



Cytosolic 5'-nucleotidase 1A is overexpressed in pancreatic cancer and mediates gemcitabine resistance by reducing intracellular gemcitabine metabolites

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ABSTRACT

Background: Cytosolic 5'-nucleotidase 1A (NT5C1A) dephosphorylates non-cyclic nucleoside monophosphates to produce nucleosides and inorganic phosphates. Here, we investigate NT5C1A expression in pancreatic ductal adenocarcinoma (PDAC) and its impact on gemcitabine metabolism and therapeutic efficacy.

Methods: NT5C1A expression was determined by semiquantitative immunohistochemistry using tissue microarrays. Gemcitabine metabolites and response were assessed in several human and murine PDAC cell lines using crystal violet assays, Western blot, viability assays, and liquid chromatography tandem mass-spectrometry (LC-MS/MS).

Findings: NT5C1A was strongly expressed in tumor cells of a large subgroup of resected PDAC patients in two independent patient cohorts (44–56% score 2 and 8–26% score 3, $n = 414$). In contrast, NT5C1A was expressed at very low levels in the tumor stroma, and neither stromal nor tumoral expression was a prognostic marker for postoperative survival. *In vitro*, NT5C1A overexpression increased gemcitabine resistance by reducing apoptosis levels and significantly decreased intracellular amounts of cytotoxic dFdCTP in +NT5C1A tumor cells. Co-culture experiments with conditioned media from +NT5C1A PSCs improved gemcitabine efficacy in tumor cells. *In vivo*, therapeutic efficacy of gemcitabine was significantly decreased and serum levels of the inactive gemcitabine metabolite dFdU significantly increased in mice bearing NT5C1A overexpressing tumors.

Interpretation: NT5C1A is robustly expressed in tumor cells of resected PDAC patients. Moreover, NT5C1A mediates gemcitabine resistance by decreasing the amount of intracellular dFdCTP, leading to reduced tumor cell apoptosis and larger pancreatic tumors in mice. Further studies should clarify the role of NT5C1A as novel predictor for gemcitabine treatment response in patients with PDAC.

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Research in context*Evidence before this study*

Pancreatic ductal adenocarcinoma is highly refractory to systemic therapies. Poor therapeutic response to gemcitabine in PDAC has been attributed in part to altered drug metabolism, in particular reduced cellular uptake or rapid enzymatic inactivation. Indeed, high expression of the human equilibrative nucleoside transporter (hENT) has been shown to predict improved patient survival following gemcitabine treatment.

The 5'-nucleotidase (NT5) family of enzymes dephosphorylates non-cyclic nucleoside monophosphates to produce nucleosides and inorganic phosphates. Interestingly, NT5C1A is a previously unrecognized gemcitabine inactivating enzyme that prevents gemcitabine activation by dephosphorylating gemcitabine monophosphate (dFdCMP). However, the detailed expression pattern in PDAC patients and the role of NT5C1A in mediating resistance to gemcitabine is currently unknown.

Added value of this study

The present study provides evidence that NT5C1A is strongly expressed in tumor cells of resected PDAC patients. Mechanistically, we show that NT5C1A is implicated in gemcitabine resistance *in vitro* and *in vivo* by decreasing the amount of intracellular cytotoxic gemcitabine metabolites. Only a small fraction of PDAC patients show NT5C1A expression in the tumor stroma, and high expression in stromal cells may contribute to improved gemcitabine sensitivity in tumor cells.

Implications of all the available evidence

We have elucidated the expression pattern and function of NT5C1A in human and mouse PDAC using several *in vitro* and *in vivo* models. Thus, our study introduces NT5C1A as gemcitabine inactivating enzyme in PDAC and paves the way for the evaluation of NT5C1A for stratified treatment approaches, both for adjuvant as well as palliative therapy regimens.

dFdU	2',2'-difluoro-2'-deoxyuridine
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
g	gram(s)
h	hour(s)
H&E	hematoxylin and eosin
HA-tag	hemagglutinin tag
hEnt	human equilibrative nucleoside transporter
HRP	horseradish peroxidase
HSP90	heat shock protein 90
ICC	immunocytochemistry
IHC	immunohistochemistry
IgG	immunoglobulin G
kg	kilogram
KPC	LSL-Kras ^{G12D/+} ;LSL-Trp53 ^{R172H/+} ;Pdx-1-Cre
LC-MS/MS	liquid chromatography tandem mass-spectrometry
MEM	Minimum Essential Media
mg	milligrams
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger ribonucleic acid
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
nM	nanomolar
ns	non-significant
NT5	5'-nucleotidase
NT5C1A	cytosolic 5'-nucleotidase 1A
PBS	phosphate buffered saline
PDAC	pancreatic ductal adenocarcinoma
pM	picomolar
PSCs	pancreatic stellate cells
qRT-PCR	quantitative real-time polymerase chain reaction
RRM1	ribonucleotide reductase M1
SEM	standard error of mean
TAM	tumor-associated macrophages
TMA	tissue microarray
TME	tumor microenvironment
U.S.	United States
WB	Western blot

Abbreviations

%	per cent
µm	micrometer
µM	micromolar
5-FU	5-fluorouracil
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
CAFs	cancer-associated fibroblasts
CC3	cleaved caspase-3
CDA	cytidine deaminase
CM	conditioned medium
day 0	day 0
DAPI	4',6-diamidino-2-phenylindole
dCK	deoxycytidine kinase
DCTD	deoxycytidylate deaminase
dFdC	2',2'-difluoro-2'-deoxycytidine
dFdCMP	2',2'-difluoro-2'-deoxycytidine monophosphate
dFdCDP	2',2'-difluoro-2'-deoxycytidine diphosphate
dFdCTP	2',2'-difluoro-2'-deoxycytidine triphosphate

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid human tumors. Due to the lack of early symptoms, around 80% of patients are diagnosed with locally advanced or metastatic disease rendering them inoperable [1–3]. Current statistics project pancreatic cancer to become the second cause of cancer-related death in the U.S. by the year 2030 [4].

Systemic treatment options have been of limited success since PDAC is intrinsically highly refractory to medical therapies. Gemcitabine monotherapy has been the standard of care for palliative and adjuvant treatment for many years without providing clinically meaningful effects on overall survival [5]. More recently, intensified chemotherapy regimens have emerged that can improve overall survival. To this end, FOLFIRINOX (FOLinic acid, 5-FU, IRINotecan and OXaliplatin) and gemcitabine plus nab-paclitaxel are available in the palliative setting [6,7], and gemcitabine plus capecitabine (a 5-FU prodrug) was shown to increase long-term survival as adjuvant treatment compared to gemcitabine alone [8]. Nevertheless, therapy regimens like FOLFIRINOX require a good performance status of the patient due to increased toxicity [6]. However, despite the availability of intensified treatment protocols, the prognosis for PDAC patients remains extremely poor in comparison with other solid malignancies with median survival times below one year in patients with advanced or metastatic disease [9].

Poor therapeutic response to gemcitabine in PDAC has been attributed in part to altered drug metabolism, in particular, reduced cellular uptake or rapid enzymatic inactivation [10–13]. Gemcitabine is a hydrophilic drug that is administered as prodrug (2',2'-difluoro 2'-deoxycytidine; dFdC) and cellular uptake is predominantly accomplished by human equilibrative nucleoside transporters (hENT). Subsequently, the prodrug dFdC is phosphorylated to mono-, di- and tri-phosphate (dFdCMP, dFdCDP and dFdCTP), blocking *de novo* DNA synthesis by inhibition of ribonucleotide reductase (RRM), or terminating DNA chains through incorporation of dFdCTP into DNA [14]. The rate-limiting activation step is the phosphorylation by deoxycytidine kinase (dCK). Inactivation of gemcitabine and its metabolites is conferred by cytidine deaminase (CDA), and other enzymes such as deoxycytidylate deaminase (DCTD) [14,15]. Moreover, molecular competition through increased levels of deoxycytidine triphosphate further reduce the effective levels of gemcitabine [16].

The 5'-nucleotidase (NT5) family of enzymes dephosphorylates non-cyclic nucleoside monophosphates to produce nucleosides and inorganic phosphates. There are seven human NT5Cs of which cytosolic NT5C1A is mainly expressed in muscle and heart [17]. Under physiological conditions, NT5C1A regulates the pool of adenosine monophosphate (AMP), which is, in turn, responsible for allosterically stimulating AMPK activity [18,19]. In addition, NT5C1A is a previously unrecognized gemcitabine inactivating enzyme that dephosphorylates gemcitabine monophosphate (dFdCMP) to the prodrug dFdC, thus potentially limiting the cytotoxicity of gemcitabine by decreasing the formation of dFdCTP [20–22].

Notably, the role of nucleotidases that may mediate drug resistance through dephosphorylation of nucleoside analogues has not been investigated in PDAC so far. We have recently described differential expression of NT5C1A in human and murine stromal and epithelial cells for the first time in PDAC [23]. However, there are currently no data for PDAC or any other solid tumor that implicates NT5C1A in therapeutic resistance to gemcitabine. Here, we explored the expression pattern and frequency of NT5C1A in two large and independent cohorts of PDAC patients and determined the role of NT5C1A in mediating resistance to gemcitabine using several *in vitro* and *in vivo* approaches.

2. Materials and methods

2.1. Cell lines and cell culture

The LSL-Kras^{G12D/+}; LSL-Trp53^{R172H/+}; Pdx-1-Cre (KPC) mouse model is a commonly used genetically engineered mouse model for pancreatic cancer [24]. Thus, murine KPC cell lines were used for this study. These cells were derived from KPC mice as previously described [24]. KPC cells were maintained in culture according to standard procedures in high glucose DMEM with phenol red, supplemented with 10% fetal bovine serum (FBS) and 1% non-essential amino acids. MEM without phenol red, supplemented with 10% FBS and 1% L-glutamine was used for human L3.6pl cells. Immortalized murine PSCs from a previous study [23] were cultured in high glucose DMEM with phenol red and supplemented with 10% FBS.

Mycoplasma tests of KPC-BL6 cell lines and of L3.6pl cells were performed prior to orthotopic transplantation and liquid chromatography tandem mass-spectrometry (LC-MS/MS), respectively.

2.2. Establishment of cell lines stably expressing NT5C1A

KPC, L3.6pl cells, and PSCs were transfected with a pSG5-vector-derivative containing the sequence of murine NT5C1A or a control plasmid without the NT5C1A insert. We followed the protocol as described by Kari et al. [25] to generate the constructs. The pSG5-vector (a kind gift of Prof. Johnsen, University Medical Center Goettingen) containing an HA-tag upstream of a multiple cloning site, a P2A-sequence and a hygromycin resistance gene was transfected into each cell line as

recently described by our group [23]. The following hygromycin concentrations (Hygromycin B Gold, InvivoGen) were used for selection: KPC1: 500 µg/ml, KPC2: 900 µg/ml, L3.6pl: 500 µg/ml, PSCs: 250 µg/ml. For maintenance, only half of these concentrations were added to the culture media.

2.3. Immunohistochemistry and immunocytochemistry

Mouse tissues were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. 4 µm sections were cut from the tissues and processed for H&E staining and immunohistochemistry using standard protocols as previously described [23]. Pictures were taken with 40× magnification with an Olympus DP27 camera and the Olympus cellSens Entry 1.12 software.

Immunocytochemistry was performed for KPC cell lines as previously described [23]. Briefly, cells were fixed with methanol, permeabilized using triton X-100 and incubated with primary antibodies for NT5C1A (Assay Biotech Cat# C15296, RRID:AB_10687827) and HA-tag (Cell Signaling Technology Cat# 3724, RRID:AB_1549585) at 4 °C overnight. Alexa Fluor®-conjugated secondary antibodies were used for detection and slides were mounted using DAPI-containing mounting solution (Vectashield®). Image acquisition was done utilizing a Leica DMi8 microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and images were extracted with equal adjustments between groups using Fiji/ ImageJ (Fiji, RRID:SCR_002285) [26]. A detailed list of all antibodies and dilutions can be found in the supplementary materials.

2.4. Tissue microarrays

Tissue microarrays (TMA) were obtained from the Department of Pathology, University Medical Center Goettingen (TMA-1, *n* = 77 patients) and the Department of Pathology, University Medical Center Erlangen (TMA-2, *n* = 337 patients) from postoperative PDAC patients. Written consent was obtained from every patient prior to tissue collection and analysis. Expression levels of NT5C1A were semi-quantitatively analyzed by an experienced pathologist at each site from score 0 (no expression) to score 3 (strong expression). Immunohistochemistry was performed using standard protocols with hematoxylin counterstaining. TMA-1 staining was performed with citrate buffer pH 6.0, NT5C1A antibody (Assay Biotech Cat# C15296, RRID:AB_10687827, 1:100), and the VECTASTAIN® ABC Rabbit Kit with ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories). Pro Taqs II Antigen Enhancer pH 9.5, NT5C1A antibody (Atlas Antibodies Cat# HPA050283, RRID:AB_2681072, 1:100) and the ImmPress Reagent Kit Anti-Rabbit IgG (Vector Laboratories) with AEC-Plus Substrate-Chromogen (Dako Denmark A/S) were used for TMA-2.

2.5. Western blot analysis

Western blot analyses were performed as previously described with 35 µg of protein [23]. Membranes were incubated with chemiluminescence substrate Plus-ECL (PerkinElmer Inc.) or Ultra-ECL (for CC3-detection) and protein bands were detected at the ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories GmbH) using Image Lab Software (RRID:SCR_014210, version 5.2.1). Quantification was performed using the rectangle volume tool of the Image Lab Software. Values were normalized to HSP90 expression and to the expression of untreated protein lysates. Murine muscle lysate was used as positive control for NT5C1A expression (3 µg). A detailed list of primary and secondary antibodies is listed in supplementary materials.

2.6. Crystal violet cell proliferation assay

The commonly used crystal violet staining [27] was performed to determine the sensitivity of transfected cell lines towards gemcitabine and

paclitaxel (both Sigma-Aldrich). To this end, cells were seeded at a density of 2000 cells (KPC cells, PSCs) and 7500 cells (L3.6pl) in 24-well plates and were allowed to attach for 24 h. Subsequently, cells were treated with increasing concentrations of gemcitabine-hydrochloride or paclitaxel, respectively. Cells were treated for six days and treatment media was renewed every other day. Finally, cells were washed with PBS, fixed with methanol and subsequently stained with crystal violet solution (0.1% in 20% ethanol). Regarding gemcitabine, three (tumor cell lines) and two (PSCs) independent experiments were performed, respectively. For paclitaxel treatment, two independent assays were carried out. Quantification of crystal violet assays was performed using 10% acetic acid to solubilize crystal violet stain and subsequent photometric measurements of diluted samples at 595 nm. Results were normalized to untreated control cells.

2.7. Preclinical *in vivo* study using a syngeneic orthotopic mouse model

All animal procedures were conducted according to institutional and national regulations. Housing conditions for the mice were at a 12 h light and 12 h dark cycle. C57BL/6-J mice (Charles River) were used for orthotopic transplantation studies. Mice were transplanted at the age of eight weeks and randomized into four groups before transplantation ($n = 7$ per group). Stably transfected syngeneic KPC-BL6 cells (+NT5C1A) and respective control cells were trypsinized, and 150,000 viable cells were mixed in an equal volume of matrigel prior to transplantation. A total volume of 40 μ l was injected into the tail of the pancreas. Small animal high-resolution ultrasound was performed as previously described [28]. In brief, a Visual Sonics Vevo 2100 High Resolution Ultrasound System, including imaging stage and anesthesia line (FUJIFILM VisualSonics Inc., Canada) was used for the study with a Vevo 2100 MicroScan Transducer MS-550-D (22–55 MHz). Mice were anesthetized with 2% isoflurane during ultrasound examinations.

Treatment with gemcitabine or saline started seven days after surgery. Mice were then sacrificed on day 14 of the treatment, with injections on day 0, 3, 7, 10, and 13. Gemcitabine-hydrochloride was administered intraperitoneally at 100 mg/kg body weight. Comparable volumes of saline were injected for control animals. Mice were weighed three times per week. As previously described, mice were sacrificed exactly 2 h after the last gemcitabine injection [29].

2.8. RNA preparation and qRT-PCR

RNA was extracted from cells using the PeqGold Total RNA kit (Peqlab GmbH) according to the manufacturer's protocol. RNA concentration was determined using a nanophotometer (INTAS P330) and 1 μ g was used for cDNA preparation. Recombinant RNasin ribonuclease inhibitor (Promega GmbH) was added to the extracted RNA samples prior to reverse transcription. M-MLV reverse transcriptase (Invitrogen) and poly(dT)₁₅ oligo primers (Roche Diagnostics GmbH) were used for cDNA preparation. Myoblast-mRNA was kindly provided by Prof. J. Schmidt, University Medical Center Goettingen and used as a positive control for human NT5C1A expression. Murine muscle RNA served as positive control for murine NT5C1A expression. TaqMan probes for NT5C1A and for β -actin are listed in supplementary materials.

2.9. Liquid chromatography tandem mass-spectrometry (LC-MS/MS)

For LC-MS/MS analysis of gemcitabine treated cells, 700,000 cells were seeded into 6-well plates. 24 h later, cells were exposed to 1 μ M of gemcitabine-hydrochloride for 2 h. Homogenates of cell pellets were analyzed for native dFdC and its triphosphate metabolite dFdCTP by LC-MS/MS according to a previously published protocol [30,31]. Three biological replicates, with 2–3 technical replicates were analyzed for gemcitabine metabolites. Serum samples from orthotopically transplanted mice were taken 2 h after administering the last

gemcitabine dose and were analyzed for 2',2'-difluoro-2'-deoxyuridine (dFdU).

2.10. Statistical analysis

GraphPad Prism (RRID:SCR_002798, version 7.03) was used for statistical analysis. Data are shown as mean \pm SEM. Statistical significance was considered for $p < .05$, with * <0.05 , ** <0.01 , and *** <0.001 . Two-tailed Student *t*-test was used for analysis, if not stated otherwise.

3. Results

3.1. NT5C1A is strongly expressed in murine and human PDAC and is not associated with overall survival

Comprehensive expression data of NT5C1A in PDAC are not available so far. To address this question, we employed two independent TMAs of resected PDAC specimens. A semi-quantitative scoring system ranging between score 0 (no expression) to score 3 (strong expression) was utilized. A relevant subgroup of patients expressed NT5C1A at high levels (score 2 and 3). 56% of all patients showed moderate expression (score 2) of NT5C1A in the epithelial compartment of the tumors and 8% expressed NT5C1A at high levels (score 3) in TMA-1 with 77 samples (Fig. 1A). A larger TMA dataset with 337 samples confirmed these findings with 44% scored with 2, and 26% of all patient samples had score 3 (Fig. 1B). Furthermore, only 13.0% (TMA-1) and 4.5% (TMA-2) of all tumors did not show immunoreactivity against NT5C1A (Fig. 1A–C). Interestingly, NT5C1A expression did not correlate with overall survival in either TMA, comparing low NT5C1A expression (score 0 and 1) with high expression levels of NT5C1A (score 2 and 3). Median survival was 18.5 vs. 15 months in TMA-1 ($p = .5$), and 17 vs. 16.4 months in TMA-2 ($p = .3$) (Fig. 1D and E). Moreover, KPC pancreatic tumors were analyzed for NT5C1A expression and robust expression could be confirmed by IHC staining in the majority of samples, and was already present in early stages of pancreatic cancer (Fig. 1F). The expression was confirmed by qRT-PCR to determine mRNA levels for NT5C1A in KPC bulk tissue. As expected no expression was found in healthy control pancreata (Fig. 1G).

3.2. NT5C1A expression in murine and human PDAC cell lines

In analogy to our published data about gemcitabine drug scavenging of stromal cells [23], we hypothesized that high levels of NT5C1A within the tumor cells might be involved in chemoresistance in PDAC, in particular gemcitabine resistance. To this end, NT5C1A may affect gemcitabine metabolism by reversing the initial phosphorylation step of dFdC to dFdCMP, resulting in decreased levels of cytotoxic dFdCTP. To test this hypothesis, NT5C1A expression was investigated in murine and human pancreatic cancer cell lines. Interestingly, murine and human pancreatic cancer cells revealed only low levels of NT5C1A expression *in vitro* compared to robust expression levels in the tumor tissue (Fig. S1A and B). Therefore, NT5C1A was stably expressed in murine KPC cell lines (KPC1 and KPC2 = KPC-BL6) and in the human L3.6pl cell line. Stable cell lines were established using a pSG5-vector derivative with subsequent hygromycin selection. NT5C1A expression in stable cell lines was validated by Western blot analysis, immunocytochemistry, and qRT-PCR analysis (Fig. 2A–D). NT5C1A and the integrated HA-tag were robustly expressed in transfected cells. Control cells that were transfected with an empty vector, were shown to express endogenous NT5C1A at low levels and did not show HA-tag expression by immunocytochemistry (Fig. 2A and B). Additionally, qRT-PCR analysis was employed to confirm mRNA expression of NT5C1A, and Western blot analysis was used to determine NT5C1A protein levels in the overexpressing cells (Fig. 2C and D).

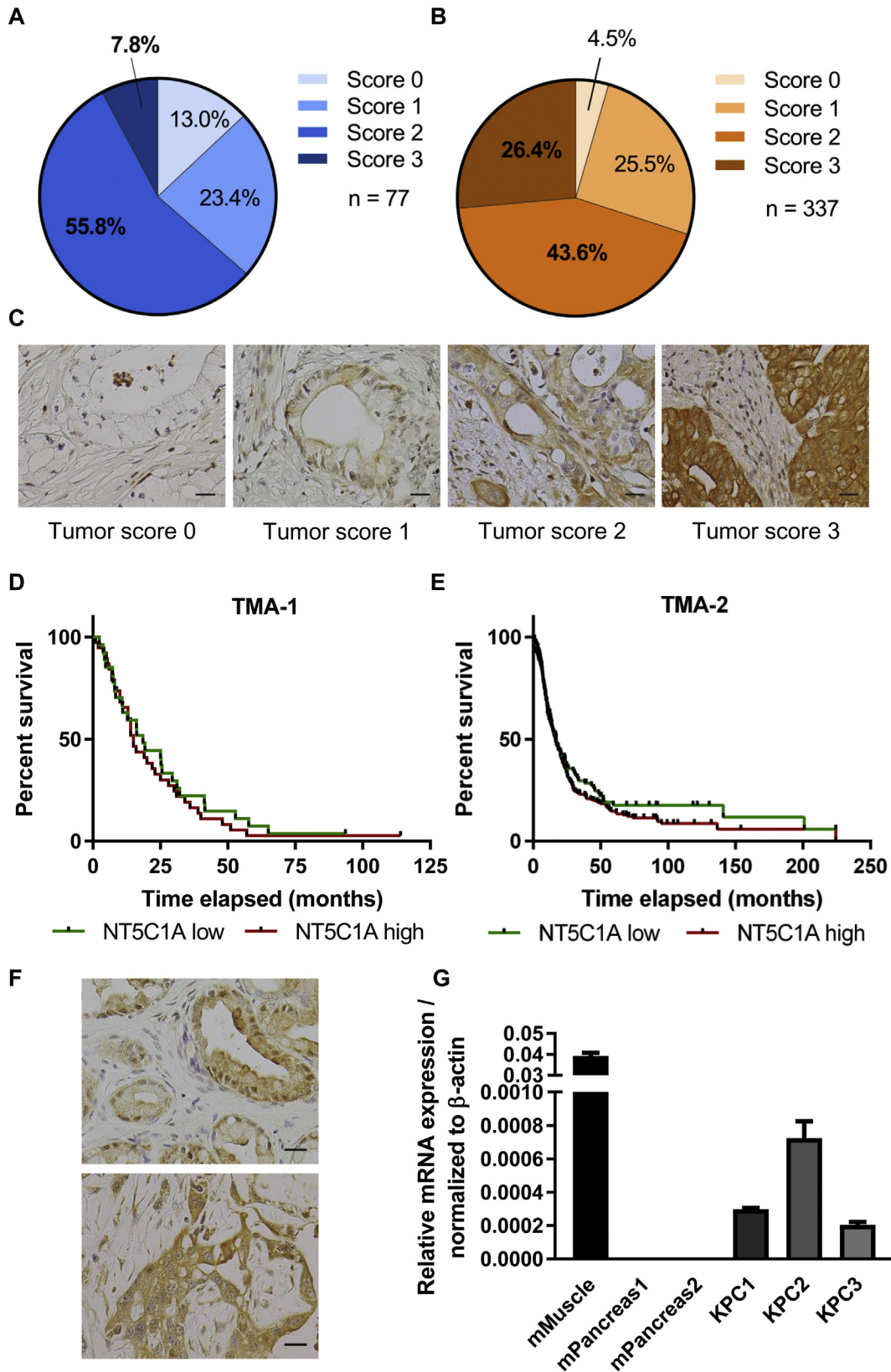


Fig. 1. Expression of cytosolic 5'-nucleotidase 1A (NT5C1A) in resected PDAC patients and in the KPC mouse model. Tissue microarrays (TMA) from A) Goettingen (TMA-1, $n = 77$) and B) Erlangen (TMA-2, $n = 337$). Score 0 = no NT5C1A expression, score 3 indicates strong intratumoral expression of NT5C1A. C) Representative images of TMA-1 for tumoral expression of NT5C1A scored with score 0, score 1, score 2, and score 3 (from left to right). Scale bars 20 μ m. D) and E) Survival analysis of patients from the Goettingen TMA cohort (D) and the Erlangen TMA cohort (E). Median survival of TMA-1 with low = 18.5 months ($n = 27$) and high = 15 months ($n = 38$; $p = .5$, log-rank test) and of TMA-2 with low = 17 months ($n = 101$) and high = 16.4 months ($n = 235$; $p = .3$, log-rank test). F) Representative images of NT5C1A immunohistochemistry in KPC mice with PanINs (upper image) and invasive carcinoma (lower image). Scale bars 20 μ m. G) NT5C1A-mRNA expression in KPC bulk tissue ($n = 3$ mice) and control normal pancreas ($n = 2$ mice). Mean \pm SEM of two technical replicates is shown for each mouse. Murine muscle was used as positive control and values were normalized to the housekeeping gene β -actin.

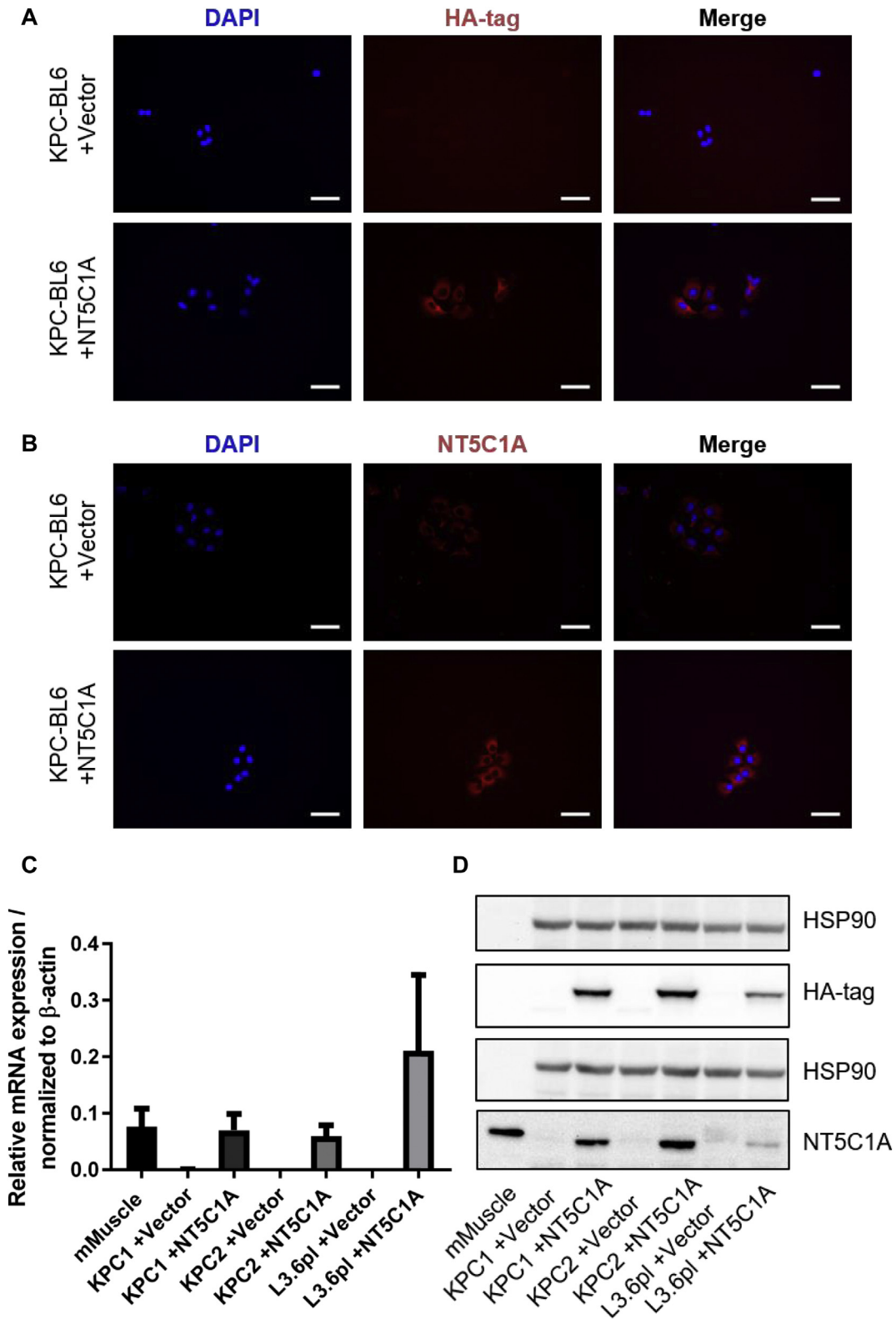


Fig. 2. Recombinant expression of NT5C1A in human and murine pancreatic cancer cell lines. A) and B) Representative images of two biological replicates of immunocytochemistry staining for NT5C1A and HA-tag. Robust staining is shown for HA-tag (A, lower panel) and NT5C1A (B, lower panel) in transfected KPC-BL6 (=KPC2) cells. No staining of HA-tag (A, upper panel) and low endogenous NT5C1A expression (B, upper panel) were detected in vector control cells (n = 2), scale bars 50 μ m. C) Quantitative RT-PCR analysis confirmed overexpression of NT5C1A in transfected cells. Diagram indicates mean \pm SEM of three biological replicates. Murine muscle sample was used as positive control and values were normalized to the housekeeping gene β -actin. D) Western blot analysis confirmed NT5C1A protein expression in transfected tumor cells with hardly any expression in vector control cells. Robust expression of HA-tag is shown in all +NT5C1A cell lines. Representative image of three independent experiments is shown. Murine muscle lysate was used as positive control for NT5C1A expression.

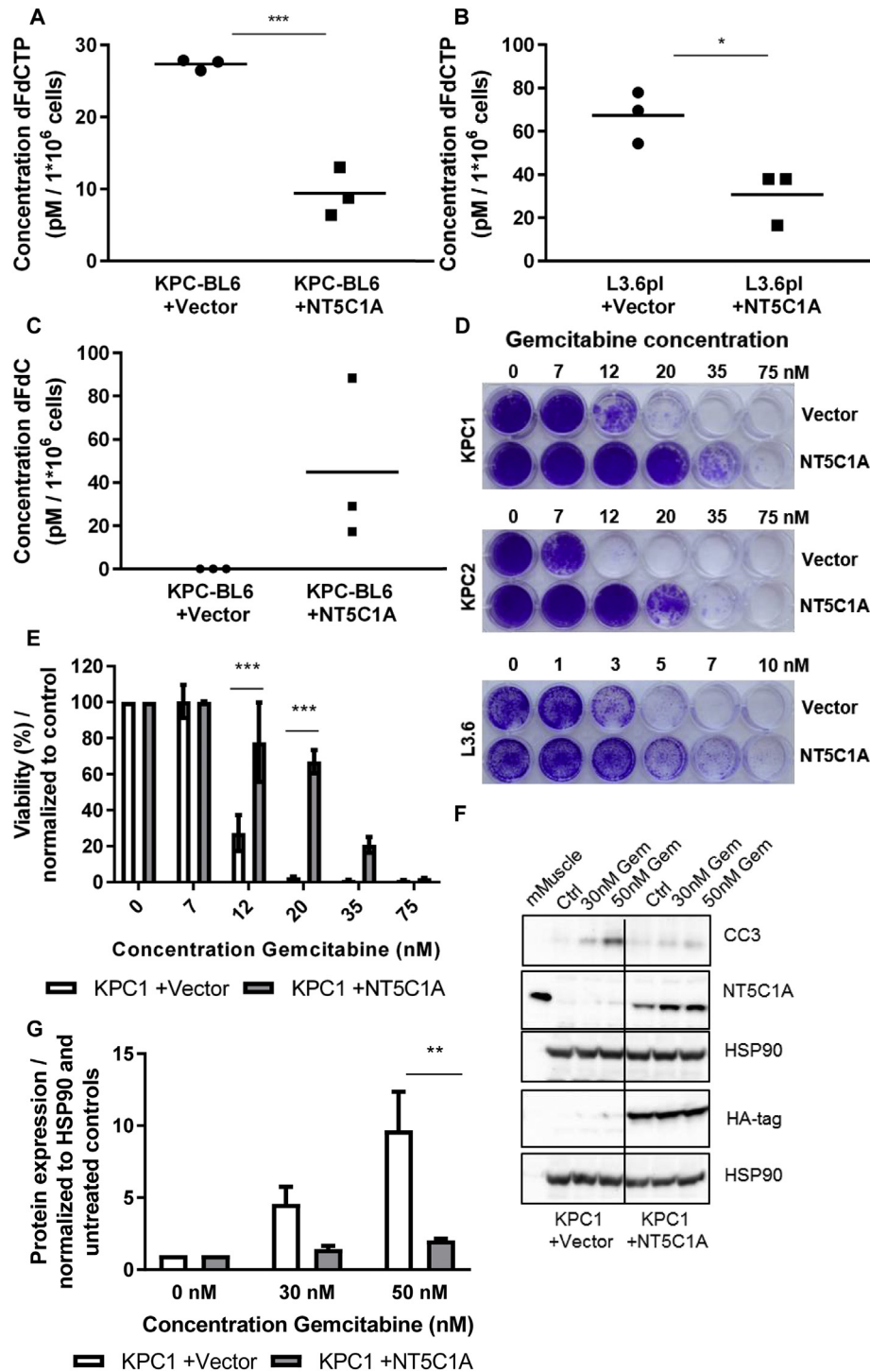


Fig. 3. High levels of NT5C1A increase chemotherapeutic resistance towards gemcitabine in pancreatic cancer cells. A) Pharmacokinetic analysis of gemcitabine metabolites in murine PDAC cells. Murine KPC-BL6 cells (A) and human L3.6pl cells (B) (+NT5C1A) and respective control cells (+vector) were simultaneously treated with 1 μ M gemcitabine-hydrochloride for 2 h. The concentration of the active gemcitabine metabolite dFdCTP (KPC-BL6: 27.3 vs. 9.4 pM per 1×10^6 cells, $p = .0008$ and L3.6pl: 67.3 vs. 30.8 pM per 1×10^6 cells, $p = .021$) and of native gemcitabine dFdC (C) were determined in homogenates of cell pellets using LC-MS/MS-analysis. Three biological replicates and the mean value are shown. Each dot represents the mean of three (KPC-BL6) or two (L3.6pl) technical replicates, respectively. All dFdC measurements of KPC-BL6 control cells were below the level of quantification, thus the values were set to zero. D) Murine and human pancreatic cancer cell lines were incubated with different concentrations of gemcitabine for six days. Crystal violet staining was more pronounced in cell lines with high NT5C1A expression. Three independent experiments, with each two technical replicates, were performed for all cell lines shown. E) Quantification of crystal violet assays for KPC1 cells using 10% acetic acid to solubilize crystal violet stain and photometric measurements at 595 nm. Results were normalized to untreated control cells. Two-way ANOVA with Sidak's multiple comparisons test was performed (12 nM: $p = .0007$, 20 nM: $p < .0001$). Graph shows mean \pm SEM. F) Western blot analysis revealed reduced cleaved caspase-3 (CC3) protein levels in NT5C1A-expressing cells following gemcitabine treatment for 24 h. Strong NT5C1A and HA-tag expression was demonstrated in the +NT5C1A-cell line. Three independent experiments were performed. G) Quantification of CC3 protein expression in these three Western blot analyses. Two-way ANOVA with Sidak's multiple comparisons test was performed (30 nM: $p = .249$, 50 nM: $p = .002$). Graph shows mean \pm SEM.

3.3. Pharmacokinetics of gemcitabine upon recombinant NT5C1A expression

NT5C1A dephosphorylates gemcitabine monophosphate (dFdCMP) to the prodrug dFdC, thus potentially limiting the cytotoxicity of gemcitabine by increasing the pool of dFdC and decreasing the amount of cytotoxic dFdCTP. To test the implication of NT5C1A on gemcitabine metabolism in pancreatic cancer we analyzed gemcitabine metabolites in cell pellets upon gemcitabine treatment using LC-MS/MS. The concentrations of the native form of gemcitabine dFdC as well as the triphosphate, the active metabolite of gemcitabine (dFdCTP) were measured using a previously established protocol [30,31]. Following NT5C1A overexpression in the murine KPC-BL6 cell line, upon 2 h of gemcitabine treatment, the dFdCTP concentration was significantly reduced compared to control cells (27.3 vs. 9.4 pM per 1×10^6 cells, $p = .0008$) (Fig. 3A). Equivalent results were seen for the human L3.6pl cell line (67.3 vs. 30.8 pM per 1×10^6 cells, $p = .021$) (Fig. 3B). Interestingly, intracellular dFdC levels were only detectable in the NT5C1A-overexpressing KPC-BL6 cells, all values in the vector control cells were below the limit of quantification (Fig. 3C). Thus, our findings suggest a significant contribution of NT5C1A towards the availability of active gemcitabine metabolites in PDAC, and possibly gemcitabine resistance.

3.4. NT5C1A overexpression confers chemotherapeutic resistance towards gemcitabine *in vitro*

We showed that NT5C1A decreases the concentration of dFdCTP *in vitro*, and thus hypothesized that NT5C1A may be an important candidate in mediating gemcitabine resistance. Using crystal violet cytotoxicity assays for two murine KPC cell lines as well as the human L3.6pl pancreatic cancer cell line, we found that NT5C1A expression reverts chemosensitivity in a concentration-dependent manner in all cell lines (Fig. 3D and E, Fig. S2A and B). In contrast, all cell lines were still sensitive to treatment with paclitaxel, which is another important chemotherapeutic agent for the treatment of pancreatic cancer patients (Fig. S3A and B). To confirm and further clarify the involvement of NT5C1A in gemcitabine resistance, we performed Western blot analysis for cleaved caspase-3 (CC3) protein levels upon gemcitabine treatment. Gemcitabine-induced CC3-levels were reduced by factor 4 following recombinant NT5C1A expression in KPC1 cells (Fig. 3F and G). Similar results were obtained for the KPC-BL6 cell line (factor 2, Fig. S2C and D). These results confirmed the impact of NT5C1A on gemcitabine resistance *in vitro*.

3.5. NT5C1A expression and function in the tumor stroma

With the objective to obtain a more comprehensive picture of the clinical situation regarding NT5C1A, we analyzed both TMAs for stromal NT5C1A expression. The same scoring system was employed with score 0 indicating no stromal expression of NT5C1A and score 3 indicating strong expression of NT5C1A. The proportion of PDAC patients without stromal NT5C1A expression was between 87.7% in TMA-1 ($n = 77$) and 47.3% in TMA-2 ($n = 330$). Low expression was detected in 11.0% (TMA-1) and 33.3% (TMA-2) of these patients. Interestingly, high scores [2 and 3] of stromal NT5C1A was only given to the samples of 1.4% (TMA-1) or 19.4% (TMA-2) of all patients (Fig. 4A–C). Consistent with the tumor data, stromal NT5C1A expression was not a prognostic marker. The median post-surgical survival time was 15.2 months for patients that did not express NT5C1A ($n = 156$) in the tumor stroma and 17.3 months for all other patients ($n = 173$; $p = .3$, TMA-2) (Fig. S4F). Due to the lack of more detailed treatment details, it remains to be answered whether stromal NT5C1A might be a predictive marker for gemcitabine response in PDAC patients. As shown by our group previously, NT5C1A is expressed at very low levels in cancer-associated fibroblasts (CAFs) and pancreatic stellate cells (PSCs) [23]. Furthermore,

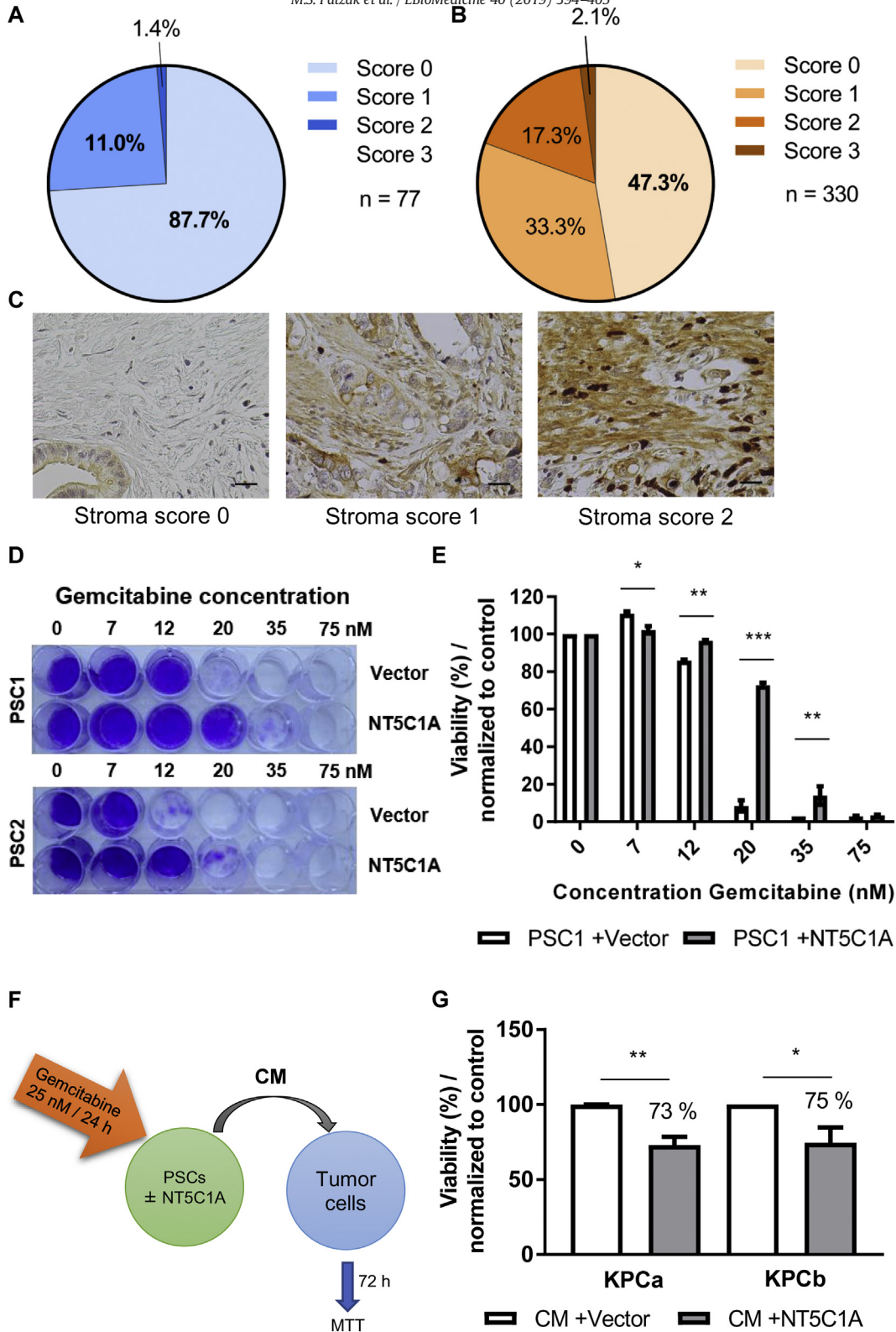
PSCs do not differ significantly regarding intracellular dFdCTP accumulation compared to CAFs [23]. Therefore, two PSC cell lines were stably transfected and + NT5C1A cells and vector control cells were analyzed by Western blot as previously shown [23], and also by immunocytochemistry and qPCR. HA-tag expression and robust expression of NT5C1A was confirmed in the +NT5C1A-cells (Fig. S4A–D). Crystal violet staining showed increased resistance of NT5C1A expressing PSCs to gemcitabine treatment (Fig. 4D and E, Fig. S4E). Considering our previously published findings that +NT5C1A-PSCs accumulate significant lower amounts of intracellular gemcitabine triphosphate [23], we hypothesized that gemcitabine availability would be significantly increased in the supernatant of NT5C1A-PSCs. To test this, MTT assays with conditioned media (CM) of transfected PSCs were performed. PSCs were incubated with 25 nM of gemcitabine-hydrochloride for 24 h and CM was then transferred to two KPC tumor cell lines (Fig. 4F). Cell viability was determined 72 h later and demonstrated significantly decreased tumor cell viability with CM of +NT5C1A-PSCs, compared to vector control cells with only endogenous levels of NT5C1A. In the two KPC cell lines the viability decreased to 73% and 75% respectively (KPCa: $p = .003$ and KPCb: $p = .047$) (Fig. 4G).

3.6. NT5C1A expression mediates chemoresistance *in vivo*

Finally, we aimed to investigate whether NT5C1A mediates chemoresistance *in vivo*. To this end, we employed a syngeneic, orthotopically transplanted mouse model using C57BL/6-J mice and stably transfected KPC-BL6 cells. Seven days after tumor cell transplantation, mice were treated with gemcitabine or saline for 14 days (Fig. S5A). High-resolution small animal ultrasound screening was performed on day 9 of the treatment to verify tumor growth upon transplantation (Fig. 5A). Intratumoral NT5C1A and HA-tag expression were confirmed by immunohistochemistry and showed robust protein levels in tumors derived from stably transfected cells (Fig. 5B). Tumor growth measured by absolute tumor weights was significantly increased following gemcitabine treatment in NT5C1A-overexpressing tumors compared to tumors derived from vector control cells (0.25 g vs. 0.37 g; $p = .03$) (Fig. 5C and D). However, the overall number of apoptotic cells within the tumor was not significantly decreased upon NT5C1A overexpression (data not shown). Moreover, serum levels of dFdU, the inactive gemcitabine metabolite, were significantly increased in mice bearing NT5C1A-overexpressing tumors with median concentrations of 14.1 vs. 17.6 μ M dFdU ($p = .009$) (Fig. 5F). These data strongly support our hypothesis that high NT5C1A expression reduces sensitivity to gemcitabine. Accordingly, and in line with the patient data demonstrating NT5C1A not to be a prognostic marker (Fig. 1D and E), we did not observe significant NT5C1A-dependent differences in tumor growth upon saline treatment of mice (0.82 g vs. 0.99 g; $p = .07$) (Fig. 5E). Additionally, comparing gemcitabine treated tumors to saline treated tumors, we show that NT5C1A expression is not induced by gemcitabine treatment (Fig. S5B).

4. Discussion

The advent of precision oncology concepts has shown extraordinary treatment responses in several tumor entities such as melanoma, lung and breast cancer. However, there are currently no established biomarkers for PDAC patients in clinical routine that would assist to select the best treatment option or to predict treatment response for individual patients [32]. Notably, several recent preclinical and clinical studies suggested that gemcitabine transport and metabolism proteins are implicated in treatment response and patient outcome and may thus serve as appropriate biomarkers to select patients for gemcitabine treatment. For example, high hENT1 and dCK protein levels have been associated with improved outcome of PDAC patients following gemcitabine treatment [10–12,33,34]. In contrast, data from large randomized



prospective clinical trials suggest that DCTD and RRM1 are not associated with survival upon gemcitabine treatment [10,34].

Here, we investigated the expression and function of NT5C1A, a previously unrecognized gemcitabine inactivating enzyme that has not been reported to be expressed in any solid carcinoma before. To the best of our knowledge, we are the first group to publish detailed expression data on NT5C1A in pancreatic cancer specimens. Interestingly, NT5C1A was robustly expressed in the epithelial compartment of

64–70% of PDAC patients, whereas 23–26% showed weak NT5C1A expression, and 5–13% were devoid of NT5C1A immunoreactivity. Although the functional implications have not been elucidated in PDAC, NT5C1A expression had no prognostic effect on overall survival of postoperative PDAC patients in our two cohorts. These results are in line with previously published data on hENT and other gemcitabine metabolizing enzymes such as DCTD that also had no prognostic effect in resected PDAC [11,34].

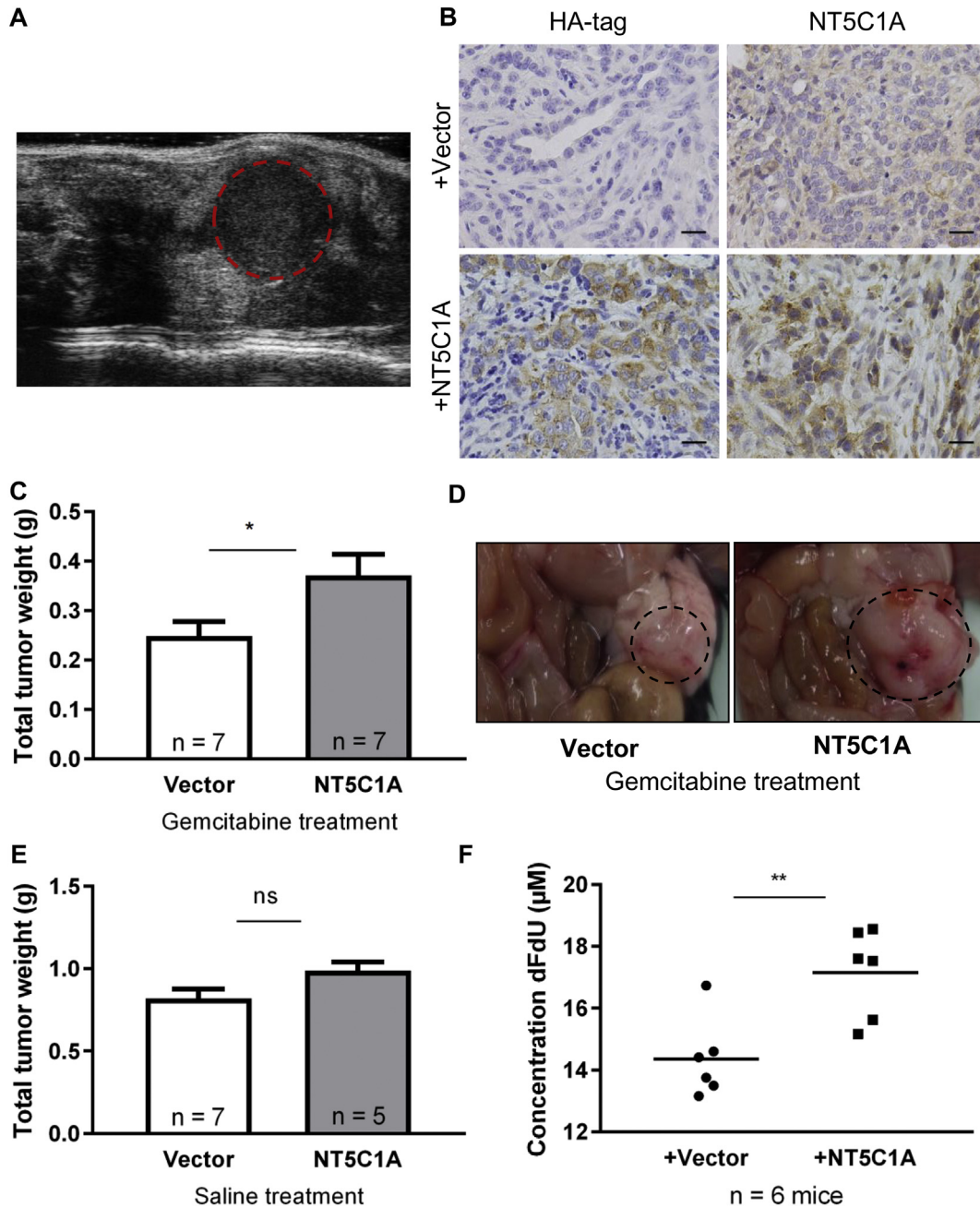


Fig. 5. NT5C1A expression mediates chemoresistance *in vivo*. A) Tumor detection was performed by small animal high-resolution sonography of orthotopically transplanted mice in all groups on day 9 of treatment. Dotted line indicates the tumor. B) Representative immunohistochemistry showing HA-tag (left) and NT5C1A (right) stainings in vector (upper panel) and NT5C1A transfected (lower panel) orthotopic tumors. Scale bars 20 µm. C) Tumor weights upon necropsy are shown with significantly increased tumor weights upon NT5C1A overexpression in the gemcitabine treated cohort ($n = 7$ each; $p = .03$). Graph shows mean \pm SEM. D) Necropsy pictures of orthotopically transplanted pancreatic tumors upon gemcitabine treatment with vector (left) and stable expression of NT5C1A (right). E) NT5C1A expression in saline treated controls did not have a significant effect on tumor growth ($p = .07$, $n = 7$ and 5, respectively). Graph shows mean \pm SEM. F) The inactive gemcitabine metabolite dFdU was measured in serum samples of gemcitabine treated mice. Significantly higher values were detected in mice with +NT5C1A tumors (median: 14.1 vs. 17.6 µM, $p = .009$, Mann-Whitney test). Single values of $n = 6$ mice per group and the mean values are shown.

Fig. 4. NT5C1A expression and function in PDAC stroma *in vitro* and *in vivo*. A) and B) Tissue microarray analysis for NT5C1A expression revealed very low expression in the tumor stroma of resected PDAC patients. A semi-quantitative scoring system indicated no stromal expression with score 0 and strong stromal expression with score 3. TMA-1 from Goettingen (A) with $n = 77$ patients (none scored with 3) and TMA-2 from Erlangen (B) with $n = 330$ patient samples. C) Representative immunohistochemistry of NT5C1A expression showing no NT5C1A expression (score 0), low expression (score 1), and robust stromal expression (score 2) of TMA-1. Scale bars 20 µm. D) Murine PSCs were treated with increasing concentrations of gemcitabine for six days and crystal violet assays were performed. The staining was more pronounced in NT5C1A expressing cell lines. Representative images of two independent experiments, with each two technical replicates, are shown. E) Crystal violet stain intensity was quantified and results were normalized to untreated control cells. Graph indicates mean \pm SEM, two-way ANOVA with Sidak's multiple comparisons test was performed (7 nM: $p < .035$, 12 nM: $p < .009$, 20 nM: $p < .0001$, 35 nM: $p = .007$). F) and G) MTT cell viability assay for KPC cell lines treated with conditioned medium (CM) of NT5C1A expressing PSCs and control CM. F) Schematic experimental overview. CM from PSCs (+NT5C1A) and control PSCs (+vector) was obtained by preincubation with 25 nM gemcitabine-hydrochloride for 24 h. Subsequently, tumor cells were treated for 72 h with CM of PSCs and viability was assessed using MTT cell viability assay. G) Tumor cell viability of two different murine KPC cell lines was significantly decreased following treatment with CM of +NT5C1A-expressing PSCs (KPCa: 73%; $p = .003$ and KPCb: 75%; $p = .047$). Graphs indicate mean \pm SEM of four biological replicates.

An unexpected finding was that murine and human pancreatic cancer cells showed dramatically reduced expression of NT5C1A when cultured in 2D. As we generated primary murine cell lines from the KPC model, in which epithelial tumor cells showed robust NT5C1A expression *in vivo*, we speculate that NT5C1A expression is regulated by signaling cues from the tumor microenvironment (TME) that are lacking during cell culture. The TME is abundant in PDAC and consists of large amounts of collagen and hyaluronic acid, activated fibroblasts and immune cells, including tumor associated macrophages (TAMs) [35]. Recent evidence from patient derived organoids (PDOs) have provided compelling insights into tumor biology and tailored therapy approaches [36,37]. This technique could be employed in future studies to understand the differential expression of NT5C1A in 2D and 3D, and possibly to use this knowledge for future patient stratification.

Notably, we provide first evidence that re-expression of NT5C1A, in both murine and human PDAC cells, reduces sensitivity to gemcitabine both *in vitro* and *in vivo*. Using LC-MS/MS techniques, we could directly demonstrate the activity of NT5C1A on gemcitabine metabolism and the subsequent reduction of tumor cell apoptosis. As expected, NT5C1A overexpression did not induce chemotherapeutic resistance to paclitaxel, whose mechanism of action is independent of intracellular phosphorylation. We had previously shown that recombinant overexpression of NT5C1A in PSCs reduced the intracellular amounts of active gemcitabine, thus, potentially limiting the described drug scavenging effect of PSCs [23]. Here, we provide a large dataset of patient samples, demonstrating not only overexpression in the majority of tumor cells but also a lack of stromal NT5C1A expression in most cases. In addition, we show for the first time that re-expression of NT5C1A in PSCs leads to an improved sensitivity of gemcitabine in tumor cells suggesting a reciprocal effect of NT5C1A, where high expression in the stroma and low expression in the tumor cells may promote chemosensitivity. As stromal cells are not present in cell culture and tumor cells express NT5C1A at low levels under 2D conditions, our observations may partly explain the conundrum that most human and murine pancreatic cancer cell lines respond very well to gemcitabine *in vitro* but fail to exert meaningful anti-tumor effects in genetically engineered mice or in human PDAC that both harbor abundant desmoplasia.

It remains to be seen whether NT5C1A has any predictive effect following gemcitabine treatment in PDAC patients, and whether a combination with hENT quantification may yield any predictive benefit over hENT or NT5C1A expression alone. In both TMA cohorts, a relevant fraction of patients did not undergo adjuvant chemotherapy. Therefore, further studies in prospectively collected, randomized and controlled clinical specimens are warranted to determine the role of NT5C1A as a potential novel predictor for gemcitabine-based therapies in PDAC.

Interestingly, a recent study provided evidence that TAMs may mediate chemoresistance in PDAC by upregulation of CDA, an important gemcitabine inactivating enzyme, in tumor cells [13]. Whether this is also true for other gemcitabine metabolizing enzymes such as NT5C1A and DCTD has not been investigated so far. Recent findings suggest multiple cross-talking pathways between tumor cells, macrophages, cancer-associated fibroblasts, and immune cells in stroma-driven chemoresistance in PDAC [38–40]. Consistently, stroma-mediated cancer cell activation correlates with widespread increases in histone acetylation at H3K9 and H3K27 residues, suggestive for profound alterations in promoter and enhancer activation levels, although the transcriptional consequences and therapeutic relevance of these findings remain largely elusive [41]. Future studies will need to address whether the dynamic changes of NT5C1A expression *in vitro* and *in vivo* may be orchestrated by epigenetic mechanisms that could also act as potential therapeutic targets.

In conclusion, we have identified NT5C1A to be highly expressed in the epithelial compartment of a relevant subgroup of PDAC patients. Mechanistically, NT5C1A mediates gemcitabine resistance by dephosphorylating gemcitabine monophosphate, thus reducing the amount

of cytotoxic gemcitabine metabolites intracellularly. Therefore, our study paves the way for the evaluation of NT5C1A for stratified treatment approaches in PDAC, both for adjuvant as well as palliative therapy regimens.

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Author contributions

M.S.P., A.N., and V.E. conceived and designed the experiments. M.S.P., V.K., F.H.H., and S.A.J. participated in the design of and performed cell culture experiments. M.S.P., E.H., S.P., and R.G.G. planned and performed animal experiments. M.S.P. did histology stainings. J.G., M.B., and J.K. provided TMA-1 slides, clinical data, and scored the stainings. C.P., R.G., P.R., and T.K. provided, stained, and scored the TMA-2. F.M.R. and D.I.J. analyzed liquid-chromatography tandem mass-spectrometry samples. M.S.P. performed data analysis. M.S.P. and A.N. assembled the data and wrote the manuscript. All authors reviewed the manuscript.

Declaration of interests

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.01.037>.

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