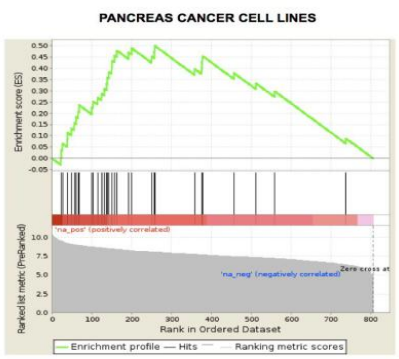
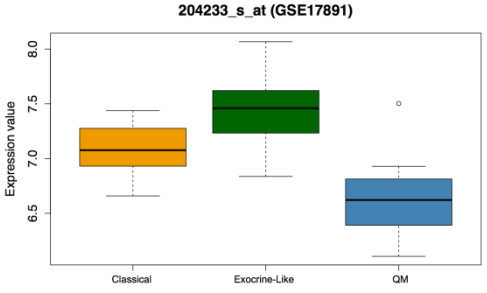


Enrichment Score (ES)	0.498
Normalized Enrichment Score (NES)	3.046
Nominal p-value	0.0
FDR q-value	0.0



	Classical-PDA	Exocrine-Like-PDA	QM-PDA
ANOVA		2,03E-06	

TUKEY MULTIPLE COMPARISONS (p adj)			
Classical			
Exocrine-Like	0,02		
QM	0,005	0,000	

C

	Classical-PDA	Exocrine-Like-PDA	QM-PDA
ANOVA		2.03E-06	
	TUKEY MULTIPLE COMPARISONS (p adj)		
Classical			
Exocrine-Like	0.02		
QM	0.05	0.00	

Figure 1. Bioinformatics analysis of CHKA expression in PDAC cell lines and tissue samples. (A) Bar plot of the cells included in the CCLE ranked by CHKA expression, assessed by 204233_s_at (left) and 204266_s_at (right) probes. A magnification of the 40 cell lines with the highest levels of CHKA is shown in the bottom panels. PDAC cell lines are marked in red. (B). Tissue Enrichment Analysis (TEA) for the PDAC cell lines included in the CCLE. PDAC cell lines are highly represented among the highest CHKA-expressing cell lines (probe 204266_s_at). (C) Box plot showing CHKA expression (probe 204266_s_at) in the three PDAC subgroups defined by Collisson et al (47) and statistical analysis of the comparison in the bottom panel.

1.2. CHKA levels in cells derived from PDAC and other tumor types.

Several reports have addressed the oncogenic properties of CHKA overexpression in many cancers (lung, breast, and bladder among others). CHKA inhibition has demonstrated anti-proliferative effects in different tumor types.

To assess CHKA overexpression in PDAC, I used a panel of 12 PDAC cell lines derived from primary and metastatic tumors. I assessed the levels of CHKA by western blotting followed by densitometry analysis. As a control I used HPDE and hTERT-HPNE cultures from immortalized, non-transformed, pancreatic cells. PDAC cells showed 3- to 11- fold higher levels of CHKA compared with HPDE and HPNE cells (Fig. 2A). This result suggests that CHKA expression correlates with malignant transformation in PDAC.

To determine whether protein levels correlated with gene expression, I measured the levels of CHKA mRNA isoform 1 by RT-qPCR in our panel of PDAC cells. (Fig.2B). This isoform is the predominant transcript expressed in the pancreas (GTEX, www.gtexportal.org/). I failed to observe a good correlation between mRNA and protein levels which suggests that post-transcriptional mechanisms participate in the regulation of CHKA in PDAC.

CHKA is important, in vitro and in vivo, for cell proliferation in bladder, breast and colon cancer (106, 107). Therefore, I compared CHKA levels between PDAC cells and cells derived from these tumors. I observed that PDAC cells (Suit2 028, Suit2 007 and SK-PC-1) expressed similar CHKA levels to other tumor types (Fig. 2C). This result suggests that CHKA might be important to sustain proliferation in PDAC.

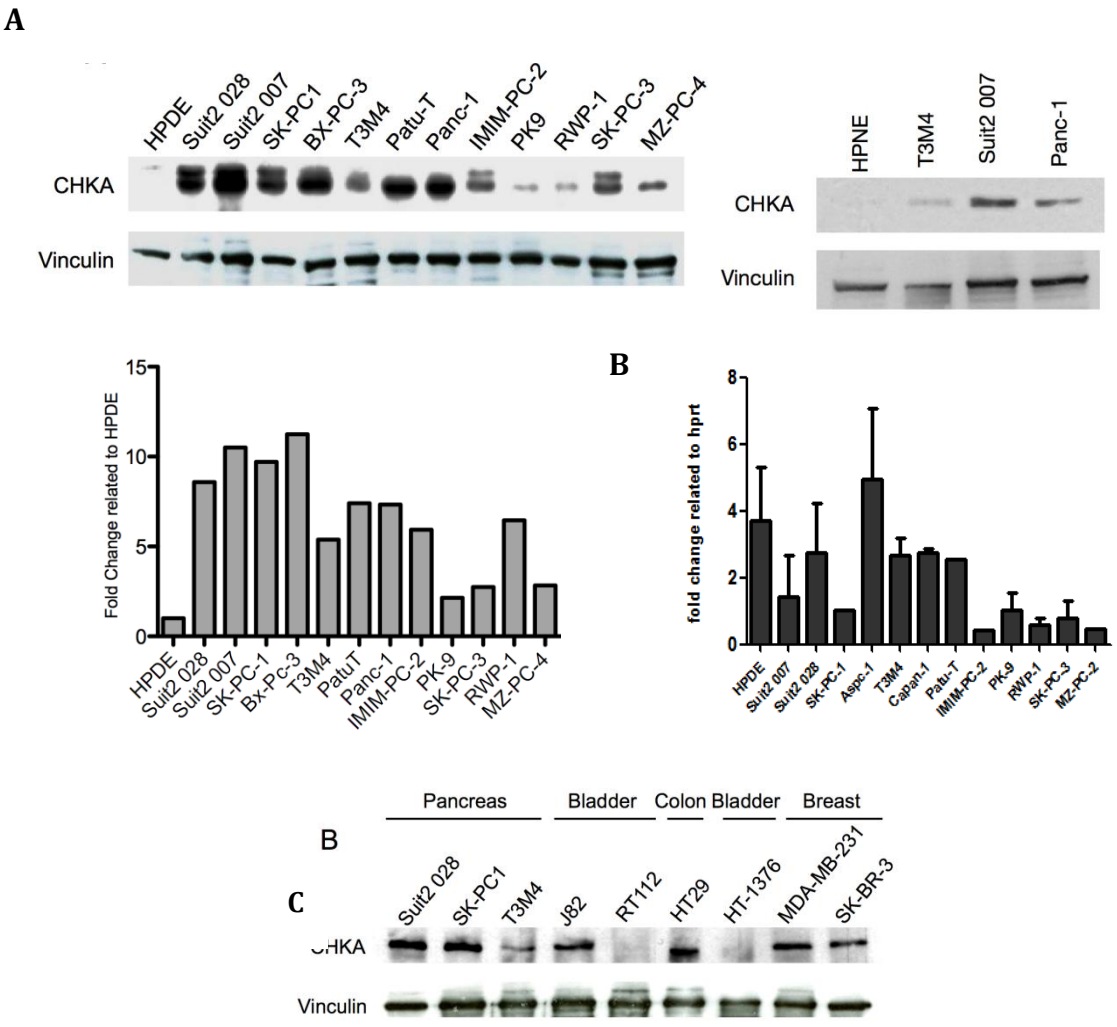


Figure 2. PDAC cells overexpress CHKA. (A). Western blot with antibodies against CHKA shows an increased expression in PDAC cells when compared with HPDE and HPNE, non-transformed pancreatic cell lines. Quantification of protein content reveals a 3- (PK-9) to 11-fold (Bx-Pc-3) difference. (B) CHKA mRNA levels in PDAC cells and their lack of correlation with protein expression in PDAC lines. (C) Comparison of CHKA levels between PDAC (Suit2 028, SK-PC-1, T3M4), bladder (J82, RT112, HT-1376), colon (HT29) and breast (MDA-MB-231, SK-BR-3) cell lines, revealing similar expression in these different tumor types.

1.3. CHKA interference reduces cell proliferation and migration.

Neoplastic cells have an accelerated proliferation rate which requires an increased metabolism to supply energy and structural components. Phosphatidylcholine, the most abundant phospholipid in eukaryotic membranes, is synthesized through the Kennedy pathway in which CHKA participates. Another main feature of neoplastic cells is the ability to migrate and reach distant organs where they form metastases.

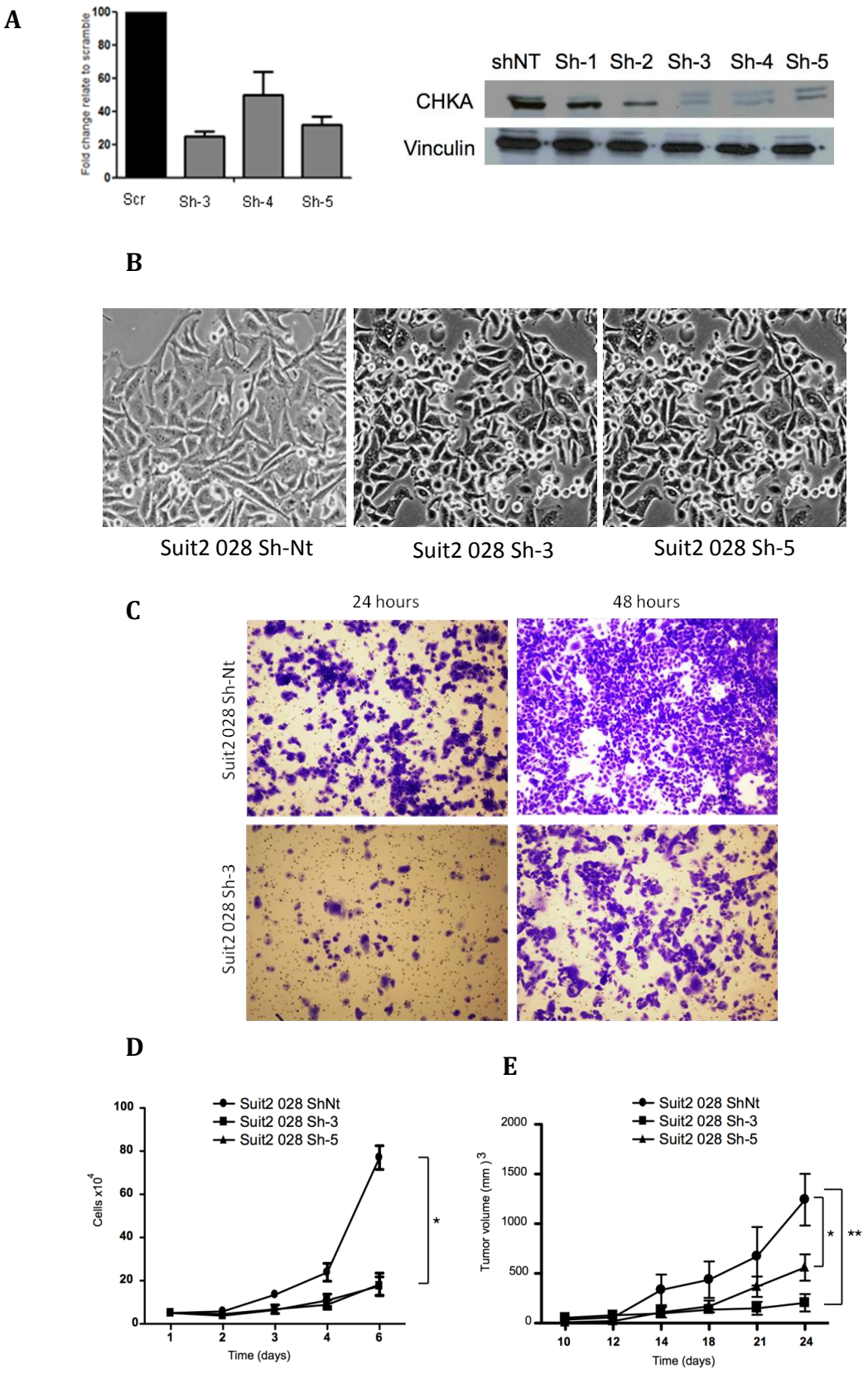


Figure 3. CHKA is necessary for cell proliferation. (A) Infection of Suit2 028 PDAC cells with lentiviruses coding for short-hairpin RNAs (shRNA) against CHKA decreases RNA and protein expression. The most effective shRNAs were 3 and 5. (B) Cell morphology is unaffected by CHKA down-regulation. (C) CHKA down-regulation reduces cell migration, (D) cell growth and (E) *in vivo* tumor volume.

CHO metabolism and CHKA have been linked with migration (203). I wanted to test whether CHKA is required to sustain these two properties in PDAC cells.

To assess whether CHKA is important to sustain proliferation of PDAC cells in vitro, I genetically down-regulated CHKA levels in Suit2 028, a pancreatic cancer cell line that expresses high levels of CHKA. I used lentiviral constructs with five different short-hairpin RNAs (shRNA) (named from Sh-1 to Sh-5) and a non-targeted shRNA as a control. Sh-3 and Sh-5 were the most effective, reducing CHKA mRNA and protein levels by up to 80% (Fig. 3A). Both shRNAs reduced CHKA levels without inducing morphological changes (Fig. 3B).

Although cells were viable after CHKA down-regulation, I observed a reduced growth and migration compared with non-targeted counterparts. Proliferation curves showed that, at day 4 after seeding, silenced Suit2 028 population was 45% of control, and at day 6 only 20% (P= 0.01) (Fig. 3D). Migration assays also revealed an important, although not significant, decrease in migration in CHKA silenced Suit2 028 at 24 and 48 hours (Fig 3C). To validate these results in vivo, I implanted control and CHKA-interfered Suit2 028 cells – using two different shRNAs - orthotopically in nude mice and followed tumor growth for three weeks. I observed a reduced tumor volume in silenced tumors compared with the controls at days 10 (P=0.004), 15 and 21 (P=0.01) after injection (Fig. 3E). Hence, CHKA expression is important to sustain cell growth and migration *in vitro* and *in vivo* and supports the importance of CHKA as a therapeutic target.

2. CHKA expression in pancreatic tissues: association with tumor differentiation and survival.

2.1. CHKA antibody specificity.

Based on published data, I selected anti-CHKA antibodies from SIGMA to determine its expression. To validate the antibody specificity using IHC, agarose blocks of formalin-fixed Suit2 028 cells infected with control or CHKA-targeting sh-lentiviruses were made. IHC analysis revealed a clear cytoplasmic staining in control cells that was reduced in interfered cells (Fig. 4). We successfully assessed CHKA antibody specificity by western-blott in silenced Suit2 028 (Fig. 3A). Therefore I be concluded that these antibodies recognize CHKA specifically.

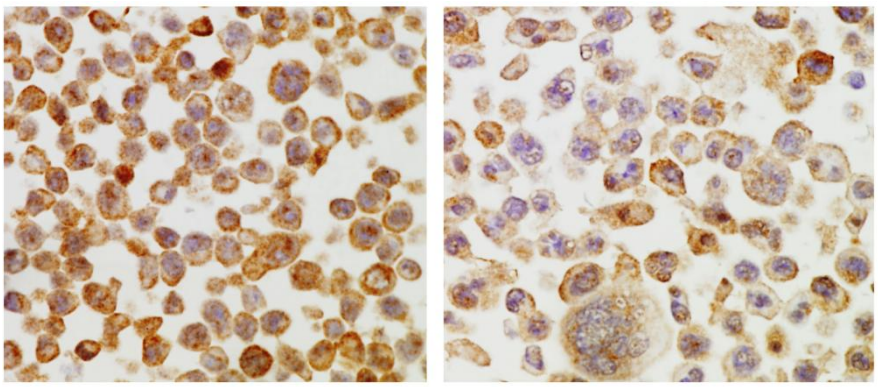


Figure 4. **Specificity of anti-CHKA antibodies.** IHC of silenced and scramble Suit2 028 cells with anti-CHKA anti-rabbit antibodies shows adequate specificity.

2.2. CHKA staining pattern in neoplastic and non-neoplastic pancreatic tissue.

To study CHKA expression in pancreas during malignant transformation I characterized its pattern of expression in normal pancreas, chronic pancreatitis tissues and in characteristic lesions of the various tumor progression steps, i.e. PanIN and invasive PDAC.

I observed that CHKA staining increased according to the degree of malignancy. Normal pancreas showed a weak cytoplasmic staining in acinar and ductal cells, while islets showed a strong cytoplasmic staining with occasional positive nuclei (Fig.5, Panel A). Stromal cells with fibroblast morphology showed sporadic cytoplasmic staining. Chronic pancreatitis samples also showed weak ductal cell staining (Fig.5, Panel B). PanIN lesions showed variable staining from moderate (Fig.5, Panel C) to strong (Fig.5, Panel D) while ductal tumor cells showed strongest staining (Fig.5, Panels E and F).

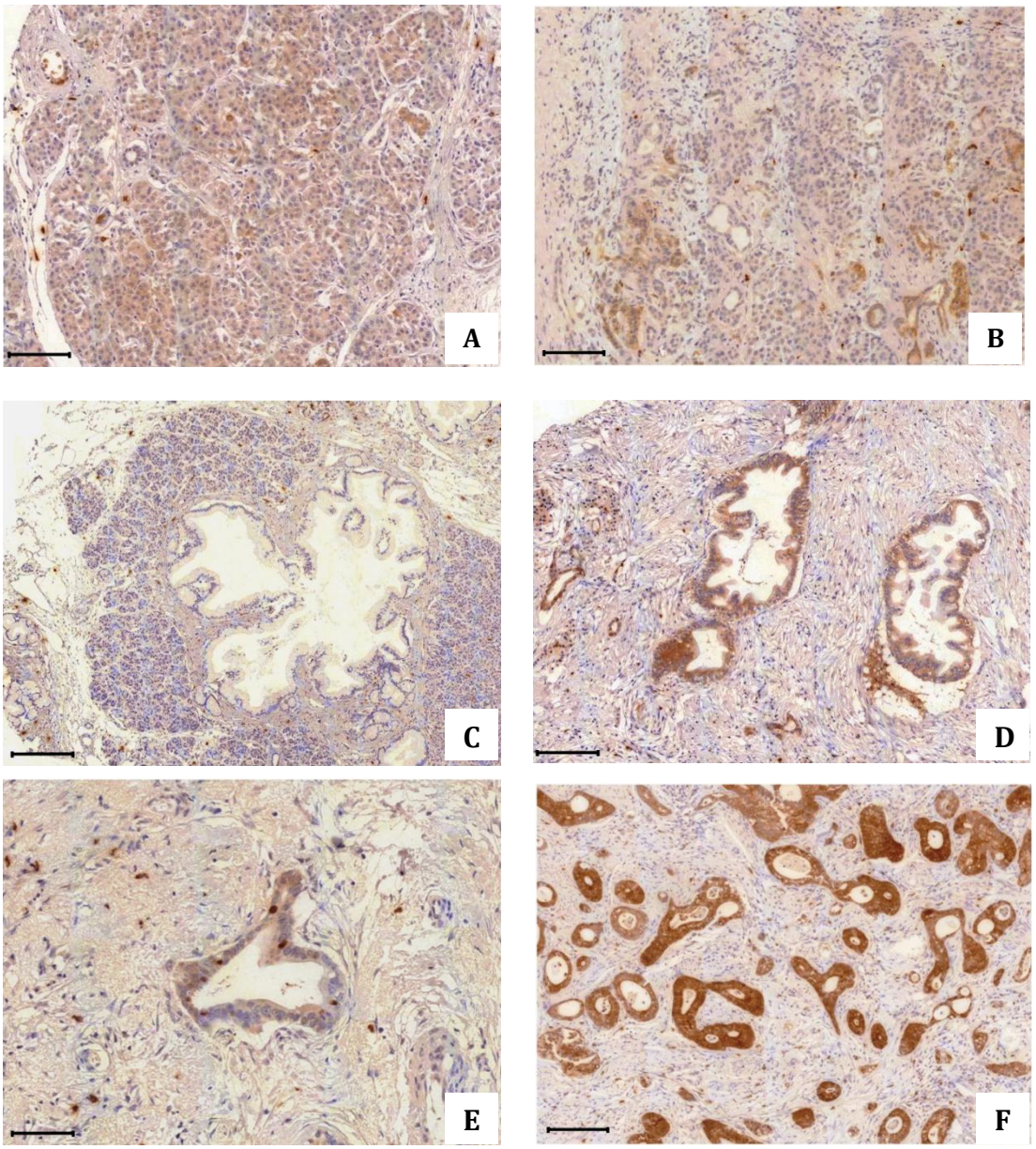


Figure 5. **CHKA expression in pancreatic tissue samples.** (A) Representative image of CHKA immunostaining in normal pancreas. (B) Representative images of staining of chronic pancreatitis samples. (C) and (D) Representative images of low grade (C) and high grade (D) PanIN lesions. (E) and (F) Representative images of CHKA staining in PDAC samples: (E) Representative image of nuclear CHKA staining. (F) Representative image of cytoplasmic CHKA staining.

2.3. Analyses of human tissue microarrays: association of CHKA expression with grade, differentiation and survival.

I analyzed CHKA expression using a human tissue microarray (TMA) containing 98 samples of PDAC, with follow-up information available for 74 patients. I describe patient characteristics in Table 1; most of the tumors were stage 3 with nodal invasion. Nearly all

tumor samples (91%) showed detectable CHKA categorized as high (32%), medium (47%) or low (21%) based on intensity (1 to 3) and percentage of staining (H-Score).

Nuclear staining was present in 43% of samples corresponding with well and moderately differentiated tumors, rather than poorly-differentiated (49% vs. 17%, *P*=0.024). This pattern correlates with improved survival (500 vs. 299 days, *P*=0.014) (Fig. 6A).

	N (%)
Gender	
Male	53 (54)
Female	45 (46)
Stage	
I	2 (2)
II	3 (3)
III	84 (86)
IV	2 (2)
Nodal status	
0	15(15)
1	44 (45)
1a	5 (5)
1b	27 (28)
Chemotherapy	
None	22 (22)
Gemcitabine	26 (27)
5-FU.	17 (17)
Other.	6 (6)

In the multivariable model, tumor grade was an independent variable associated with survival. Patients with moderately and well-differentiated tumors showed a longer survival compared with those with poorly differentiated (432 vs. 284 days, *p*=0.002)(Fig. 6B). If we consider moderately- and well-differentiated tumors as a single group, we found a positive correlation between nuclear CHKA staining and longer survival (540 vs. 303 days, *P*=0.015) (Fig. 6C). Moreover this correlation increased when considering only patients with moderately differentiated tumors (575 vs. 299 days, *P*=0.002) (Fig. 6D).

Table 1. PDAC patient´s characteristics.

In patients with undifferentiated tumors, there was no association between CHKA expression and outcome (262 vs. 253 days, no statistical difference) (Fig. 6E). Regarding the stroma, the small number of cases showing CHKA expression prevented us from establishing an association with survival (Fig. 6F). We can conclude that, in PDAC, CHKA nuclear localization correlates with a differentiated state and better prognosis.

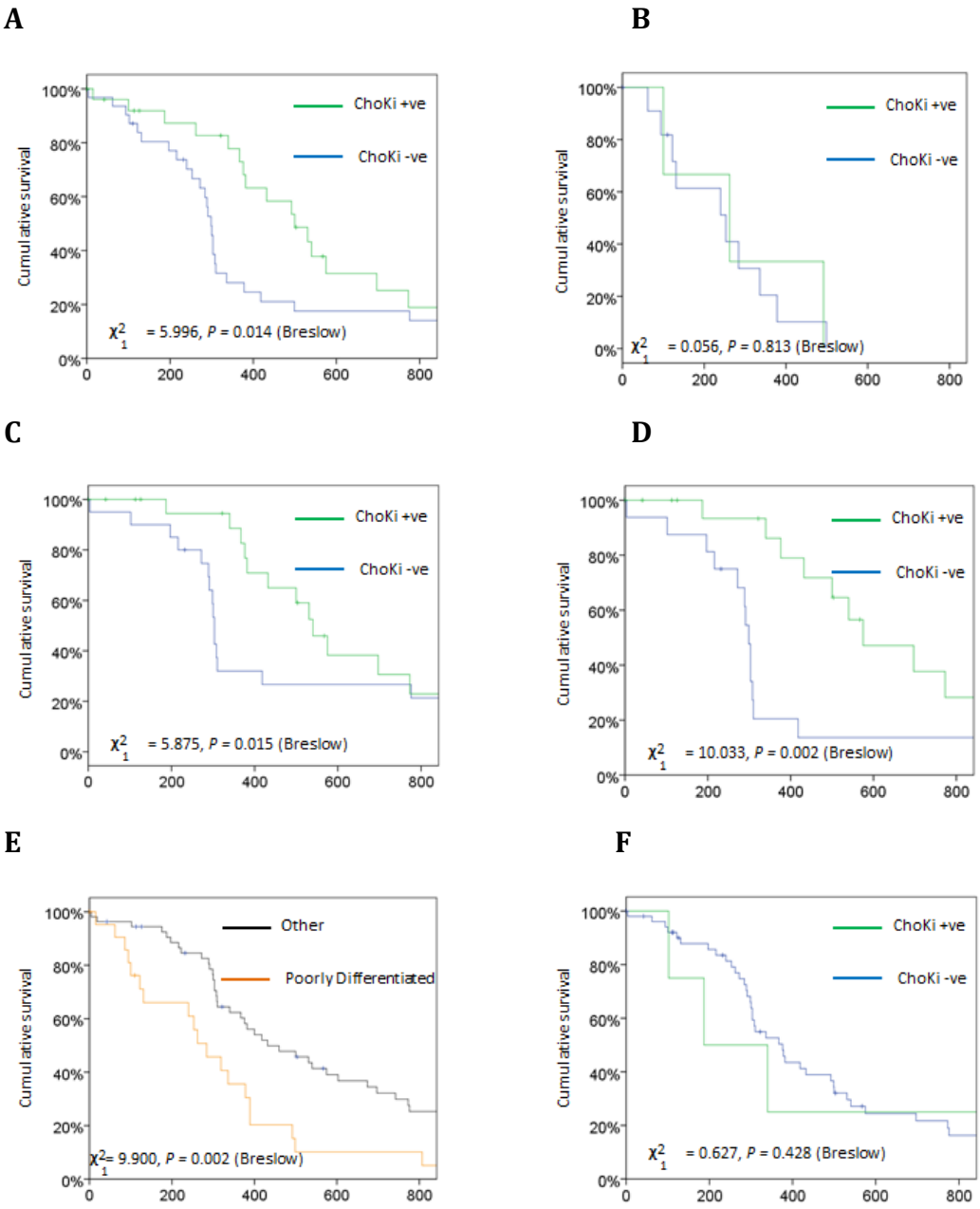


Figure 6. Kaplan-Meier analysis of survival according to CHKA expression. Survival curves showing outcomes in different CHKA staining patterns: (A) Nuclear CHKA in all PDAC samples, in (B) poorly, (C) non-poorly and (C) only in moderately differentiated tumors differentiated tumors. (E) Correlation between tumor grade and survival and (E) between stromal staining and survival.

3. Biological effects of CHKA inhibition, using MN58b, in PDAC cells.

3.1 MN58b has anti-proliferative activity.

MN58b is a first generation CHKA inhibitor with anti-tumoral activity *in vitro* and *in vivo*. CHKA catalyzes the phosphorylation of CHO to PCHO, which is a readout of CHKA activity. I treated IMIM-PC-2 cells with 0.5 µM of MN58b and quantified PCHO levels. The decrease in PCHO levels upon CHKA inhibition shows that CHKA is a target of MN58b (Fig. 7A).

To analyze the effect of MN58b on cell proliferation, I used colony-formation assays using four PDAC cell lines (SK-PC-1, Suit2 028, IMIM-PC-2 and RWP-1) and two different doses of MN58b (1 µM or 5 µM). At 1 µM, MN58b strongly reduced the number of colonies and at 5 µM colony-formation was practically abolished (Fig. 7B).

Viability experiments confirmed the cytotoxic effect of MN58b on nine different PDAC cell lines, with IC50s ranging from 0.23 to 3.2 µM (Table 2). Interestingly, there was a direct correlation between CHKA protein levels and MN58b sensitivity ($R^{2=}$ 0.88); cells with higher CHKA levels were more sensitive to MN58b (Fig. 7C). This suggests that CHKA expression may predict the response to MN58b. To further explore this hypothesis, CHKA levels were genetically modulated in two PDAC cell lines (Suit2 007 and Suit2 028) using two different shRNAs. CHKA down-regulation led to increased resistance to MN58b in interfered cells (Table 3).

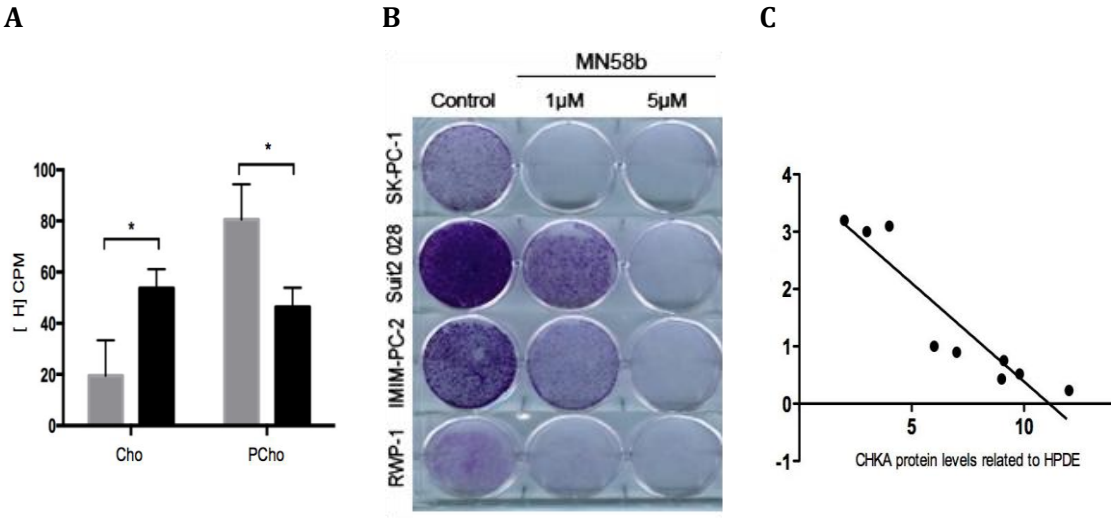


Figure 7. MN58b is cytotoxic for PDAC cells and this effect correlates with CHKA levels. (A) CHKA inhibition with MN58b leads to reduced CHKA activity and PCHO production in IMIM-PC-2. (B) Effect of MN58b on colony formation in four PDAC cell lines (SK-PC-1, Suit2 028, IMIM-PC-2, RWP-1) expressing different CHKA levels. (C) Correlation between CHKA protein levels in PDAC cells and IC50 to MN58b.

Cell line	IC50 (MN58b)
SK-PC-1	0.23
Suit2 028	0.52
Suit2 007	0.43
IMIM-PC-2	0.75
T3M4	0.9
Panc-1	1
PSC	3
RWP-1	3.1
SK-PC-3	3
MZ-PC-4	3.3

Table 2. IC50 of different PDAC cell lines.

MN58b IC50(μM)	CHKA-Nt	CHKA Sh-2
Suit2 028	1.8	7.1
Suit2 007	0.6	1.7

Table 3. Sensitivity of Suit2 028 and Suit2 007 after CHKA silencing.

To compare the sensitivity of PDAC with other tumor types, I used two bladder cancer cell lines (RT112 and MGH-U3). The IC50s obtained for both cell lines (0.42 and 1.2 μM, respectively) were in the range sensitivity of PDAC cells (Fig. 8A).

Targeting the stroma might be relevant from the therapeutic point of view. A reduced stroma may facilitate the uptake of cytotoxic drugs by the tumor. As I mentioned in the introduction, pancreatic stellate cells (PSC) place a major role in determining the nature of the tumoral stroma and participate in PDAC chemo-resistance (48, 49). We tested their sensitivity to MN58b: PSC cells had an IC50 of 3 μM (Fig. 8B). This result indicates that MN58b can also have a cytotoxic effect on PSC at the concentrations at which it is active on some PDAC cells (SK-PC-3 or RWP-1).

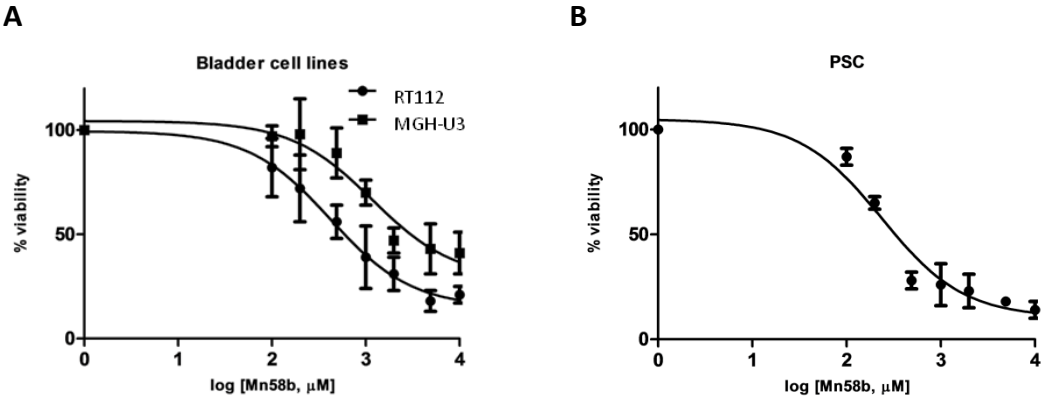


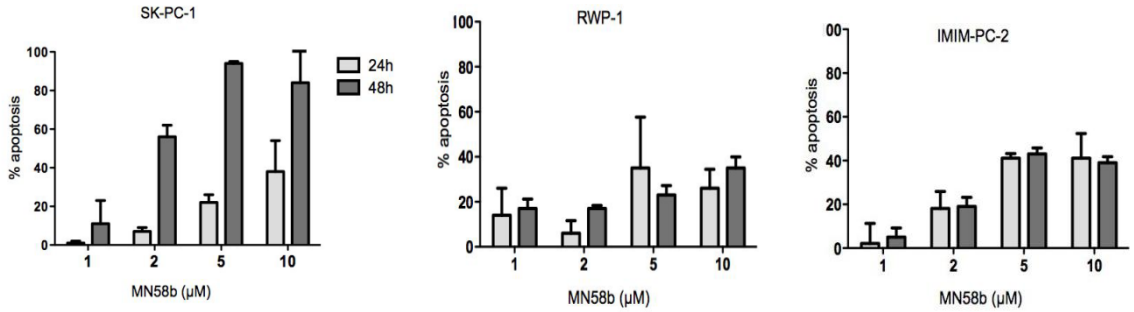
Figure 8. **Antiproliferative effect of MN58b in bladder cancer cell lines and PSC** (A) MN58b dose-response curve in two bladder cell lines (RT112, MGH-U3) and (B) PSC.

3.2. MN58B induces apoptosis and impairs proliferation.

To unravel the mechanism of action of MN58b, I treated three PDAC cell lines with increasing doses of MN58b (from 1 to 10μM) and measured Annexin V expression (an

early apoptosis marker). At 5 µM and 48 hours the proportion of Annexin V-positive cells ranged from 30% (RWP-1) to 90% (SK-PC-1), suggesting that apoptosis is one of the mechanisms of toxicity in response to MN58b. In SK-PC-1 and Suit2 007 cells, I observed a correlation between CHKA expression levels and Annexin V positivity, supporting the idea that CHKA is a predictive marker for MN58b response (Fig. 7A). I confirmed the apoptotic response with a different technique, i.e. measuring the cleavage of Caspase-9 by western blotting: similar results were obtained (Fig. 9B). I conclude that the cytotoxic effect of MN58b in PDAC cells is mainly through apoptosis.

A



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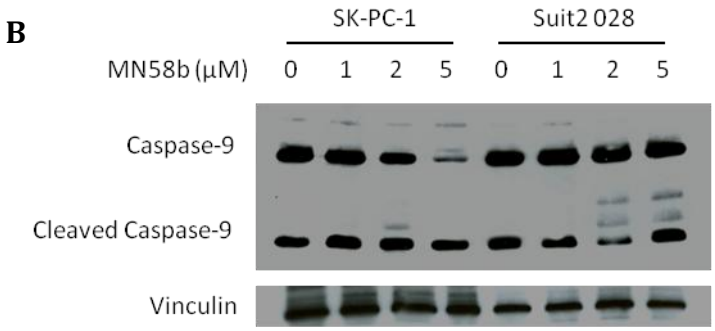
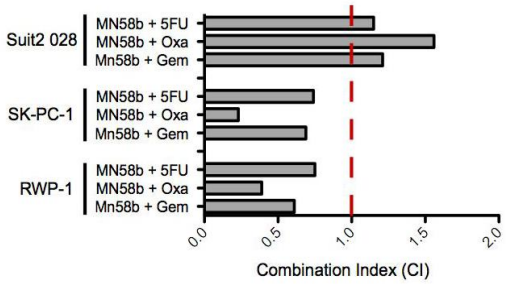


Figure 9. NM58b induces apoptosis in PDAC cells. (A) Expression of Annexin V in three PDAC cell lines upon treatment with increasing concentrations of MN58b. (B) Western blotting against Caspase-9 in two PDAC cell lines treated with increasing concentrations of MN58b.

3.3. MN58b synergizes with several chemotherapeutic agents.

Gemcitabine, a nucleotide analogue, is the gold standard treatment for advanced PDAC. The combination of 5-Fluorouracil and Oxaliplatin has also demonstrated activity against PDAC, but with significant toxicity. I analyzed whether MN58b synergizes with these drugs. For that purpose I treated three PDAC cell lines (SK-PC-1, Suit2 028, and RWP-1) expressing different levels of CHKA with MN58b alone or in combination with either Gemcitabine, Oxaliplatin or 5-Fluorouracil.



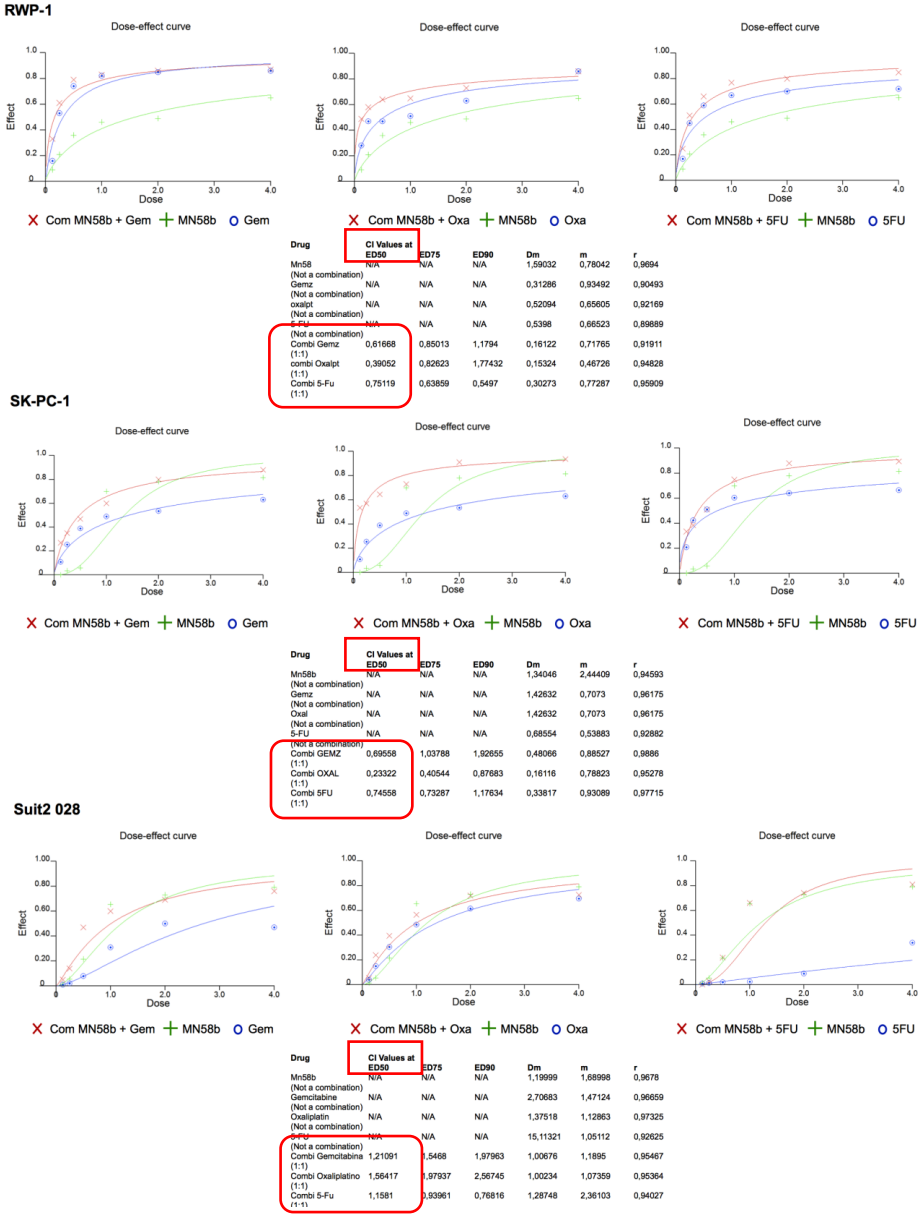


Figure 10. MN58b synergizes with gemcitabine, oxaliplatin and 5-FU in SK-PC-1 and RWP-1. (A). Representative graph showing CI of the different combinations in the three PDAC cell lines. MN58b synergizes with the three drugs in SK-PC-1 and RWP-1 but no effect is seen in Suit2 028. (B). Dose-effect curves of the mentioned combinations.

I measured the synergism calculating the combination index (CI) that determines the effect of each drug separately at a range of doses. In Suit2 028 cells, none of the combinations tested showed increased effects when compared with single agent MN58b. In the other two cell lines MN58b showed synergism in combination with the three drugs, especially in combination with oxaliplatin (SK-PC-1 CI=0.23; RWP-1 CI=0.39) (Fig. 10A and B). These findings support the potential of using MN58b in combination with other chemotherapeutic drugs.

3.4. Gemcitabine-resistant PDAC cells are more sensitive to MN58Bb.

Primary and acquired resistances contribute to the limited efficacy of PDAC treatment. Few therapeutics options remain after Gemcitabine failure. I have demonstrated the synergistic effect of MN58b with Gemcitabine *in vitro*. To assess whether MN58b could be of value in gemcitabine-resistant tumors, I compared the sensitivity to MN58b in parental and Gemcitabine-resistant Suit2 007 cells: the IC50 of both lines were 3.14 μM and 0.77 μM, respectively (Fig. 11), supporting the notion that MN58b could represent a valuable treatment strategy for Gemcitabine-resistant tumors.

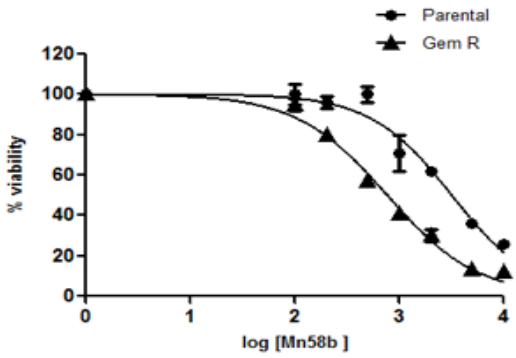


Figure 11. MN58b dose-response curves for parental and gemcitabine-resistant Suit2 007 cells.

3.5. Down-regulation of CHKA sensitizes cells to gemcitabine, oxaliplatin and 5-fluorouracil.

I showed how MN58b synergizes with different chemotherapeutic agents *in vitro* and we wondered whether CHKA inhibition *per se* might sensitize cells to other cytotoxic drugs. To test this hypothesis we silenced CHKA in Suit2 007 cells and treated them with Gemcitabine, Oxaliplatin and 5-FU. We calculated the IC50 of the drugs in non-targeted and CHKA-silenced Suit2 007 cells. The results illustrate that CHKA silencing sensitizes Suit2 007 cells to all the drugs tested (Table 4). Therefore, we can conclude that CHKA levels predict the response to MN58b and other drugs.

N58b IC50 (μM)	CHKA ShNt	CHKA Sh1	CHKA Sh2	pShNt *vs* Sh1	pShNt *vs* Sh2
Gemcitabine	0.78	0.14	0.13	<0.001	<0.001
Oxaliplatin	19	3.8	5.2	<0.001	<0.001
5-FU	40.9	15.5	10	<0.001	<0.001

Table 4. Changes in drug sensitivity to gemcitabine, oxaliplatin and 5-fluorouracil after down-regulation of CHKA using lentiviral shRNAs.

4. Resistance to MN58b is mediated by the up-regulation of the ABCB transporters 1 and 4.

4.1. Generation and characterization of IMIM-PC-2 cells resistant to MN58b.

Acquisition of resistance is a common feature among tumors and the reason for treatment failure and cancer progression. Resistance is classified as primary or acquired. Because of its relevance from the therapeutic point of view, I decided to focus on the possible mechanisms of acquired resistance to MN58b.

To generate a drug-resistant cell line, IMIM-PC-2 cells were treated initially with a low dose of MN58b. Cells were passaged every 72 hours and subcultured in medium containing 20% increasing doses of MN58b. After 9 months of treatment resistant cells (IMIM-PC-2-R) were isolated and exposed to 10 μM of MN58b. An equal number of colonies between control and treated cells (Fig. 12A) was observed and the IC50 of IMIM-PC-2-R cells was 156 μM, 40-fold higher that parental (Fig. 12B).

I characterized resistant cells from a pharmacological and biological standpoint. No differences in choline kinase enzymatic activity (Fig. 12C) were found, however, IMIM-PC2-R cells showed a reduced choline uptake compared with parental cells (Fig. 12D).

Choline metabolism is essential to sustain lipid synthesis and this might impact on cell proliferation. IMIM-PC-2-R cells displayed a low proliferation rate compared with parental cultures and this effect was already detectable at day 4 after seeding (Fig. 12E). Finally, I analyzed migration capacity of parental and resistant cells at different time points. At 24 hours the differences were minimal; by contrast, at 48 hours the resistant cells showed a clear reduction (90%) in their migration capacity compared with the parental cells (Fig. 12F). Acquisition of resistance is thus related with lower choline metabolism, reduced uptake and decreased cell proliferation and migration.

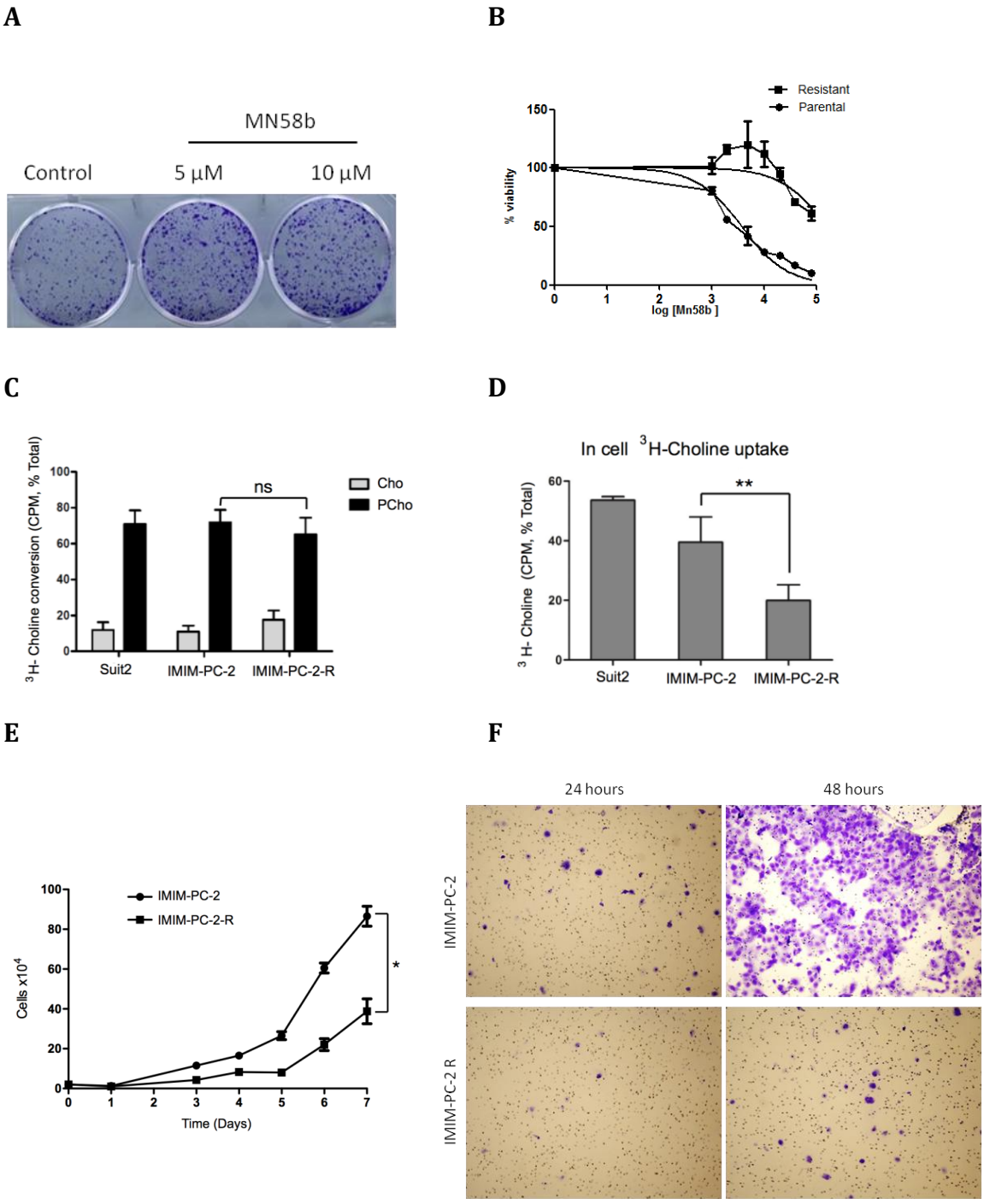


Figure 12. IMIM-PC-2-R cells show a decreased proliferation rate and choline uptake. (A) Colony-formation assay with parental and 5 and 10 µM MN58b resistant IMIM-PC-2. (B) IC50 for parental and resistant IMIM-PC-2. (C) CHO to PCHO conversion rate after resistance acquisition. (D) Comparison of Choline uptake in Suit2 028, parental and and IMIM-PC-2-R cells. (E) Proliferation and (F) cell migration changes in IMIM-PC-2-R.

4.2. Transcriptomic profiling of IMIM-PC-2 resistant cells.

I wanted to explore MN58b resistance mechanisms in PDAC cells. Hence, I extracted RNA from parental and resistant cells and analyzed their transcriptomic profile using next generation sequencing (RNA-seq).

I performed principal component analysis (PCA) and demonstrated that cells cultured in the two conditions (wild-type vs. resistant) grouped independently (Figure 13A). I found a total of 3956 genes statistically deregulated in IMIM-PC-2-R compared with their parental counterparts: 2353 up-regulated (with log2 fold-changes ranging from 8.5 to 0.7) and 1603 down-regulated (with log2 fold-changes ranging from and 9.9 to 0.41). Although GSEA analysis failed to reveal any significantly deregulated pathway, I found that two of the 5 most up-regulated genes in IMIM-PC-2-R - with a log2-fold change of 8.5 and 6.1, respectively- were the ATP-binding cassette (ABC) transporters 1 and 4 (ABCB-1 and ABCB-4), both members of the multi-drug resistant protein family (MDR) that participates in the acquisition of drug resistance in many tumors (Fig 13B).

These results were validated by RT-qPCR and showed an almost 700-fold up-regulation of ABCB-1 and ABCB-4 mRNA in IMIM-PC2-R cells compared with parental cells (Fig. 13C). I confirmed this up-regulation at the protein level by western blotting using a specific antibody (Fig. 13D), although the differences were not as dramatic. Acute treatment of IMIM-PC-2 with MN58b did not produce changes in ABCB-1 or 4 expression (Fig. 13E and F).

These results indicate that the up-regulation of the transporters ABCB-1 and 4 may participate in the mechanism of resistance to MN58b.

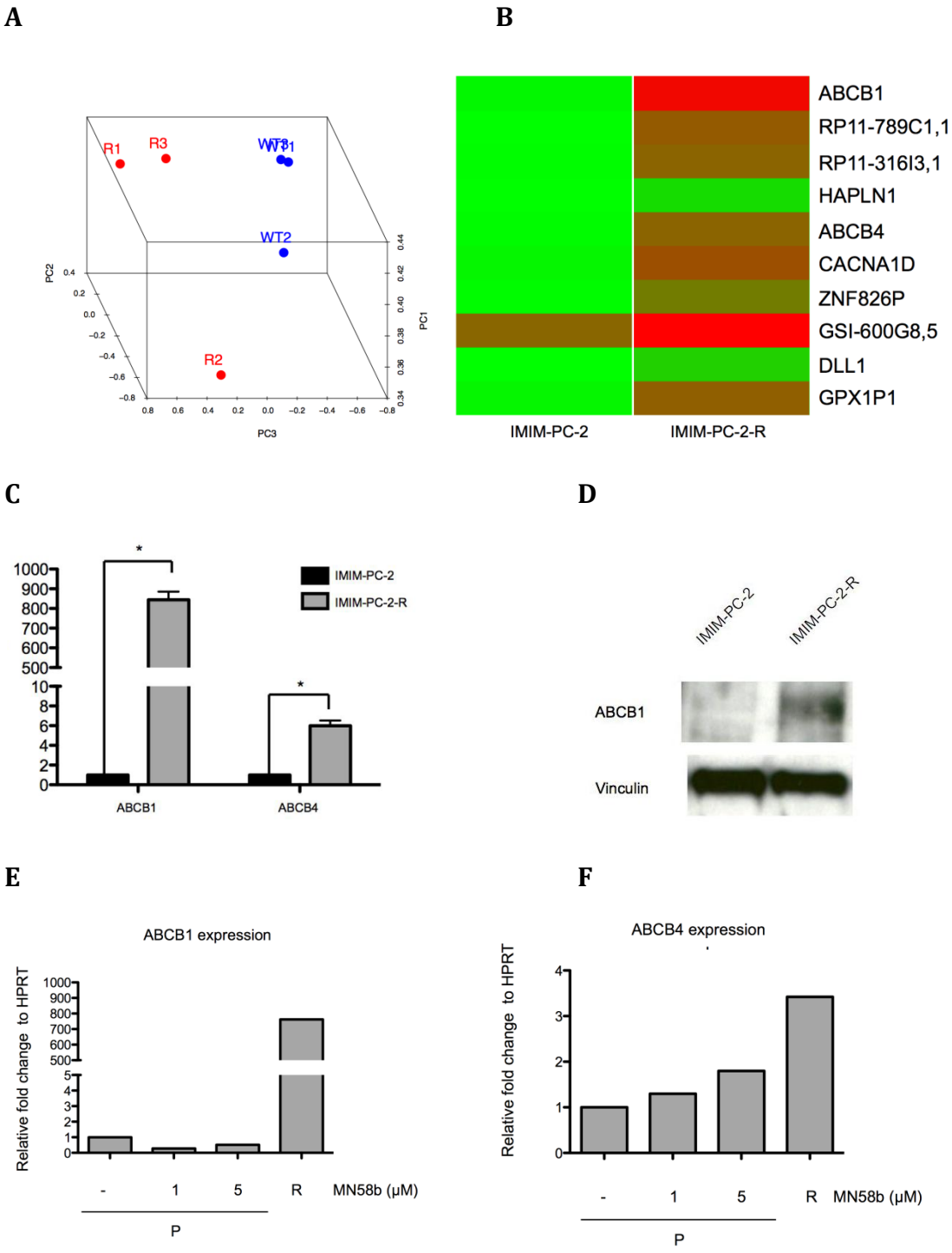


Figure 13. Up-regulation of ABCB transporters 1 and 4 is responsible for acquired resistance to MN58b in IMIM-PC-2-R cells. (A) 3D scatterplot of transcriptome analyses reveals two differentiated clusters corresponding to parental and resistant cells. (B) Among the ten most significantly up-regulated genes in IMIM-PC-2-R cells we find ABCB-1 and ABCB-4, two members of the MDR family. (C) Results from RNA-Seq analyses were confirmed by RT-qPCR and (D) western blotting. (E and F). Effect of acute treatment of IMIM-PC-2 cells with MN58b on the expression of ABCB-1 and ABCB-4 mRNA, assessed by RT-qPCR with IMIM-PC-2-R for comparison.

4.3 Functional activity of the ABCB transporters in IMIM-PC-2 resistant cells

I assessed the functional activity of ABCB-1 and ABCB-4 transporters using calcein-AM. Once incorporated into the cell and metabolized, calcein-AM is converted into the fluorescent dye calcein, detectable by fluorescence microscopy. ABCB transporters extrude calcein and reduce its intracellular concentrations thus informing about transporter activity. Intracellular calcein measurements after calcein-AM treatment were 3-fold lower in IMIM-PC-2-R cells compared with parental counterparts, confirming the functionality of the ABCB transporters in resistant cells (Fig. 14A).

A

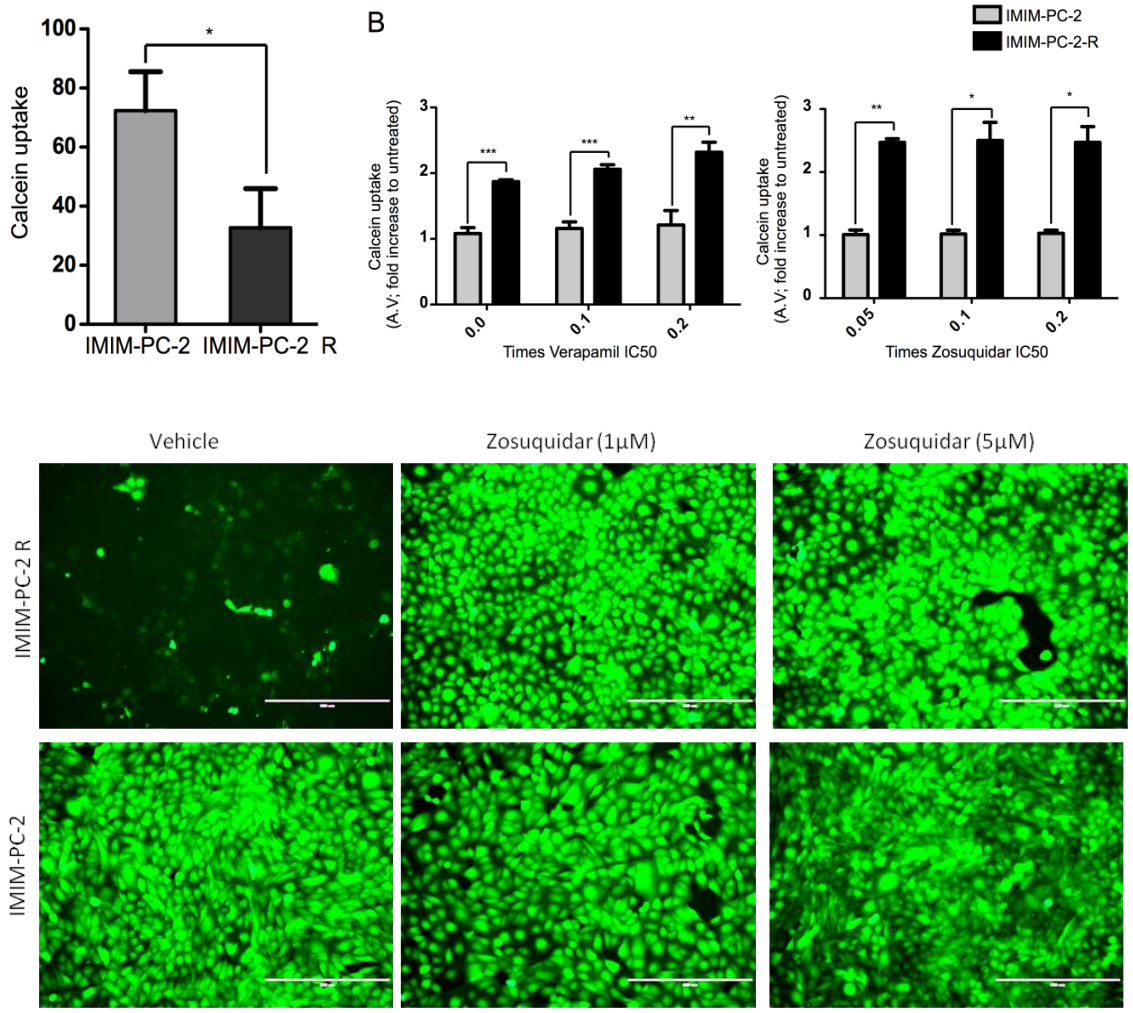


Figure 14. Blockade of ABCB transporters increases intracellular calcein uptake and resensitizes IMIM-PC-2-R cells to MN58b. (A) Calcein uptake is reduced by approximately 50% in IMIM-PC-2-R cells as a consequence of ABCB overexpression. Treatment of IMIM-PC-2-R cells with verapamil and zosuquidar (B), both inhibitors of MDR proteins, increases calcein uptake, as can also be appreciated by inmunofluorescence.

4.4 ABCB inhibition sensitizes R IMIM-PC-2 cells to MN58b.

To examine how ABCB transporters participate in the resistance to MN58b, I inhibited their activity and analyzed whether MN58b-resistant cells become drug sensitive. I used two drugs to block ABCB-1 pumping activity; verapamil, a calcium channel blocker, and Zosuquidar, an ABCB-1 specific inhibitor. Pre-treatment of IMIM-PC-2-R with both drugs increased calcein up-take 2-3 fold (Fig 14 B and C) and sensitized IMIM-PC-2-R to MN58b with IC50 values in the range of parental cells (Table 5). We can conclude that ABCB inhibitors sensitize IMIM-PC2-R cells to MN58b.

IC50 MN58b (μM)	PARENTAL	RESISTANT
Control	2	22
0.1 [IC50 Verap]	2	2.7
0.25 [IC50 Verap]	1.8	2
Control	2.5	53.8
0.1 [IC50 Verap]	2.6	4.6
0.25[IC50 Verap]	3.1	4.7

Table 5. Sensitivity of parental and IMIM-PC-2-R cells to MN58b after verapamil treatment.

Discussion

PDAC remains one of the most lethal neoplasias with a 5 year survival of approximately 5% and its mortality rate has remained unaltered in the last 50 years (131, 134). Therefore, it is essential to find new therapeutic targets and understand the mechanisms implied in drug-resistance. The extensive biological and genetic knowledge gathered in the last few years through genome sequencing studies has -unfortunately- revealed a paucity of direct oncogenic targets. However, the rich biology of PDAC is stimulating extensive research on other aspects such as the role of the stroma, the immune system/inflammation, and metabolic pathways. Increasing attention has been paid during the last decade to metabolic adaptations during malignant transformation (57), mainly regarding glucose and glutamine metabolism (204, 205). Among the latter, there is a metabolic reprogramming of glutamine mediated by KRAS, the hallmark genetic alteration in PDAC (206). Until now, little importance has been given to lipidic metabolism.

In this work, I have focused on the study of choline metabolism. CHO is, through the Kennedy pathway, the main source of PC, the most abundant phospholipid in eukaryotic membranes. During malignant transformation, there is an increase of total choline-containing phospholipid metabolites and a switch from GPC to PC, the precursor of PC (207). CHKA is one of the key enzymes in the Kennedy pathway and it catalyzes the phosphorylation of CHO. CHKA plays an important role in malignant transformation through its oncogenic cooperation with RhoA and it is an important source of second messengers such as DAG. Pharmacological inhibition of CHKA has shown anti-proliferative effect in a variety of transformed cell lines and in vivo models but nothing is known regarding its involvement in pancreatic tumors (107, 124, 127, 208).

1. CHKA is consistently overexpressed in PDAC cell lines and its down-regulation decreases oncogenic properties.

In this work, I show that CHKA is a potentially important target in pancreatic cancer, based on three types of data: 1) the available information in public databases regarding CHKA expression in PDAC; 2) the expression of CHKA in PDAC cell lines and 3) the effects of CHKA inhibition and down-regulation in cultured PDAC cells and *in vivo* models.

I first mined the public databases for evidence of CHKA involvement in PDAC. We found that it is overexpressed in many PDAC cell lines, such as PaTuT and Capan. It is also differentially expressed in the three subtypes defined by Collisson et al (47): classical (adhesion-associated and epithelial genes with KRAS addiction and GATA6 overexpression), exocrine-like (expressing digestive enzyme transcripts) and quasi-mesenchymal (mesenchyme genes). The classical subtype shows the highest expression of

CHKA while the lowest CHKA levels are observed in QM-subtype. This observation is in agreement with the well-known connection between RAS proteins and CHKA through Ral-GDS and PI3K (96).

To get more insight into the importance of CHKA in PDAC, I compared its expression in transformed and non-transformed cultured PDAC cells and also with cultured cells derived from other neoplasias. CHKA protein expression was 3 to 12-fold higher in transformed PDAC cells than in non-transformed ductal cells (HPDE and HPNE) and the levels were similar to those found in bladder, colon and breast cancer cells. This strengthens the role of CHKA in PDAC development and indicates that PDAC cells have increased CHKA levels compared to non-transformed PDAC cells, within the range of those tumors in which CHKA role is relevant in patients, such as bladder, where CHKA expression is associated with tumor size, metastatic spread and poor survival (106) and breast carcinomas, 40% of which have an increased CHKA activity (209).

The fact that mRNA levels were not consistently increased in transformed versus non-transformed PDAC cells, and the absence of correlation between protein and genomic levels, suggest post-transcriptional mechanisms in the regulation of CHKA in PDAC. Alternative splicing is a well-known post-transcriptional modification for CHKA resulting in two isoforms, 1 and 2. In the recent years several studies have reported a low correlation between transcript and protein levels suggesting that post-transcriptional modifications have a much more important role in biological processes than we generally consider, specially with proteins involved in transcriptional processes in which quantifying only mRNA expression might not always suffice to reflect the situation at the protein level (210). Although classically elevated oncogene levels correlates with tumor dedifferentiation and aggressiveness, reports from Collisson et al suggest that increased levels of genomic CHKA lack any association with dedifferentiation; CHKA levels are higher in differentiated (classical and exocrine) than dedifferentiated (QM) PDAC subtypes. Unfortunately they did not compared CHKA levels between PDAC and non-tumorogenic pancreatic tissue.

After I studied whether this enhanced expression of CHKA in PDAC cell lines was related with malignant transformation or was only the result of a high metabolic rate associated with *in vitro* cell proliferation. For that purpose, I tested the effects of CHKA genetic down-regulation on two main properties of malignant cells, proliferation and migration. CHKA inhibition reduced PCHO formation, cell proliferation and cell migration as expected. Also,

tumors generated in xenografts with CHKA-silenced cells were smaller in volume than those of non-silenced cells, suggesting a link between CHKA and tumor aggressiveness.

The connection between some of the major pro-survival pathways (MAPK and PI3K) and the Kennedy pathway has been widely studied as summarized in Fig.D1. CHKA is stimulated by RAS and PI3K through RHO GTPases (96, 211-213), and, on the other hand, it has become clear that CHKA - through the production of the second-messenger PA - is a key activator of MAPK and PI3K/AKT signaling (86, 214). CHKA down-regulation directly decreases cell proliferation by attenuating major survival pathways (MAPK/PI3K), and indirectly by reducing the activity of the remaining CHKA due to the attenuation of RAS and PI3K -direct CHKA activators-. CHKA down-regulation can also affect cell proliferation through the decrease of important cell cycle regulators such as Cyclin A1 (121). While I did not study these mechanisms in PDAC cells, it is likely that they contribute to the effects we have observed.

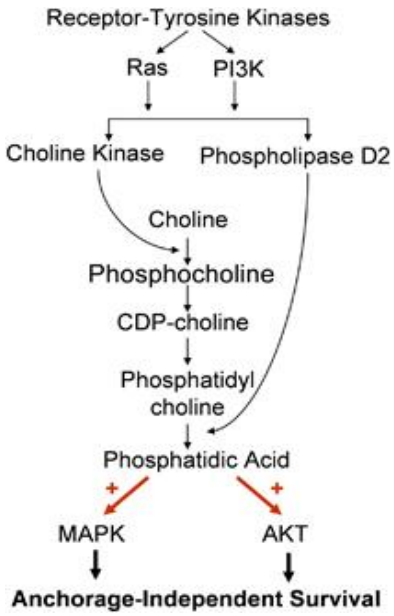


Figure D1. CHKA is required for the activation of the PI3K/AKT and MAPK signaling pathways. PCHO serves as an essential metabolic reservoir for the production of PC, the major phospholipid constituent of membranes and substrate for the production of lipid second messengers. In particular, phosphatidic acid, generated from the cleavage of phosphatidylcholine by the RAS and PI3K target phospholipase D2, has emerged as a key activator of the MAPK and AKT signaling pathways. *Adapted from Yalcin et al.*

Migration was also impaired upon CHKA knock-down, supporting the importance of choline metabolism in this process. Endometrial differential 3 (EID3), the enzyme that hydrolyzes glycerophosphocholine (GPC) to produce gycerol-3-phosphate (G3P) and

choline, is required for cell migration, and its down-regulation decreases migration by disruption of the PKC alpha signaling pathway (115) and down-regulation of the β1 integrin subunit required for basal membrane attachment (203). Stewart et al. described EDI3 up-regulation in primary ovarian and endometrial cancers and its correlation with metastasis and worse prognosis (115). Therefore, it is conceivable that CHKA inhibition might reduce tumor dissemination through down-regulation of proteins related with the epithelial to mesenchymal transition. Also, Granata et al have reported a decrease in migration and invasion after CHKA down-regulation. Their genomic and ingenuity pathways analyses revealed 476 genes and 25 pathways differentially expressed in CHKA silenced cells (121); among the biological functions associated with the most affected networks they found that cell movement, growth and proliferation were decreased contrary to cell death, that was increased. Microarray analysis in breast cancer cells after CHKA down-regulation also detected changes in 33 proliferation-related genes (120). Moreover, CHKA-silenced cells showed reduced oriented actin stress fibres and focal adhesion sites that enables them to migrate.

Genetically CHKA down-regulated PDAC cells are viable, possibly due to incomplete CHKA silencing, which is in accordance with previous reports in ovarian cancer cell lines that, upon 80% silencing by siRNA transfection, showed a 35% reduction of cell proliferation at day 3 (121). These studies failed to observed apoptotic effects, pointing to the differential requirements of CHKA levels to sustain proliferation and to induce apoptosis.

These results suggest that CHKA expression is important for the oncogenic properties of PDAC cells, as in other tumors (215), and therefore can be a reasonable therapeutic target for PDAC. Our results are in accordance with Penet et al who recently described the elevation of PC and tCho in cultured PDAC cells and tumor-derived xenografts (216) due to CHKA, CHT1, and CTL1 overexpression.

2. CHKA expression is enhanced in tumors, where its nuclear localization is a marker of differentiation and prognosis.

After validating the specificity of our antibody using CHKA down-regulated cultured cells with two different shRNAs, we analyzed whether tumors overexpress CHKA and whether CHKA expression associates with clinical-pathological features. Tissue analyses confirmed an enhanced expression of CHKA in PDAC samples in comparison with normal pancreas and chronic pancreatitis; staining revealed prominent cytoplasmic CHKA expression in

>90% of tumor samples which was undetectable in normal pancreas and pancreatitis samples. This result shows for the first time that PDAC samples overexpress CHKA similarly to prostate cancer (110), endometrial cancer (104) and other tumors.

CHKA is generally considered to be a cytoplasmic protein (215). However, we observed that 40% of PDAC samples showed nuclear immunoreactivity with anti-CHKA antibodies. Given the specificity analyses performed, we are confident that these results indeed reflect that CHKA can be localized in the nucleus as well as in the cytoplasm. Moreover, nuclear CHKA expression was correlated with tumor differentiation: 17% of poorly differentiated tumors showed nuclear CHKA versus 49% of moderately- and well-differentiated tumors ($p=0.024$). Contractor et al have also described nuclear CHKA in prostate cancer using immunohistochemistry, although with a lower frequency than us, only in 1 out of 20 samples. They also found that CHKA nuclear expression was more commonly associated with benign prostate tissue than with pre-malignant PIN lesions (110).

Our analysis confirms that, in our case series, tumor differentiation constitutes a prognostic marker. The fact that CHKA nuclear expression correlated with tumor differentiation suggested an association with patient survival. In fact, subjects with PDAC whose tumors showed nuclear CHKA expression had a median overall survival of 500 days compared to 299 days for those lacking nuclear expression. Interestingly, among patients with moderately differentiated tumors, CHKA nuclear expression is a prognostic marker. Therefore, CHKA nuclear expression may allow the distinction of subgroups with distinct recurrence risks among those with moderately differentiated tumors. These results need to be validated in independent series; if so, they might contribute to stratify surgically-resected patients for adjuvant treatment. CHKA up-regulation has already been described as worse outcome prognostic marker in early non-small lung cancer (109).

The mechanisms and biological significance of nuclear CHKA are still poorly understood. CHKA could be phosphorylated and translocated to the nucleus with other proteins, such as ERK. It is appealing to hypothesize that CHKA might have different roles depending on its subcellular location. In the cytoplasm, CHKA could act as an oncoprotein promoting an increased synthesis of PC and second messengers involved in survival pathways. In the nucleus, CHKA could modulate the activity of transcription factors involved in differentiation as is the case of pyruvate kinase. The M2 isoform of pyruvate kinase is present in the cytoplasm as a tetramer that catalyzes the formation of pyruvate from phosphoenolpyruvate while in the nucleus it is active as a dimer and it has protumorigenic

activity through STAT3 phosphorylation (217). As in the case of pyruvate kinase, the differential localization and function of CHKA might be related to its structure and participation in multimolecular complexes. CHK occurs as a homodimeric (α/α, β/β) and heterodimeric (α/β) form. (90). Only the α- isoform is crucial for PC synthesis and is responsible for malignant transformation. It is possible that the α/α dimer is predominantly expressed in the cytoplasm of PDAC tumors, therefore contributing to its malignant potential, whereas the α/β dimer might be predominantly nuclear, having the opposite role. Further experiments using specific CHKA antibodies, subcellular fractionation, and proteomics should contribute to solve this question.

3. The antitumor effects of MN58b on PDAC cells.

For pharmacological inhibition, we chose MN58b - a specific CHKA inhibitor whose selectivity has recently been shown in vivo by magnetic resonance spectroscopy (218) and which is also specific to CHKA in PDAC cells, as we have shown by the reduction of PCHO formation upon MN58b treatment. MN58b anti-neoplastic activity in vivo is due mainly to two main mechanisms: the intracellular increase of ceramide, a pro-apoptotic molecule (219), and an exacerbated endoplasmatic reticulum stress response through CHOP proteins (129) (Fig. D2).

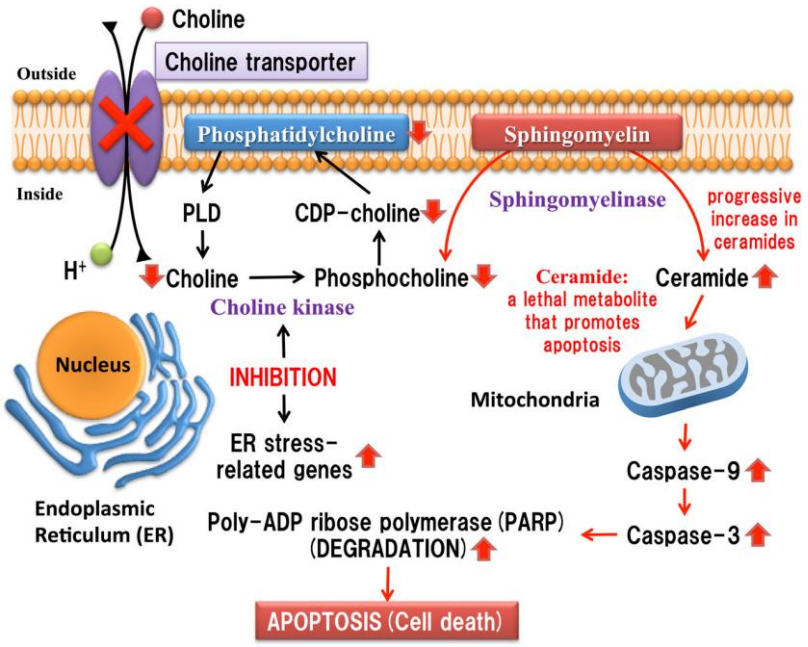


Figure D2. Schematic illustration of the mechanisms causing cell death by MN58b.

We showed that induction of apoptosis -measured by increased caspase 9 activation and Annexin V staining- in PDAC cells treated with MN58b explains its cytotoxic effect. We found a direct association between CHKA levels and drug sensitivity: PDAC cell lines with higher CHKA levels were the most sensitive to MN58b, suggesting a role of CHKA as a predictive marker of response to MN58b, further supported by the resistance resulting from CHKA knockdown in MN58b-sensitive cells. Also Hernando et al. reported that, mice with CHKA-overexpressing bladder tumors responded better to MN58b than non-expressing ones (106). We propose that this may be due to the phenomenon of oncogene addiction which postulates that some tumors rely mainly on the expression of one oncogene to sustain its growth and survival and, therefore, its inhibition has dramatic consequences for the cell (220).

This finding is relevant given the heterogeneity of tumor response to targeted therapies. Predictive marker identification is one of the main goals in drug development, as they provide opportunities for patient stratification in order to achieve higher activity and avoid unnecessary toxicities (3). Although several drugs are used for PDAC treatment (gemcitabine, 5-FU, oxaliplatin, irinotecan, nab-paclitaxel and erlotinib) no predictive markers have been described for any of them and usually CT scans after two or three cycles of chemotherapy reveal treatment efficacy. We believe that, if feasible, efforts should be made not only in the development of new therapies but also in the description of their predictive markers in order to ensure treatment efficacy from the beginning and avoid unnecessary toxicities. This is even more important for PDAC patients which have a short overall survival and a quick deterioration in performance status and, in many occasions, are not able to receive more than one treatment line.

Interesently, we demonstrate that CHKA levels not only predict response to MN58b but also to classical chemotherapeutics agents in PDAC cells; CHKA down-regulation enhanced sensitivity to gemcitabine, oxaliplatin and 5-FU, suggesting that high levels of CHKA render cells more resistant to conventional treatments. In fact, breast cancer cells with higher CHKA levels are more resistant to 5-FU and CHKA silencing makes them more sensitive (116). Finally Granata et al have recently shown that enhanced CHKA levels contribute to a drug resistant phenotype in epithelial ovarian cancer cells and transfection with CHKA siRNA increased their sensitivity to cisplatin, paclitaxel and doxorubicin (121).

Drug combination is a common therapeutic strategy in cancer with two main advantages over monotherapy: it enhances treatment efficacy - the simultaneous use of drugs with

different mechanisms of action blocks different pathways implied in tumor progression - and decreases toxicity. Combinations of gemcitabine with different chemotherapeutic agents (gemcitabine plus erlotinib, gemcitabine plus nab-paclitaxel and 5-FU plus oxaliplatin and irinotecan (FOLFIRINOX) (NCCN guidelines, 2015)), are currently used in the clinic in the treatment of metastatic disease and have demonstrated improved efficacy in patients with metastatic PDAC. Therefore overall patient survival has increased from 6 months (gemcitabine monotherapy) to 8.5 months (gemcitabine + nab-paclitaxel) or 11.1 months (FOLFIRINOX) (177) (173). Here, I show the additive or synergistic effect on cell death between MN58b and gemcitabine, oxaliplatin and 5-fluorouracil in RWP-1 and SK-PC-1 cells. Previous reports support the efficacy of MN58b in combination with other drugs: MN58b synergizes with 5-FU in cultured colorectal cells and xenografts through the down-regulation of thymidylate synthase and thymidine kinase, two of the enzymes involved in the metabolism of 5-FU (221). The evidences for synergy in experiments silencing CHKA or combining MN58b with gemcitabine, 5-FU, and oxaliplatin indicate that these drug combinations merit further preclinical and clinical attention.

What is the basis for the synergy between MN58b and other drugs and the influence of CHKA levels on drug sensitivity? Why should the pharmacological or genetic inhibition of CHKA enhance drug action? Granata et al show that the increased sensitivity after CHKA down-regulation is due to a decrease in glutathione and cysteine content and to the increase in intracellular levels of reactive oxygen species, which alters antioxidant cellular defenses (121, 222) and renders cells sensitive to other injuries. The role of CHKA in the synthesis of crucial components of cellular membranes such as PC implies that probably CHKA inhibition alters membrane composition and/or properties and thus contributes to modulate the permeability of cells to antitumor drugs. Finally, CHKA down-regulation decreases PC production which - through its breakdown by phospholipase D2 - is an important source of mitogenic signals - such as diacylglycerol, lyso-phosphatidylcholine and phosphatidic acid - that activates PI3K/AKT and MAPK pathways (84, 86). The attenuation of these pathways, involved in cell survival, probably renders cells less capable of recovering after external stress such as drug treatment. Drugs that inhibit the PI3K/AKT/mTOR pathway - such as temsirolimus or everolimus - are used in the clinic (223) and their combination with CHKA inhibitors could be an interesting therapeutic strategy.

Two of the main questions regarding PDAC treatment refer to the role of the stroma and the therapeutic options after gemcitabine failure. Stroma surrounding tumoral cells

(named as "the desmoplastic reaction") is thought to be one of the main reasons accounting for PDAC chemoresistance as it hampers drug penetration (55). Pancreatic stellate cells (PSC) play a critical role in stroma formation and turnover and, therefore, the concept has emerged that new therapeutic strategies should not only target tumor cells but also pancreatic stellate cells and more generally the stroma. MN58b has cytotoxic effect over stellate cells, which makes it even more promising against PDAC. MN58b, through its direct effect on PSC and stroma, could facilitate the penetration of other chemotherapeutics in PDAC.

We have observed that gemcitabine-resistant Suit2 007 PDAC cells are approximately four times more sensitive to MN58b than non-resistant Suit2 007 cells. Gemcitabine has two main antiproliferative mechanisms: it is a nucleotide analogue which substitutes cytidine during DNA replication, leading to apoptosis, and it also targets ribonucleotide reductase (RNR), blocking deoxyribonucleotide formation. The vast majority of PDAC show either primary or acquired resistance to gemcitabine through a variety of mechanisms, possibly also explaining their enhanced sensitivity to MN58b. Gemcitabine-resistant cells show increased expression of hypoxia-inducible factor 1α (HIF-1α) (224), which binds directly to the endogenous promoter of CHKA and increases CHKA levels (94), thus sensitizing them to MN58b. Gemcitabine-resistant cells also reduce reactive oxygen species (ROS) by increasing glutathione (GSH) production through an increase in NF-kβ-Nrf2 activity (225). This might be mediated by CHKA: in fact, Granata et al showed that CHKA down-regulation decreases glutathione and increases intracellular reactive species (222). Finally, gemcitabine-resistant cells show an overactivation of the MAPK/ERK signaling pathway and MAPK/ERK inhibition using lenalidomide, an immunomodulatory agent, restores gemcitabine sensitivity (226). CHKA inhibition also decreases MAPK/ERK activity through the decrease of phospholipase D and phosphatidic acid and it renders gemcitabine-resistant cell more sensitive to MN58b (86). The possible inter-relatedness of these mechanisms also merits further attention in future studies.

Gemcitabine, alone or in combination with nab-paclitaxel, is the common first-line treatment for PDAC patients (NCCN guidelines, 2015). Although there are other drugs with cytotoxic activity against PDAC (oxaliplatin, 5-fluorouracil, irinotecan), the toxicity of the FOLFIRINOX regime makes it impossible for many patients to receive further treatments. This, combined with the fact that targeted therapies usually have lower toxicity than classical chemotherapeutics, opens the question of whether CHKA inhibitors could be a reasonable therapeutic approach after gemcitabine progression.

4. Mechanisms and behavior of PDAC cells with acquired resistance to MN58b.

To better understand the mechanisms of resistance to MN58b, we generated the resistant cell line IMIM-PC-2-R, which has an IC50 30-fold higher than the parental cultures. These cells showed a reduction in CHO uptake, decreased proliferation, and reduced migration capacities. Resistant cells expressed similar levels of CHKA compared with their wild-type counterparts and similar enzymatic activity, measured as the rate of conversion from CHO to PC.

CHO uptake takes place through four major families of transmembrane proteins: high-affinity choline transporters (CHTs), intermediate-affinity choline transporter-like proteins (CTLs), organic cation transporters (OCTs) (with low affinity for choline) and organic cation/carnitine transporters (OCTNs). Choline uptake and CHKA expression are rate-limiting steps in choline phospholipid metabolism and crucial for malignant proliferation, as shown by decreased cell proliferation after down-regulation of CTL1 in lung cancer (227).

We set to identify the mechanisms of resistance of IMIM-PC-2 cells through a genome wide transcriptomics analysis. We found a down-regulation of 28 genes belonging to the solute-carrier gene (SLC) superfamily. The SLC superfamily includes 55 gene families with 362 genes that encode for a huge variety of proteins involved in transport, including passive transporters, symporters and antiporters, with affinity for a wide range of substrates such as inorganic cations/anions, amino acids and metal ions (228). Several SLC transporters are responsible for choline transport: SLC5A7 is a bona fide CHT, SLC44 is a CTL, and SLC22 belongs to the OCT subgroup. SLC44A2 and SLC22A17/18 transcripts were down-regulated in IMIM-PC-2-R cells. By contrast, the expression of transcripts coding for CHKA and other enzymes of the Kennedy pathway were not affected. These results suggest that cells adapt to CHKA chronic inhibition by reducing choline uptake through choline transporter down-regulation.

IMIM-PC-2-R cells functionally resemble CHKA-silenced Suit2 028 cells: they display reduced cell proliferation, migration and tumor formation. This phenotype is achieved through different mechanisms (reduced choline uptake in IMIM-PC-2-R versus reduced CHKA expression in Suit2 028 cells), leading to decreased PCHO and PC formation which, as discussed before, are essential to generate intermediates that activate survival and

proliferation pathways (86). We also found genes related with tumor invasion and metastasis among those most down-regulated in IMIM-PC-2-R cells. In fact, the most down-regulated gene is CEACAM6 whose expression promotes EMT and is associated with worse prognosis in PDAC (229).

We next investigated the mechanisms of MN58b resistance in IMIM-PC-2. Ramirez de Molina et al described the up-regulation of acid ceramidase (ASAH1) as a mechanism of resistance to MN58b in non-small cell lung cancer (NSCLC). ASAH1 inhibition restored the sensitivity to the drug (219). Contrarily, we found that ASAH1 was down-regulated in resistant cells indicating that this is not the mechanism of resistance in our model. We found among the 10 most up-regulated genes in IMIM-PC-2-R the multi-drug resistance (MDR) proteins ATP-binding cassette sub-family B member 1 (ABCB-1), also known as permeability glycoprotein 1 (Pgp-1), and member 4 (ABCB-4). Overexpression of these important candidates was demonstrated at the protein level. MDR transmembrane proteins are endowed with the ability to extrude drugs and we demonstrated their functionality in IMIM-PC-2-R cells using calcein uptake experiments. Therefore, we postulated that ABCB-1 inhibition should restore sensitivity to MN58b. We assayed this hypothesis using competitive inhibitors, either lacking specificity for several transmembrane proteins (Verapamil) or those specific for ABCB-1/Pgp-1 (Zosuquidar). I showed that inhibiting pump activity using these two drugs resulted in re-sensitization of IMIMPC- 2-R cells to MN58b, which provides formal proof of the causal relationship between MDR overexpression and drug resistance. Using ABCB1 and 4 specific siRNA has also shown to restore cell sensitivity in paclitaxel resistant cells (230).

MDR up-regulation is a well-known mechanism of acquired resistance to a variety of chemotherapeutic agents (vinblastine, etoposide and doxorubicin) (231). However it is also involved in intrinsic drug resistance: in ovarian cancer, a subgroup of cells with adult stem cell characteristics, called side population, showed up-regulation of Pgp-1, and resistance to paclitaxel leading to tumor progression. Its inhibition re-sensitizes cells to paclitaxel (232). Enhanced MDR expression is also a characteristic of cancer stem cells (CSC) and a major mechanism of chemoresistance.

Blocking Pgp-1 showed promising results *in vitro*; in leukemia cells, zosuquidar restores sensitivity to anthracyclins (190). Although two reports showed the antitumoral efficacy of Zosuquidar in phase I and II trials (196, 233), no clinical improvement was demonstrated in phase III clinical trials: among patients with newly diagnosed acute

leukemia, the addition of zosuquidar to cytarabine and daunorubicin showed no improvement in overall survival, probably due to compensation by other efflux proteins (BCRP, MRP1) that are not inhibited by zosuquidar (234). The simultaneous up-regulation of multiple drug efflux pumps is not unique to IMIM-PC-2-R cells and can be a significant problem despite the fact that, *in vitro*, the blockade of Pgp-1 seems to be sufficient to restore MN58b sensitivity. The use of less specific MDR inhibitors might be advantageous to overcome resistance. Verapamil, a well-known calcium antagonist used for the treatment of arterial hypertension and some cardiac disorders, has been known to be a Pgp-1 modulator for more than thirty years (235) but, despite its promising *in vitro* activity, few *in vivo* studies have been carried out probably because of its non-specific mechanism of action. In recent years, some studies have shown that when administered by arterial infusion combined with chemotherapy it improves outcomes in advanced gastric, hepatocarcinoma, colorrectal and lung cancer patients (236-239). Recently, some authors have explored the possibility of delivering doxorubicin and verapamil into the tumor using hydrogel nanoparticles. They observed an increase of intracellular concentration and cytotoxic activity of doxorubicin (240). Therefore, it seems that after the initial failure of molecules directed against Pgp-1 it is time to reconsider the efficacy of less specific Pgp-1 blockers.

Conclusions

1. PDAC cells and human pancreatic tumors overexpress choline kinase alpha to sustain cell proliferation.

2. In pancreatic tumors, choline kinase alpha localizes both in the cytoplasm and in the nucleus. Nuclear expression associates with the outcome of patients with well and moderately-differentiated tumors.

3. In pancreatic cancer cells, *in vitro* choline kinase inhibition using MN58b causes cell death by apoptosis. MN58b has synergistic or additive effects with gemcitabine, oxaliplatin and 5-Fluorouracil.

4. Choline kinase alpha levels constitute a predictive marker of the *in vitro* response to MN58b and its down-regulation sensitizes pancreatic cancer cells to gemcitabine, oxaliplatin and 5-FU.

5. Gemcitabine-resistant Suit2 007 pancreatic cancer cells show increased sensitivity to MN58b in comparison with non-resistant Suit2 007 cells.

6. Acquired resistance to MN58b associates with decrease choline uptake, which attenuates the malignant phenotype of cells.

7. Acquired resistance to MN58b is due to the up-regulation of Pgp-1 and ABCB-4 and can be reverted with the Pgp-1 inhibitors Verapamil and Zosuquidar.

Conclusiones

1. Colina quinasa alfa está sobreexpresada en líneas celulares de PDAC y en tumores de páncreas y es necesaria para la proliferación celular.

2. En los tumores de páncreas, colina quinasa alfa se halla en el citoplasma y en el núcleo. Su expresión nuclear constituye un factor pronóstico en los tumores bien y moderadamente diferenciados

3. En células de cáncer de páncreas, la inhibición *in vitro* de colina quinasa alfa mediante MN58b produce la muerte celular por apoptosis. MN58b posee un efecto sinérgico o aditivo con gemcitabina, oxaliplatino y 5-FU.

4. Los niveles de colina quinasa alfa constituyen, *in vitro*, un factor predictivo de respuesta al MN58b y el descenso de sus niveles sensibiliza a las células de cáncer de páncreas a gemcitabina, oxaliplatino y 5-FU.

5. Las células de cáncer de páncreas resistentes a gemcitabina Suit2 007 muestran una mayor sensibilidad al MN58b que las no resistentes.

6. La adquisición de resistencia a MN58b se asocia con un descenso en la captación de colina que atenúa el fenotipo maligno de las células.

7. La sobreexpresión de Pgp-1 y ABCB-4 es responsable de la adquisición de resistencia al MN58b que puede ser revertida mediante los inhibidores de Pgp-1 Verapamilo y Zosuquidar

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Choline Kinase Alpha (CHKα) as a Therapeutic Target in Pancreatic Ductal Adenocarcinoma: Expression, Predictive Value, and Sensitivity to Inhibitors

José M. Mazarico[1], Victor J. Sánchez-Arévalo Lobo[1], Rosy Favicchio[2], William Greenhalf[3], Eithne Costello[3], Enrique Carrillo-de Santa Pau[1], Miriam Marqués[1], Juan C. Lacal[4], Eric Aboagye[2], and Francisco X. Real[1,5]

Abstract

Choline kinase α (CHKα) plays a crucial role in the regulation of membrane phospholipid synthesis and has oncogenic properties *in vitro*. We have analyzed the expression of CHKα in cell lines derived from pancreatic ductal adenocarcinoma (PDAC) and have found increased CHKα expression, associated with differentiation. CHKα protein expression was directly correlated with sensitivity to MN58b, a CHKα inhibitor that reduced cell growth through the induction of apoptosis. Accordingly, CHKα knockdown led to reduced drug sensitivity. In addition, we found that gemcitabine-resistant PDAC cells displayed enhanced sensitivity to CHKα inhibition and, *in vitro*, MN58b had additive or synergistic effects with gemcitabine, 5-fluorouracil, and oxaliplatin, three active drugs in the treatment of PDAC. Using tissue microarrays, CHKα was found to be overexpressed in 90% of pancreatic tumors. While cytoplasmic CHKα did not relate to survival, nuclear CHKα distribution was observed in 43% of samples and was associated with longer survival, especially among patients with well/moderately differentiated tumors. To identify the mechanisms involved in resistance to CHKα inhibitors, we cultured IMIM-PC-2 cells with increasingly higher concentrations of MN58b and isolated a subline with a 30-fold higher IC_{50}. RNA-Seq analysis identified upregulation of ABCB1 and ABCB4 multidrug resistance transporters, and functional studies confirmed that their upregulation is the main mechanism involved in resistance. Overall, our findings support the notion that CHKα inhibition merits further attention as a therapeutic option in patients with PDAC and that expression levels may predict response. *Mol Cancer Ther; 15(2); 1–11. ©2016 AACR.*

Introduction

Pancreatic adenocarcinoma (PDAC) is the fourth cause of cancer-related death in the Western world, with a 5-year survival of <5%. During the last two decades, PDAC-related deaths have only decreased marginally (1). There are multiple reasons for this poor outcome. A majority of cases with PDAC are diagnosed at an advanced stage, with either local (26%) or distant metastases (53%), and only 20% of patients are candidates for surgery with curative intent (2). There is also a lack of effective therapies for advanced disease. Gemcitabine has been the gold-standard treatment for metastatic disease. The combination of 5-fluorouracil (5-FU), oxaliplatin, and irinotecan (FOLFIRINOX) and the combination of gemcitabine with nab-paclitaxel and gemcitabine with erlotinib have shown increased antitumor activity (3–6), but there is a need to identify new therapeutic targets and drugs.

In the last years, there has been a renewed interest in exploiting the metabolic reprogramming that cells undergo upon malignant transformation (7). One promising metabolite deregulated in cancer is choline, an essential nutrient of the B vitamin family that is necessary for the synthesis of phosphatidylcholine. Phosphatidylcholine is the most abundant phospholipid of the eukaryotic cell membrane. Tumors show an active choline metabolism manifested by an increase of phosphocholine and total choline metabolites required to sustain cell growth and transformation (8). Phosphatidylcholine is also the precursor of important second messengers and mitogenic signals, such as diacylglycerol or arachidonic acid, through its hydrolysis by phospholipases. The first enzyme of the Kennedy pathway, choline kinase, phosphorylates free choline and generates phosphocholine. Two enzymes have been identified in mammals: choline kinase α (CHKα), of which two isoforms are generated by alternative splicing, and choline kinase β (CHKβ; ref. 9). CHKβ has not been reported to be oncogenic (10), but CHKα has been proposed to participate in initiation and progression of several tumors (11–14). These

[1]Epithelial Carcinogenesis Group, Cancer Cell Biology Programme, Spanish National Cancer Research Center-CNIO, Madrid, Spain. [2]Comprehensive Cancer Imaging Centre, Department of Surgery & Cancer, Imperial College London, Hammersmith Hospital, London, United Kingdom. [3]The NIHR Liverpool Pancreas Biomedical Research Unit, Liverpool, United Kingdom. [4]Division of Translational Oncology, Department of Oncology, Fundación Jiménez Díaz, Madrid, Spain. [5]Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.

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Corresponding Authors: Francisco X. Real, Centro Nacional de Investigaciones Oncológicas, Melchor Fernández Almagro 3, Madrid 28029, Spain. Phone: 349-1732-8000; Fax: 349-1224-6923; E-mail: preal@cnio.es; and Víctor J. Sánchez-Arévalo Lobo, E-mail: vjsanchez@cnio.es

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findings render CHKα an attractive therapeutic target. Several choline kinase inhibitors (CHKI) have been developed from hemicholinium-3. MN58b, a first-generation CHKI-competitive inhibitor, has antiproliferative and antitumoral activity *in vitro* and *in vivo* (11, 15–18). Second-generation CHKIs are in phase I clinical trials (19).

We aimed to assess the importance of CHKα in PDAC and to test the efficacy of its pharmacologic inhibition as a single agent or in combination with the drugs most commonly used in this tumor. CHKα levels are predictive of drug sensitivity and CHKα downregulation sensitizes cells to other anticancer drugs. We have identified a novel mechanism of acquired resistance to CHKIs, resulting from the upregulation of multidrug resistant (MDR) proteins.

Material and Methods

Cell culture

The following PDAC cell lines were used: SK-PC-1, SK-PC-3, and IMIM-PC-2 (20); Suit2 007, Suit2 028, T3M4, and PATU 8988 T (M. Buchholz, University of Marburg, Germany); Panc-1, RWP-1, BxPC-3 (ATCC); PK-9 (C. Iacobuzzio-Donahue, Memorial Sloan Kettering Cancer Center, New York, NY); MZ-PC-4 (University of Zürich, Switzerland). Oncogene-immortalized HPDE (M. Tsao, University Health Network, Toronto, Canada) and hTERT-immortalized human ductal pancreatic cells (HPNE, T. Gress) were used as controls (21). Nonpancreatic cells used were J82, RT112, and HT1376 (bladder; F. Radvanyi, I. Curie, Paris, France); HT-29 (colon; A. Zweibaum, INSERM, Villejuif, France); human embryonic kidney (HEK)–293T (ATCC); SK-BR-3, MDA-MB-231 (breast; ATCC). The identity of the lines was confirmed using short tandem repeat (STR) fingerprinting. Cells were cultured in DMEM supplemented with 10% FBS, 1% Na pyruvate, and 1% penicillin/streptomycin. All cells were free of mycoplasma contamination. Control nontargeting or CHKα-targeting lentiviral particles were produced in HEK-293T cells using Sigma Mission plasmids following the manufacturer's instructions. The interfering sequences were:

Sh-1: GTACCGGGTGTTACTTGCAGGTACTTTGCTCGAGCAAAGTACCTGCAAGTAACACTTTTTTG;
Sh-2: GTACCGGGCAGATGAGGTCCTGTAATAACTCGAGTTATTACAGGACCTCATCTGCTTTTTTG;
Sh-3: CCGGGCGATTAGATACTGAAGAATTCTCGAGAATTCTTCAGTATCTAATCGCTTTTT;
Sh-4: CCGGCCAAGAAACAACAGCTCCATTCTCGAGAATGGAGCTGTTGTTTCTTGGTTTTT;
Sh-5: CCGGGCCAAGATTTCATCTATTGAACTCGAGTTCAATAGATGAAATCTTGGCTTTTT.

To produce viral particles, psPAX2 and VSVG packaging plasmids were used. Virus-containing supernatant was collected 24 hours after transfection, filtered, and used to infect the corresponding cells in the presence of hexadimethrine bromide polybrene (5 μg/mL; Sigma). Two rounds of infection were performed within 24 hours; cells were selected for 48 hours in medium containing puromycin (2 μg/mL; Sigma).

Generation of MN58b-resistant cell lines

To generate MN58b-resistant IMIM-PC-2 cells, MN58b was added starting at 0.1 μmol/L. Control IMIM-PC-2 cells were cultured without drug. MN58b concentration was increased by 50% weekly at each passage of the cells (split 1:3 when confluent); final concentration was 8 μmol/L.

To generate gemcitabine-resistant PDAC cell lines, an incremental dose approach was used. The starting concentration was 35 nmol/L 2′-deoxy-2′,2′-difluorocytidine monohydrochloride (Eli Lilly Ltd). Gemcitabine concentration was maintained constant and was increased 1.5- to 2-fold at each cell passage. The final concentration was 250 nmol/L. Resistant cells were authenticated by STR profiling.

Growth and viability assays

Cells (5×10^4 per well) were seeded in triplicate in 6-well plates, trypsinized, and counted. To determine viability, cells (2×10^4 per well) were seeded in 24-well plates. After 24 hours, medium was removed and MN58b was added; after 72 hours, cells were fixed with 3% formaldehyde, washed twice with PBS, and incubated with 0.5% crystal violet in 25% methanol; crystal violet was eluted with 10% acetic acid and the OD590 nm was determined. To assess colony formation, cells (5×10^4 per well) were seeded in 6-well plates and medium was replaced 24 hours later with medium containing MN58b. After 72 hours, cells were processed as described earlier.

Apoptosis assays

Cells (5×10^4 per well) were seeded in 6-well plates in the presence of increasing concentrations of MN58b. After 24 and 48 hours, cells were washed with PBS, resuspended in Annexin V binding buffer, and incubated with APC-Annexin V (BD Biosciences) in the dark for 15 to 20 minutes. DAPI was added for 15 minutes, and viability was assessed in a FACS Canto II flow cytometer (BD Biosciences). Results were quantified using FlowJo software (FlowJo version 7.6.1, TreeStar Inc.).

Drug synergy assays

Cells (2×10^4 per well) were seeded in 24-well plates. After 24 hours, medium was removed and drugs (MN58b, gemcitabine, oxaliplatin, and 5-FU) were added for 72 hours, alone or in combination, at a range of doses according to the previously estimated IC_{50} for each drug. Viability was determined and the combination index (CI) values were calculated using the Chou and Talalay method (22) with the Calcusyn software (Biosoft). A CI of 0.9–1.1 indicates an additive effect, a CI <0.9 indicates synergy, and a CI >1.1 indicates antagonism.

Calcein-AM assay

Cells (2×10^4 per well) were seeded in black 96-well plates. After 24 hours, calcein-AM uptake and conversion to fluorescent calcein were determined using the Invitrogen calcein-AM assay kit according to the manufacturer's instructions (Invitrogen). Fluorescence was determined at 490/520 nm using a luminometer reader (PHERAstar).

Choline uptake

A pulse-chase in cell assay was used to determine choline uptake. ^{3}H-choline chloride was added to the culture media for 60 minutes; the media was then removed and the wells washed 3× with PBS. Cells were lysed and choline uptake was assessed as the amount of ^{3}H present in the lysates, as determined using a scintillation counter.

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Choline kinase activity

Free ^{3}H-choline was added to the reaction mix ($MgCl_2$ 10 mmol/L, KCl 100 mmol/L, ATP 500 μmol/L, and Tris pH 7.5 100 mmol/L), and its phosphorylation was determined from the amount of ^{3}H-choline converted to ^{3}H-phosphocholine using a modified Bligh and Dyer assay (23). The use of passive lysis buffer ensured enzymatic activity in the lysates before ^{3}H-choline chloride was added. The reaction was stopped at 60 minutes by the addition of methanol/chloroform to effectively initiate the lipid extraction step. Phase extraction using tetraphenylborate then separated choline from phosphocholine, and the amount of ^{3}H in each fraction was determined using a scintillation counter.

Immunoblotting

Cells were lysed in NP-40 buffer (25 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% NP-40) supplemented with protease inhibitor cocktail. Proteins (20 μg) were fractionated, transferred to nitrocellulose, and incubated with mouse monoclonal antibody AD8 recognizing CHKα (1:1,000 dilution; ref. 24) or anti-rabbit anti-CHKα polyclonal antiserum (Sigma, HPA024153; 1/100). After washing, peroxidase-labeled anti-mouse Ig (Amersham Pharmacia Biotechnology) was added. Mouse anti-vinculin (Sigma) served as control. Reactions were developed using enhanced chemiluminescence (Amersham Pharmacia Biotechnology).

RT-qPCR

Total RNA was isolated using the GenElute Mammalian Total RNA kit (Sigma). Samples were treated with DNase I (Ambion) and converted to cDNA using TaqMan reverse transcription reagents (Applied Biosystems). Quantitative PCR was performed using SYBR-green mastermix in a Prism 7900 HT instrument (Applied Biosystems). The following primers were used for the detection of CHKα mRNA isoform 1: AAAGAGGGATCCGAACAAGC (forward) and AGTGACCTCTCTGCGAGAATG (reverse); ABCB1: CTGTGAAGAGTAGAACATGAAG (forward) and TTGCACCTCTCTTTTATCTG (reverse); and ABCB4: GAGGTCAAAAACAGAGGATTG (forward) and CCTTTTCACTTTCAGTATCCAG (reverse). Reactions were performed in triplicate; expression levels were normalized to individual hypoxanthine phosphoribosyltransferase mRNA values using the $\Delta\Delta C_t$ method (25).

In vivo subcutaneous tumorigenic assay

Suit2 028 CHKα-silenced (Sh-3 and Sh-5) and their nontarget counterparts (shNt) cells were grown in 6- to 8-week-old female BALB/c Nu/Nu mice (Charles River or Harlan Laboratories). Cells (2×10^6, 100 μL in PBS) were injected subcutaneously, growth was monitored using an electronic caliper, and volumes calculated using the formula ($L \times W^2 \times 0.5$). Mice were housed in IVC cages; animal work was performed by licensed investigators in accordance with the United Kingdom Home Office Guidance on the Operation of the Animal (scientific procedures) Act 1986 and Amended 2012 in line with the EU Directive 2010/63.

IHC

Tissue microarrays (TMA) containing duplicate nonmalignant and malignant cores from 96 patients with PDAC treated at the Royal Liverpool University Hospital (Liverpool, Merseyside, United Kingdom) were manufactured following good laboratory practice standards. Cores were taken from tumor regions identified by an experienced pathologist. Samples were collected under ethical committee approval for characterization of tumor markers for chemotherapy from the Liverpool (Adult) Research Ethics Committee (07/H1005/87).

Sections were incubated in boiling water for 10 minutes in citrate buffer pH 6. Slides were incubated at 4°C overnight with rabbit anti-CHKα polyclonal antiserum HPA024153 (1/100). After rinsing in PBS, horseradish peroxidase–conjugated anti-rabbit Ig antibody (DAKO) was added for 1 hour. Bound antibody was revealed with diaminobenzidine, and sections were counterstained. *H*-score was calculated as the product of intensity (scale 0–3) and percentage of positive cells; samples were classified as high (upper quartile), medium (two mid-quartiles), or low (lower quartile) CHKα staining.

Antibody specificity was demonstrated using Suit2 028 cells infected with control or CHKα-specific shRNA lentivirus (Supplementary Fig. S1).

CHKα expression and patient characteristics/variables

The clinical variables considered were age, gender, stage, grade, date of surgery, chemotherapy, and survival. The Kaplan–Meier method applying the log-rank test was used to estimate the differences in overall survival. Multivariate analyses including the above variables were performed.

RNA-Seq and bioinformatics analysis

RNA from parental and MN58b-resistant cells was purified using TRIzol. Integrity was assayed on an Agilent 2100 Bioanalyzer (Supplementary Table S1). PolyA+ fractions were purified and randomly fragmented, converted to double-stranded cDNA, and processed by end-repair, dA-tailing, and adapter ligation (Illumina "TruSeq Stranded mRNA Sample Preparation Part # 15031047 Rev. D"). Adapter-ligated library was completed by 10 PCR cycles with Illumina PE primers. The purified cDNA template library was applied to an Illumina flow cell for cluster generation (TruSeq cluster generation kit v5) and sequenced on Genome Analyzer IIx (GAIIx) with SBS TruSeq v5 reagents following the manufacturer's instructions (SingleRead 1 × 40 bases). Image analysis and per-cycle base calling was performed with Illumina Real Time Analysis software (RTA1.13). Conversion to FASTQ was performed with CASAVA-1.8 (Illumina). These files contain only reads that passed "chastity" filtering (flagged with a N, for *NOT filtered* in the sequence identifier line). "Chastity" parameter measures signal contamination in raw data and allows flagging unreliable reads. Quality check was done via fastqc (v0.9.4, Babraham Bioinformatics). Raw reads were aligned to the reference genome hg19/GRCh37 with tophat[1] (version 2.0.4) using the following parameters: –bowtie1, –max-multihits 5, –genome-read-mismatches 1, –segment-mismatches 1, –segment-length 19, –splice-mismatches 0, and –library-type fr-firststrand. Gene expression levels (fragments per kilobase of exon per million fragments, FPKM) were quantified with cufflinks[2] (version 2.0.2), as annotated in Ensembl version GRCh37.65, with the following parameters: -N, –library-type fr-firststrand, -u.

Sample correlation, principal component analysis, and differential gene expression

FPKM correlations and PCA clustering of samples were carried out with the R (version 2.14.1) functions cor() and prcomp. Differential gene expression analysis was performed with the

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cuffdiff function included in cufflinks[2] (version 2.0.2) with parameters: -N, –library-type fr-firststrand, -u.

CHKα expression in cancer cell lines

CHKα expression values were obtained from the Cancer Cell Line Encyclopedia (CCLE; ref. 26). The method of Subramanian and colleagues (27) was adapted to carry out a tissue enrichment analysis (TEA) with the CCLE PDAC lines. Cell lines were preranked by CHKα expression for each of the probes included in the microarray (204266_s_at and 204233_s_at) to calculate an enrichment score for the PDAC subgroup. The score reflects how often PDAC lines appear at the top or bottom of the ranked dataset (27).

CHKα expression in pancreatic tumor tissues

Expression values for CHKα were downloaded from the Gene Expression Omnibus series GSE15471 (probes 204266_s_at and 204233_s_at; ref. 28). Samples were classified as classical-, exocrine-like– and quasi-mesenchymal–PDA (QM-PDA) as defined by Collisson (29). One-way ANOVA was used to assay differences in CHKα expression between PDAC categories and Tukey test was used to test pairwise comparisons. The R (version 2.14.1) functions aov, TukeyHSD, and boxplot were used for statistical analyses and graphs; *P* values < 0.05 were considered significant.

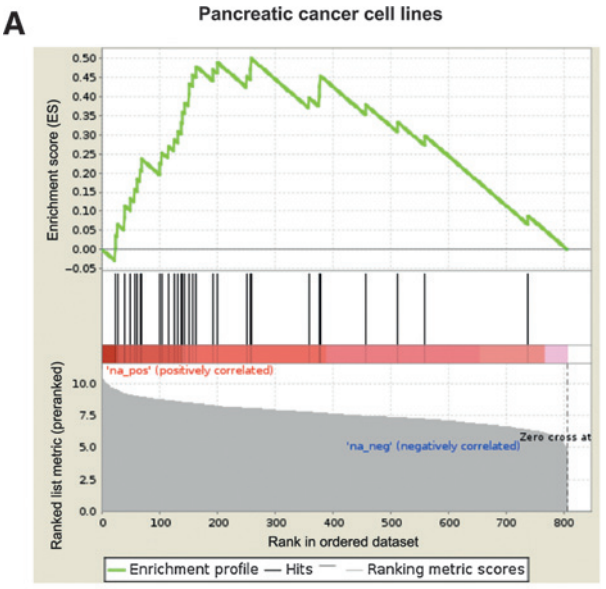
Results

PDAC cells overexpress CHKα

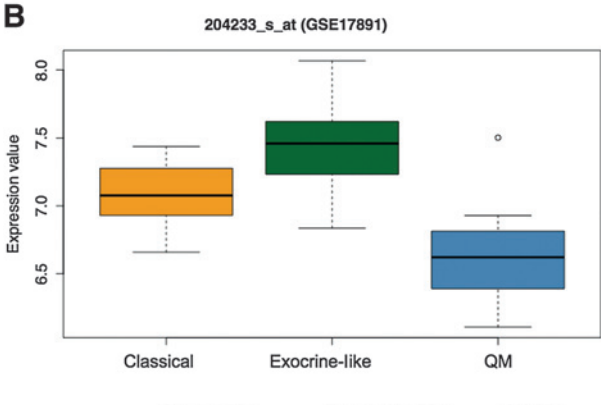
To obtain an overall view of the expression of CHKα in PDAC, we applied a similar analysis as in GSEA–gene set enrichment against a preranked gene list, but we replaced genes by cell lines (TEA). The public data from the CCLE were used. Cell lines derived from PDAC are significantly enriched among those displaying high level of expression of CHKα (Fig. 1 and Supplementary Fig. S2). This enrichment was significant for both CHKα probes for which information is available through CCLE (Fig. 1 and Supplementary Fig. S2). In addition, we assessed whether CHKα expression is associated with the three PDAC categories recently defined by Collisson and colleagues (29). As shown in Fig. 1B, samples in the QM group express lower levels of CHKα mRNA than classical or exocrine-like samples, suggesting a relationship between CHKα expression and cell differentiation.

We then used western blotting to compare CHKα expression in 12 PDAC cell lines and two immortalized pancreatic ductal cell lines (HPDE and hTERT-HPNE). PDAC lines showed 3- to 11-fold higher levels of CHKα compared with HPDE or HPNE cells (Fig. 2A and Supplementary Fig. S3). CHKα levels in PDAC cells were comparable with those found in bladder, colon, and breast cancer cell lines (Fig. 2B). We did not find a good correlation between isoform 1 mRNA levels, assayed using RT-qPCR, and protein. This isoform is the predominant transcript expressed in the pancreas (30), suggesting that posttranscriptional mechanisms participate in the regulation of CHKα in PDAC.

To study the requirement of CHKα for proliferation, we used RNA interference with different sh-lentiviruses in high-expressing Suit2 028 cells. A partial CHKα knockdown was achieved with Sh-3 and Sh-5 (Fig. 2C). We did not observe morphologic changes or cell death (data not shown), possibly because of the incomplete silencing. However, a significant reduction in the growth rate of



Enrichment score (ES)	0.498
Normalized enrichment score (NES)	3.046
Nominal *P* value	0.0
FDR *q* value	0.0



	Classical-PDA	Exocrine-like-PDA	QM-PDA
ANOVA	2.03E-06		
Tukey multiple comparisons (P_{adj})			
Classical			
Exocrine-like	0.02		
QM	0.05	0.00	

Figure 1.
Bioinformatics analysis of CHKα expression in PDAC cell lines and tissue samples. A, TEA of the expression data of PDAC lines in the CCLE. PDAC lines are highly represented among the highest CHKα-expressing cells (probe 204233_s_at). B, box plot showing CHKα expression (probe 204266_s_at) in the PDAC subgroups defined by Collisson and colleagues and statistical analysis of the comparison (bottom).

silenced cells was observed *in vitro* (Fig. 2D). Suit2 028 cells, CHKα-silenced with two different lentiviral shRNAs and nontargeted controls, were subcutaneously injected in nude mice. A significantly reduced growth of xenografts was observed with both shRNAs at days 10 and 15 after injection (Fig. 2D). We conclude

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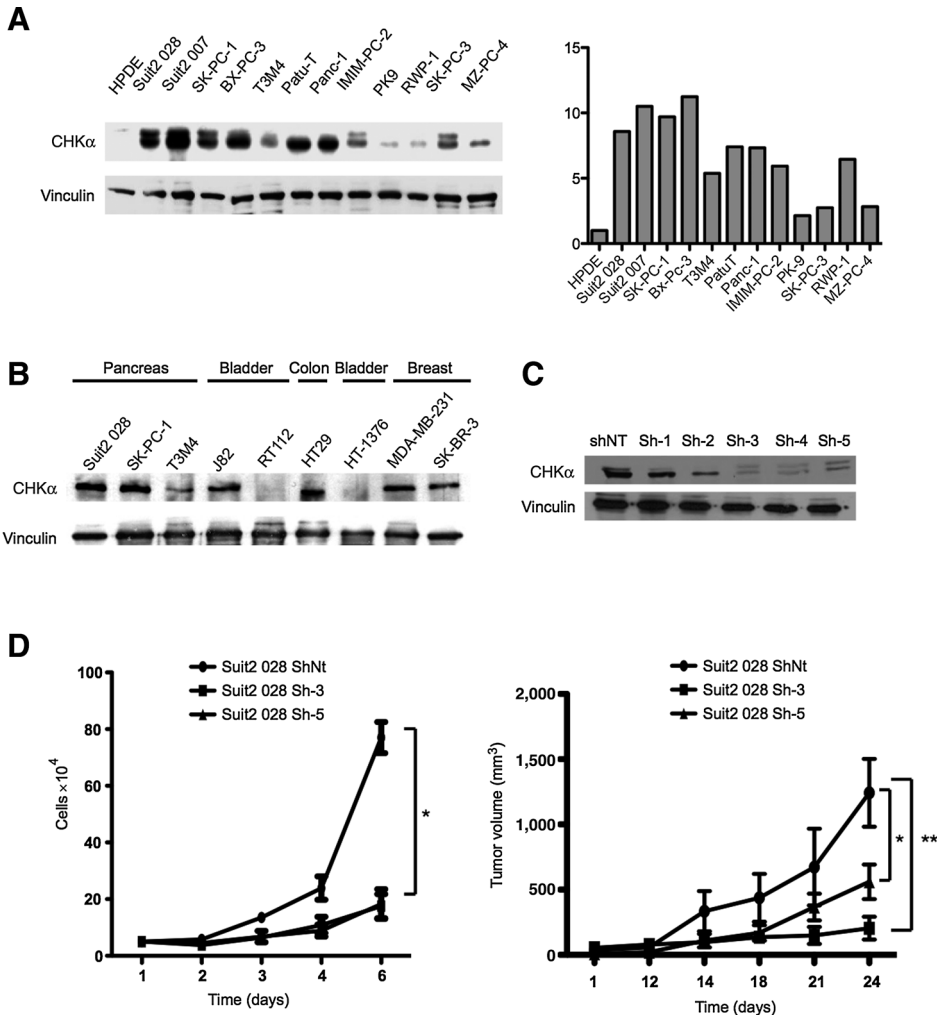

Figure 2.
CHKα is overexpressed in cultured PDAC cells, and it is important to sustain cell proliferation. A, CHKα expression by western blotting in PDAC cell lines and nontransformed HPDE pancreatic cells; quantification was performed by densitometry. B, CHKα expression in a panel of nonpancreatic cells. C, CHKα expression in Suit2 028 cells infected with 5 different shRNA lentiviruses (Sh-1 to Sh-5) or a scrambled (Nt) sequence. D, growth of control and CHKα-silenced Suit2 028 cells *in vitro* (left) and *in vivo* as subcutaneous xenografts (right).

that, as in other tumor types, CHKα is overexpressed in PDAC cells and is important to sustain cell growth.

CHKα expression in pancreatic tissues: association with tumor differentiation and survival

In nonneoplastic pancreas, weak cytoplasmic staining of acinar and ductal cells was observed. Islet cells showed stronger cytoplasmic staining and, occasionally, positive nuclei (Fig. 3A, top left). A low proportion of stromal cells with a fibroblast morphology showed cytoplasmic staining as well. We analyzed CHKα expression using a human PDAC TMA containing predominantly stage III tumors with nodal invasion (Supplementary Table S2). Chronic pancreatitis samples showed weak ductal cell staining (Fig. 3A, top middle), whereas ductal tumor cells showed stronger staining (Fig. 3A bottom, middle and right). PanIN lesions showed variable staining from moderate to strong (Fig. 3A). The majority (91%) of tumor samples showed detectable CHKα expression that was categorized as high (32%), medium (47%), or low (21%) according to intensity and percentage of staining. Nuclear staining was present in 43% of samples and was associated with well/moderately differentiated tumors (49% vs. 17%, $P = 0.024$; Fig. 3A).

Follow-up information was available for 74 patients. There was no statistical association between H-score and survival, but nuclear staining was associated with improved survival (500 vs. 299 days, $P = 0.014$; Fig. 3B, left) in all samples, and especially among moderately differentiated tumors (575 vs. 299 days, $P = 0.002$; Fig. 3B, right). In the multivariable model, grade, but not CHKα nuclear staining, was an independent variable associated with improved survival.

The antiproliferative activity of the CHKαI MN58b is associated with CHKα expression levels

MN58b selectively inhibits CHKα; accordingly, we observed a decrease in the synthesis of phosphocholine from choline in IMIM-PC-2 cells (Fig. 4A). We analyzed the effects of MN58b on the growth of four PDAC cell lines (SK-PC-1, Suit2 008, IMIM-PC-2, and RWP-1). MN58b had a marked effect on colony formation at 1 μmol/L, and growth was completely abolished at 5 μmol/L in all the cell lines tested (Fig. 4B). In a panel of 12 PDAC cell lines, the IC_{50} of MN58b ranged from 0.23 to 3.2 μmol/L. We found a direct relationship between CHKα protein expression and MN58b sensitivity ($R^2 = 0.88$; Fig. 4B and Supplementary Table S3). CHKα knockdown in Suit2 028 and Suit2 007 cells was associated with an increase in the IC_{50} (Supplementary Table S4).

To determine the mechanism of action of MN58b, we treated PDAC cells with increasing concentrations of MN58b (1–10

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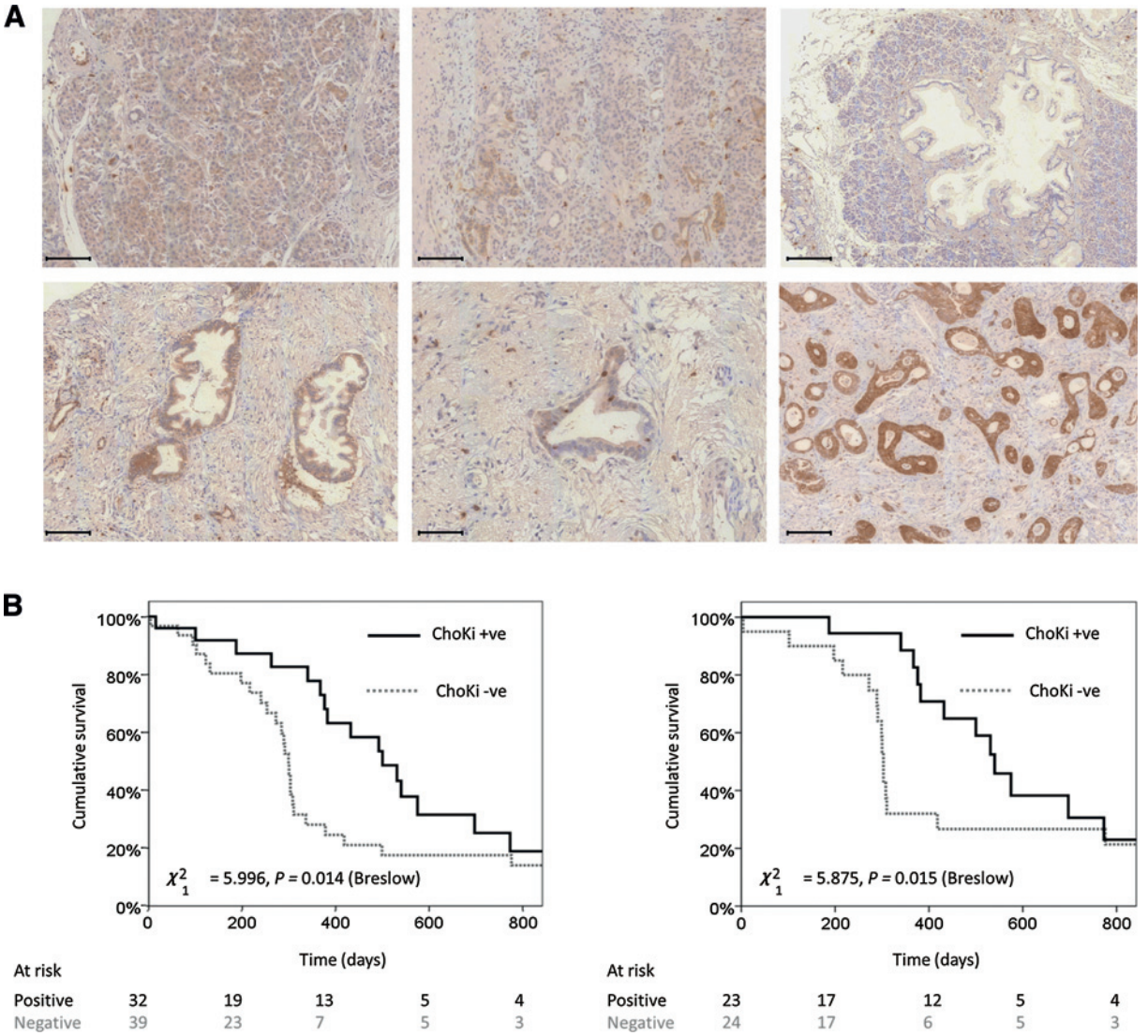

Figure 3.
CHKα expression in pancreatic tissue samples. A, representative images of CHKα immunostaining in normal pancreas (top left), chronic pancreatitis (top middle), PanIN (low grade, top right; high grade, bottom left), and PDAC (bottom, middle and right). Bottom, middle and right, nuclear and cytoplasmic CHKα, respectively. B, Kaplan–Meier survival curves of a cohort of 74 patients (left) with PDAC showing that nuclear CHKα staining correlates with better outcome. The same analysis, restricted to patients with moderately differentiated tumors, is shown at right.

μmol/L) for 24 or 48 hours and analyzed apoptosis through Annexin V. There was a direct correlation between CHKα expression and the percentage of Annexin V–positive cells at 48 hours (Fig. 4C). The induction of apoptosis was confirmed through the analysis of cleaved caspase-9 by western blotting; a dose–response relationship was observed (data not shown). Therefore, MN58b induces apoptosis and this response correlates with CHKα expression. These results suggest that CHKα could be a predictive marker of response to MN58b.

Combination effects of MN58b and chemotherapeutic agents

Both primary and acquired resistances contribute to the limited efficacy of gemcitabine in the treatment of PDAC. We used parental and gemcitabine-resistant Suit2 007 cells to assess the relationship between resistance and MN58b sensitivity. The IC_{50} of MN58b for parental and resistant cells was 3.14 μmol/L and 0.77 μmol/L, respectively, supporting the notion that MN58b could be a therapeutic alternative in gemcitabine-resistant tumors.

To test the synergism of MN58b with other chemotherapeutic agents active in PDAC therapy (5), we treated PDAC cells (SK-PC-1, Suit2 028, and RWP-1) expressing variable levels of CHKα with gemcitabine, oxaliplatin, or 5-FU plus MN58b at concentrations lower than the IC_{50}. The synergism was measured as CI. In Suit2 028 cells, none of the combinations tested showed increased effects. In the other two cell lines, MN58b showed an additive effect in combination with gemcitabine and 5-FU, and synergism in combination with oxaliplatin (SK-PC-1, CI = 0.23; RWP-1, CI = 0.39; Fig. 4D and Supplementary Fig. S4). These findings support the use of MN58b in combination with other chemotherapeutic drugs.

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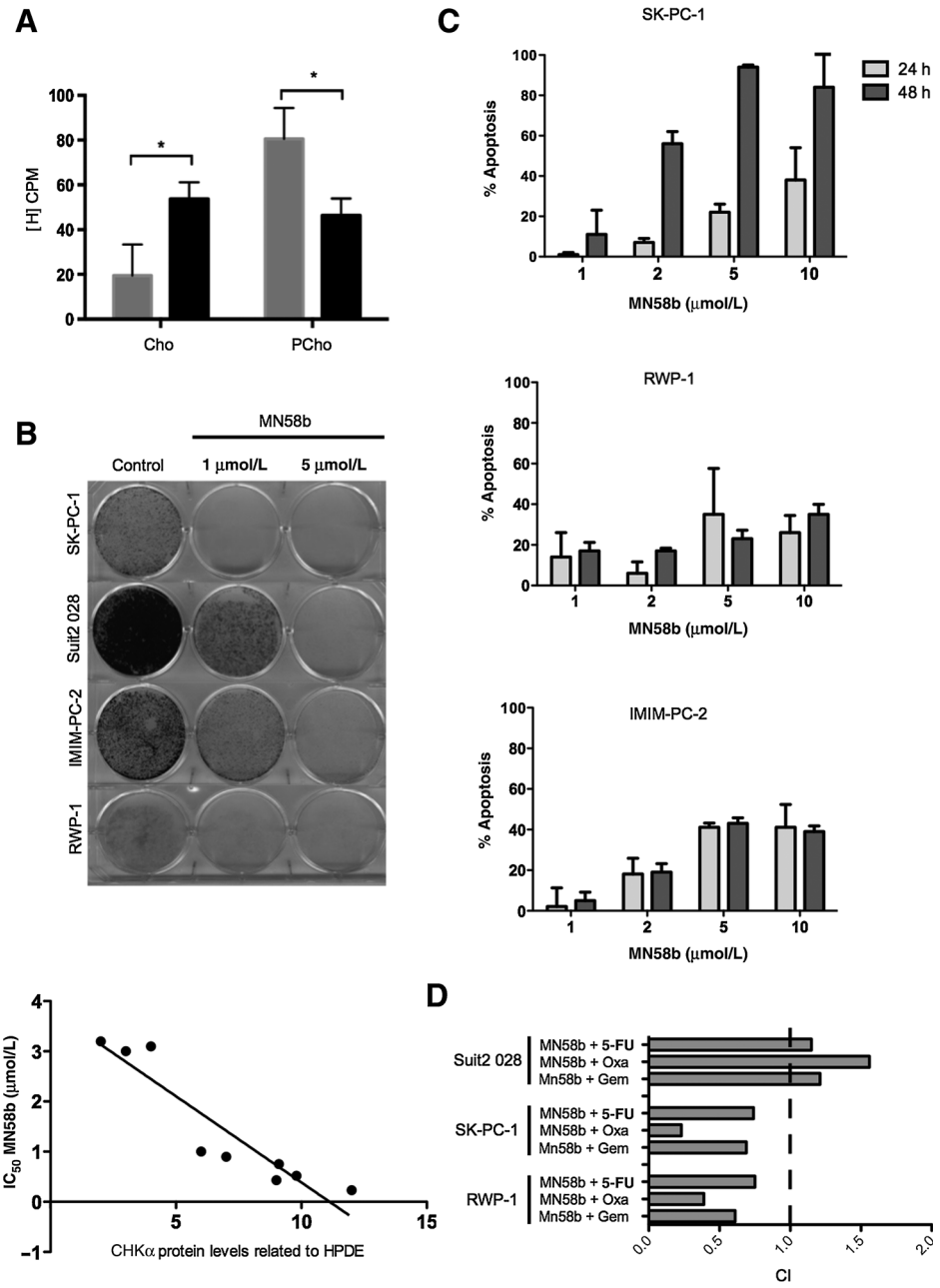

Figure 4.
CHKα levels are associated with the apoptotic response to MN58b. A, treatment of IMIM-PC-2 PDAC cells with MN58b reduces phosphocholine (PCho) synthesis. B, colony formation using four PDAC cell lines treated with increasing MN58b concentrations (top). Inverse correlation between levels of CHKα and MN58b IC_{50} (bottom). C, apoptosis, measured by Annexin V staining and flow cytometry, in three PDAC cell lines treated with MN58b for 24 or 48 hours. D, synergistic effects of MN58b combined with gemcitabine (Gem), oxaliplatin (Oxa), and 5-FU on PDAC cell lines. Cho, choline.

Resistance to MN58b is mediated by the upregulation of the ABCB transporters 1 and 4

To assess the mechanisms involved in the acquisition of resistance to CHKI, we generated an MN58b-resistant line from parental IMIM-PC-2 cells by continuous culture with increasing drug concentrations. After 9 months of treatment, IMIM-PC-2-R cells were established; their IC_{50} was 156 μmol/L, approximately 30-fold higher than that of parental cells. Colony-forming capacity of IMIM-PC-2-R cells was not affected by treatment with 10 μmol/L MN58b (Fig. 5A). IMIM-PC-2-R displayed a lower baseline proliferation rate than parental cells (Fig. 5A) as well as reduced choline uptake (approximately 50%; Fig. 5B). However, CHKα enzymatic activity was similar in resistant and parental cells (Fig. 5C).

To investigate the mechanisms of resistance, we performed RNA-Seq of both lines. Scatterplot3d library was used to visualize the first 3 components accounting for 99.8% of the variance between the samples (Supplementary Fig. S5A). GSEA pathway analysis of differentially expressed genes did not reveal any significantly deregulated pathway. However, 2 of the 5 top upregulated genes in IMIM-PC-2-R cells were members of the MDR protein family of ATP-binding cassette (ABC) transporters 1 and 4 (ABCB1 and ABCB4; Supplementary Fig. S5B). Results from the transcriptome analysis were validated by RT-qPCR, showing a 700-fold upregulation of ABCB1 and ABCB4 in the resistant cells compared with the parental ones (Fig. 5D). The overexpression of the transporters was confirmed at the protein level, although the fold change was more modest (Fig. 5D). ABCB1 and ABCB4 mRNA expression was not

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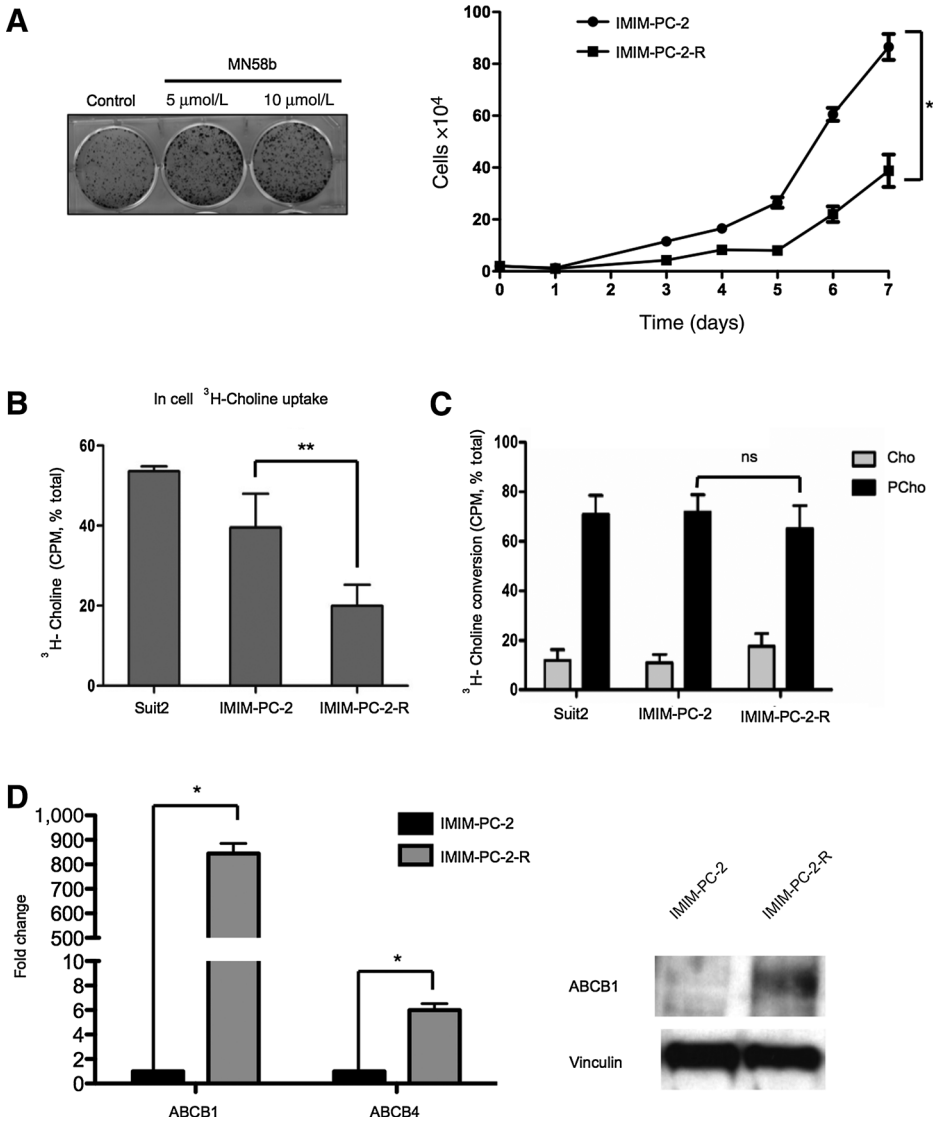

Figure 5.
Generation and characterization of MN58b-resistant IMIM-PC-2 cells: overexpression of ABCB transporters. A, generation of MN58b-resistant IMIM-PC-2 cells (left). Growth curves of parental versus IMIM-PC-2-R cells (right). B, choline (Cho) uptake in parental and IMIM-PC-2-R cells. C, *in vitro* choline kinase activity in parental versus IMIM-PC-2-R cells. D, validation of ABCB1 and ABCB4 overexpression in parental IMIM-PC-2-R cells by qRT-PCR (left) and western blotting (ABCB1; right). PCho, phosphocholine.

affected by acute treatment with MN58b (Supplementary Fig. S6), supporting the notion that selection pressure from chronic exposure to the drug is required for their upregulation.

The functional activity of the transporters was assessed using calcein-AM, an ABCB1 substrate that is converted to the fluorescent dye calcein in cells. IMM-PC-2-R cells showed 3-fold less intracellular calcein than their parental counterparts (Fig. 6A). To demonstrate that MDR proteins are responsible of the resistance, we inhibited their activity using verapamil, an L-type calcium channel blocker of the phenylalkylamine class, and zosuquidar, an ABCB-specific inhibitor. Pretreatment with both drugs increased calcein uptake in IMIM-PC-2-R cells 2- to 3-fold (Fig. 6B and C) and also reduced its IC_{50} to values in the range of parental IMIM-PC-2 (Supplementary Table S5).

Discussion

PDAC is one of the most chemoresistant tumors, and new targets and drugs are urgently needed. Metabolic reprogramming has emerged as a new hallmark of cancer providing opportunities for therapy (7). Beyond the extensively studied metabolic pathways involving glucose and glutamine, recent attention has focused on choline metabolism. Choline is not only important for cell proliferation, as it is the main source of phosphatidylcholine, but it also plays an important role in transformation through its cooperation with RhoA (31). Here, we have addressed the importance of CHKα in PDAC using both chemical and genetic inhibition, and we have tested the potential of MN58b, a CHKα inhibitor, against PDAC cells *in vitro*. We have also identified and characterized ABCB transporter upregulation as a new mechanism of acquired resistance to MN58b.

A recent study has shown high levels of choline and PC in PDAC cell lines and tumors, supporting the importance of CHKα as a potential metabolic target (32). Using bioinformatic analyses and a panel of cell lines, we show that CHKα is overexpressed in PDAC versus nontransformed HPDE pancreatic ductal epithelial cells, as has been reported in lung, breast, colorectal, and bladder cancer cells (11, 14), and that PDAC lines rank among those

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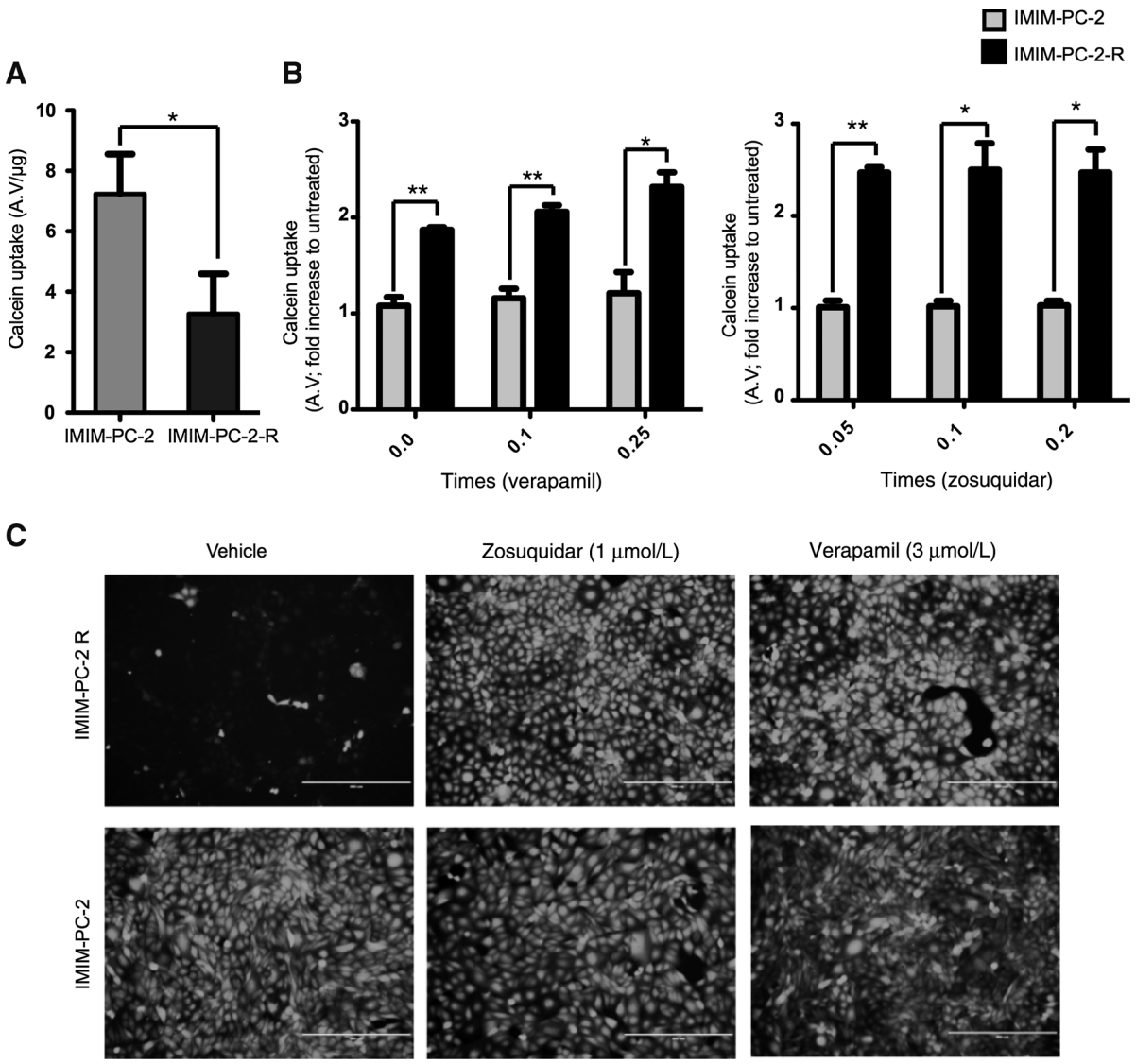

Figure 6.
Pharmacologic modulation of ABCB transporter activity restores sensitivity of IMIM-PC-2-R cells to MN58b. A, calcein uptake in parental and IMIM-PC-2-R cells. B, changes in calcein uptake in parental and IMIM-PC-2-R cells treated with variable concentrations of verapamil or zosuquidar. C, representative images of calcein uptake in parental and IMIM-PC-2-R cells treated with verapamil or zosuquidar.

expressing highest CHKα mRNA. The relationship between CHKα expression levels and tumor aggressiveness has not been extensively analyzed, but recent data in lung and bladder cancer support an association both using tumor samples and xenografts in mice (14, 33). Unlike in these reports, we did not find a good correlation between mRNA and protein levels in PDAC cells. To determine whether overexpression of CHKα results from a high metabolic rate or is required for cell growth, we inhibited the enzyme using MN58b, a specific CHKα inhibitor whose selectivity has recently been shown *in vivo* by magnetic resonance spectroscopy (34), and by genetic knockdown. CHKα inhibition by both strategies led to decreased cell proliferation, possibly due to reduced PI3K/AKT and MAPK signaling (35).

Immunohistochemical analysis of human PDAC samples revealed prominent cytoplasmic staining in >90% of samples. A similar high frequency of CHKα expression has been reported in prostate cancer (36) and in other tumor types, but this is the first report on PDAC. We found prominent nuclear CHKα staining in >40% PDAC samples, as was also recently reported in 1 of 20 prostate cancer samples of high Gleason score as well as in one prostatic intraepithelial neoplasia lesion (36). It has been hypothesized that CHKα could be phosphorylated and translocated to the nucleus with other proteins, such as ERK. Nuclear staining was associated with overall improved survival, although not independently of grade. The specificity of the antibody used was validated using knockdown experiments in cultured cells. These results require confirmation in independent series. The potential relevance of the nuclear localization of CHKα is supported by the association with patient survival, particularly among well/moderately differentiated tumors, unlike the cytoplasmic expression.

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More work is required to elucidate the function of CHKα in relationship to its localization. Whereas in the cytoplasm it can act as an oncoprotein promoting an increased synthesis of phosphatidylcholine and second messengers for survival pathways in more aggressive tumors, its nuclear distribution is associated with more differentiated tumors with less aggressive clinical behavior. Interestingly, we also found that high levels of CHKα mRNA are found in PDAC classified as "classical" or "exocrine" than in those that are "quasi-mesenchymal".

Pharmacologic inhibition of CHKα resulted in apoptosis, with a clear association between CHKα levels and drug sensitivity. The IC_{50} values obtained for PDAC cells are similar to those reported in other tumors, including bladder cancer (14). Our findings point to the potential value of CHKα levels as a predictive factor of response to inhibitors and are substantiated by the resistance resulting upon CHKα knockdown in sensitive cells. The relevance of factors predictive of drug response is becoming apparent as precision approaches are being applied in the clinics. Obviously, these *in vitro* results need to be validated *in vivo*.

Gemcitabine has been the mainstay of therapy in metastatic PDAC and remains an important drug, but tumors rapidly become resistant. MN58b is effective in gemcitabine-resistant cells, suggesting that CHKα inhibition may be effective as second-line treatment in patients progressing after treatment with this drug. Combination chemotherapy, such as FOLFIRINOX (5), has shown antitumor activity in PDAC. The evidence for additive/synergistic effects in experiments combining MN58b with gemcitabine, 5-FU, and oxaliplatin indicates that these combinations merit preclinical and clinical attention. The role of CHKα in the synthesis of crucial components of cellular membranes suggests that altered membrane composition or properties contribute to modulate the permeability of cells to antitumor drugs.

Because acquisition of drug resistance is the most common reason for therapeutic failure in oncology, it is important to establish mechanisms of resistance early on during drug development. We have generated a PDAC line with an IC_{50} 30-fold higher than the parental one and we have identified a novel mechanism of MN58b resistance through MDR gene overexpression. Inhibiting pump activity using verapamil, resulting in resensitization of IMIM-PC-2-R cells to MN58b, provides formal proof of the causal relationship between overexpression and resistance.

In conclusion, CHKα expression is deregulated in PDAC cells. Our results support the notion that CHKα represents a feasible therapeutic option in this tumor, alone or in combination with other chemotherapeutic agents, and anticipates new mechanisms of resistance. CHKα expression levels may be a predictive marker of response, associated with specific PDAC molecular subtypes.

Disclosure of Potential Conflicts of Interest

J.C. Lacal has ownership interest (including patents) in TCD Pharma SL. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J.M. Mazarico, V.J. Sánchez-Arévalo Lobo, J.C. Lacal, E. Aboagye, F.X. Real

Development of methodology: J.M. Mazarico, V.J. Sánchez-Arévalo Lobo, E. Carrillo-de Santa Pau, M. Marqués, E. Aboagye

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Mazarico, R. Favicchio, W. Greenhalf, E. Costello, M. Marqués

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Mazarico, V.J. Sánchez-Arévalo Lobo, R. Favicchio, E. Carrillo-de Santa Pau, M. Marqués, F.X. Real

Writing, review, and/or revision of the manuscript: J.M. Mazarico, V.J. Sánchez-Arévalo Lobo, W. Greenhalf, E. Costello, J.C. Lacal, E. Aboagye, F.X. Real

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Mazarico, F.X. Real

Study supervision: J.M. Mazarico, V.J. Sánchez-Arévalo Lobo, E. Aboagye, F.X. Real

Other (obtaining financial support): F.X. Real

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