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

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Abstract

Choline kinase \(\alpha\) (CHK\(\alpha\)) plays a crucial role in the regulation of membrane phospholipid synthesis and has oncogenic properties in vitro. We have analyzed the expression of CHK\(\alpha\) in cell lines derived from pancreatic ductal adenocarcinoma (PDAC) and have found increased CHK\(\alpha\) expression, associated with differentiation. CHK\(\alpha\) protein expression was directly correlated with sensitivity to MNS58b, a CHK\(\alpha\) inhibitor that reduced cell growth through the induction of apoptosis. Accordingly, CHK\(\alpha\) knockdown led to reduced drug sensitivity. In addition, we found that gemcitabine-resistant PDAC cells displayed enhanced sensitivity to CHK\(\alpha\) inhibition and, in vitro, MNS58b had additive or synergistic effects with gemcitabine, 5-fluorouracil, and oxaliplatin, three active drugs in the treatment of PDAC. Using tissue microarrays, CHK\(\alpha\) was found to be overexpressed in 90% of pancreatic tumors. While cytoplasmic CHK\(\alpha\) did not relate to survival, nuclear CHK\(\alpha\) 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 CHK\(\alpha\) inhibitors, we cultured IMIM-PC-2 cells with increasingly higher concentrations of MNS58b 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 CHK\(\alpha\) inhibition merits further attention as a therapeutic option in patients with PDAC and that expression levels may predict response. Mol Cancer Ther; 15(2); 323–33. ©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 (33%), 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 \(\alpha\) (CHK\(\alpha\)), of which two isoforms are generated by alternative splicing, and choline kinase \(\beta\) (CHK\(\beta\); ref. 9). CHK\(\beta\) has not been reported to be oncogenic (10), but CHK\(\alpha\) has been proposed to participate in initiation and progression of several tumors (11–14). These
findings render CHKα an attractive therapeutic target. Several choline kinase inhibitors (CHKI) have been developed from hemicholinium-3. MN58b, a first-generation CHKα-inhibitor, has antiproliferative and antitumor 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 PATLI 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 Zurich, Switzerland). Oncogene-immortalized HPDE (M. Tsoo, University Health Network, Toronto, Canada) and hTERT-immortalized human ductal pancreatic cells (HPNE, T. Gress) were used as controls (21). Nonpancreatic cells used 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, T. Gress) were used as controls (21). Nonpancreatic cells used were J82, RT112, and HT1376 (bladder; F. Radvanyi, I. Curie, T. Gress) were used as controls (21). Nonpancreatic cells used were J82, RT112, and HT1376 (bladder; F. Radvanyi, I. Curie, T. Gress) were used as controls (21). Nonpancreatic cells used were J82, RT112, and HT1376 (bladder; F. Radvanyi, I. Curie, T. Gress) were used as controls (21).

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.

Drug synergy assays

Cells (5 × 10⁴ per well) were seeded in 6-well plates, trypsinized, and counted. To determine viability, cells (2 × 10⁴ 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 × 10⁴ 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.

To produce viral particles, psPAX2 and pSVG 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).

To generate 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.

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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 mmol/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 tetrathenylborate 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 \(\mu\)g) were fractionated, transferred to nitrocellulose, and incubated with mouse monoclonal antibody AD8 recognizing CHK\(_{2}\) (1:1,000 dilution; ref. 24) or anti-rabbit anti-CHK\(_{2}\) polyclonal antibody (Sigma, HPA024153; 1/100). After washing, peroxidase-labeled antimouse 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 CHK\(_{2}\) mRNA isoform 1: AAAGAGGGATCCGGAA-CCTCTCTTTTATCTG (reverse); and ABCB4: GAGGTCAAAAACAGGAGATG (forward) and AGTGACCTCTCTGCGAGAATG (reverse); and ABCB1: CTGTGAAGAGTAGAACATGAAG (forward) and TTGCA-CAAGC (forward) and AGTGACCTCTCTGCGAGAATG (reverse). Reactions were performed in triplicate; expression levels were normalized to individual hypoxanthine phosphoribosyltransferase mRNA values using the \(\Delta\Delta\)Ct method (25).

In vivo subcutaneous tumorigenic assay

Suit2 028 CHK\(_{2}\)-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 \(\times\) 10\(^5\), 100 \(\mu\)L 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 EUI 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/H11005/87).

Sections were incubated in boiling water for 10 minutes in citrate buffer pH 6. Slides were incubated at 4°C overnight with rabbit anti-CHK\(_{2}\) polyclonal antisera (Sigma, 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. 

RNA-Seq and bioinformatics analysis

RNA from parental and MN58b-resistant cells was purified using TRIzol. Integrity was assessed 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 v3) and sequenced on Genome Analyzer Ix (GAIIx) with SBS TruSeq v5 reagents following the manufacturer’s instructions (SingleRead 1 \(\times\) 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/GRCCh37 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
Cuffdiff function included in cufflinks² (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 pre-ranked 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 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 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

![Figure 1](image-url)

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).
that, as in other tumor types, CHK\(\alpha\) is overexpressed in PDAC cells and is important to sustain cell growth.

**CHK\(\alpha\) 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 CHK\(\alpha\) 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 CHK\(\alpha\) 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. 3B, right). In the multivariable model, grade, but not CHK\(\alpha\) nuclear staining, was an independent variable associated with improved survival.

The antiproliferative activity of the CHK\(\alpha\)I MN58b is associated with CHK\(\alpha\) expression levels

MN58b selectively inhibits CHK\(\alpha\); 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 (SR-PC-1, Suit2 008, IMIM-PC-2, and RWP-1). MN58b had a marked effect on colony formation at 1 \(\mu\)mol/L, and growth was completely abolished at 5 \(\mu\)mol/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 \(\mu\)mol/L. We found a direct relationship between CHK\(\alpha\) protein expression and MN58b sensitivity (\(R^2 = 0.88\); Fig. 4B and Supplementary Table S3). CHK\(\alpha\) 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
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 IC50 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 IC50. 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.
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 IC50 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...
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-specfic inhibitor. Pretreatment with both drugs increased calcein uptake in IMM-PC-2-R cells 2- to 3-fold (Fig. 6B and C) and also reduced its IC50 to values in the range of parental IMM-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

Figure 5.
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.
More work is required to elucidate the function of CHKα in relationship to its localization. Whereas in the cytoplasm it can act as an oncprotein 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 IC50 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 IC50 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 re sensitization 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.

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Acknowledgments

The authors thank the members of the ECG laboratory and the Canceralia consortium for valuable discussions, C. Blanco and J. Hernández-Losa for help with the analysis of synergy, and G. Gómez from CNIO Bioinformatics Unit for help with the CCLE data analysis.

Grant Support

This work was supported, in part, by grants from the EU 7th Framework Programme (Project 289737-CANCERALIA, to F. X. Real, E. Costello, and E. Aboage); Ministerio de Economía y Competitividad (SAF2011-29699; to J. C. Lacal); Instituto de Salud Carlos III (RTICC RD12–0036–0034; to F. X. Real), COST Action EU-Pancreas BM120 (to J. M. Mazarico); and by the NIHR Liverpool Pancreas Biomedical Research Unit, Cancer Research UK, and Pancreatic Cancer UK (to E. Costello and W. Greenhalh). RTICC is supported, in part, by European Regional Funds. J.M. Mazarico was supported, in part, by a Rio Hortega Fellowship from the Instituto de Salud Carlos III, Madrid, Spain.

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Received March 16, 2015; revised October 23, 2015; accepted November 17, 2015; published OnlineFirst January 14, 2016.

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Choline Kinase in Pancreatic Ductal Adenocarcinoma


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doi:10.1158/1535-7163.MCT-15-0214

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