Na^+/K^+-ATPase subunit α3 expression is associated with the efficacy of digitoxin treatment in pancreatic cancer cells

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Abstract. The alpha subunits (ATP1A1-3) of Na^+/K^+-ATPase binds digitoxin with varying affinity. The expression levels of these subunits dictate the anticancer effects of digitoxin. In the present study, three pancreatic cancer cell lines, AsPC-1, Panc-1 and CFPAC-1, were used to investigate the effects of digitoxin in relation to the expression of the subunits ATP1A1 and ATP1A3. Cell viability and intracellular calcium concentrations was measured in relation to the gene and protein expression of ATP1A1 and ATP1A3. Digitoxin was used to treat the cells at concentrations of 1-100 nM, and the intracellular calcium concentrations increased in a concentration-dependent manner in the Panc-1 and in the CFPAC-1 cells with treatment at 100 nM. In the AsPC-1 cells only the supraphysiological concentration of digitoxin (100 nM) resulted in a decrease in the number of viable cells (unviable cells increased to 22%), whereas it had no effect on intracellular calcium levels. The number of viable Panc-1 and CFPAC-1 cells decreased after digitoxin treatment at 25-100 nM (unviable Panc-1 cells increased to 33-59%; unviable CFPAC-1 cells increased to 22-56%). Digitoxin treatment also affected the transcriptional expression of the *ATP1A1* and *ATP1A3* subunits. In Panc-1 cells, *ATP1A3* gene expression was negatively associated with the digitoxin concentration (25-100 nM). In the AsPC-1 and CFPAC-1 cells, the expression of the *ATP1A1* gene increased in the cells treated with the 100 nM digitoxin concentration. The protein expression of ATP1A1 and ATP1A3 was not altered with digitoxin treatment. The basal protein expression of ATP1A1 was high in the AsPC-1 and CFPAC-1 cells, compared to the Panc-1 cells, in contrast to the basal expression of ATP1A3, which was higher in the Panc-1 cells, compared to the other pancreatic cancer cells used. On the whole, the present study demonstrates that the high expression of ATP1A3 renders pancreatic cancer cells more susceptible to digitoxin-induced cell death. The findings suggest that the expression of ATP1A3 may be used as a marker for tumor sensitivity to digitoxin treatment, where a high expression of ATP1A3 is favorable for the anticancer effects of digitoxin.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a 5-year survival rate of <5% (1,2). Novel complementary treatments are warranted since current treatments are often inefficient and are associated with severe side-effects (3). Data from epidemiological studies have suggested positive therapeutic effects using cardiac glycosides in the treatment of various types of cancer, and may thus prove to be promising as a complementary treatment to current PDAC therapeutics (4-7).

Cardiac glycosides are natural compounds most well known for their cardiovascular effects. Digitoxin is one of the most extensively used cardiac glycosides and it inhibits the membrane receptor Na^+/K^+-ATPase by binding to its α-subunit (8). The Na^+/K^+-ATPase consists of three parts, the β-subunit β1-3, the α-subunit with its four isoforms α1-4 (ATP1A1-4), and accessory proteins FXYD 1-7 (9). The α- and β-subunits are differentially expressed depending on the type of tissue (10). ATP1A1 is expressed in almost all tissues (11), ATP1A2 is mainly expressed in the heart, brain and skeletal muscles, ATP1A3 mostly in nervous and muscle tissue, and ATP1A4 is only expressed in spermatozoa (12,13). Binding experiments with various cardiac glycosides have demonstrated that ATP1A2 and ATP1A3 have a higher affinity to digitoxin than ATP1A1 (9,10,14). A higher affinity indicates a more effective blockage by digitoxin. The values for digitoxin binding constants, dissociation constant (K_D; inverse to affinity), are 38 nM for ATP1A1 and 14 nM for ATP1A3 (9).

The blockage of the Na^+/K^+-ATPase pumping function leads to an increase in the intracellular concentration of sodium ions which, in turn, forces the Na^+/Ca^{2+}-exchanger (NCX) to transport Na^+ out of the cell and Ca^{2+} into the cell (15). As a result, the intracellular Ca^{2+} concentrations increase (16). In non-excitable cells under basal conditions, the intracellular concentrations of Ca^{2+} are maintained at a low homeostatic level, with a balance of influx and efflux of Ca^{2+} (17). Maintaining a low intracellular concentration of calcium (Ca^{2+}) is essential for the majority of eukaryotic cells,

since a high persistent intracellular concentrations of Ca^{2+} is harmful and trigger apoptosis (18).

A deregulation of the α-subunits is often observed in cancer cells, with an upregulation of ATP1A3 and the downregulation of ATP1A1, such as in colorectal cancer and renal cell carcinoma (13,19). Cancer cells have been shown to be more sensitive to digitoxin than normal cells, where nanomolar concentrations of digitoxin induce the apoptosis of cancer cells (8,20-22). It was thus hypothesized that the increased sensitivity of cancer cells is dependent on the altered expression of the α-subunits of Na^+/K^+-ATPase.

To investigate this hypothesis, the present study we used three well-characterized commercially available pancreatic cancer cell lines (AsPC-1, Panc-1 and CFPAC-1). The basal gene expression of the three α-subunit isoforms of Na^+/K^+-ATPase, *ATP1A1*, *ATP1A2* and *ATP1A3,* as well as the protein levels of ATP1A1 and ATP1A3 were measured. The effects of digitoxin treatment at 1-100 nM (therapeutic concentrations, 10-40 nM) were evaluated by examining the changes in the expression of ATP1A1 of ATP1A3, intracellular Ca^{2+} levels and cell viability.

Materials and methods

Cells and cell culture. The present study used three pancreatic cancer cell lines, AsPC-1 (CRL-1682), Panc-1 (CRL-1469) and CFPAC-1 (CRL-1918) (ATCC; LGC Standards GmbH). The AsPC-1 cells were grown in RPMI-1640 medium supplemented with 1% HEPES and 1% sodium pyruvate, the Panc-1 cells in Dulbecco's modified Eagle's medium (DMEM) with 1% L-glutamine, and the CFPAC-1 cells in DMEM. Media were also supplemented with 10% FBS and 1% penicillin-streptomycin (PEST). All cell culture media and reagents were purchased from MilliporeSigma. All incubations were performed at 37˚C in 5% CO_2. PCR was used to test the cell lines for mycoplasma infection (using the LookOut® Mycoplasma PCR Detection kit; cat. no. MP0035, MilliporeSigma).

Seeding and treatment with digitoxin. For digitoxin (cat. no. D5878, MilliporeSigma) treatment, 5,000 cells were seeded in 100 µl complete growth medium in 96-well plates and incubated at 3˚C with 5% CO_2 for 20 h to a sub-confluent monolayer. Following 20 h of incubation, new media (100 µl) containing digitoxin was added to the cells. The control cells only received new media. The cells were further incubated at 37˚C with 5% CO_2 for 48 h. The concentrations of digitoxin used in all experiments were as follows: 0 nM (controls), and 1, 10, 25, 40 and 100 nM (human therapeutic range, 10-40 nM).

Cell viability assay. The analysis of cell viability was performed using the colorimetric method CellTiter 96® AQueous One Solution Cell Proliferation assay (MTS) assay (Promega Corporation). Following 48 h of incubation at 37˚C with 5% CO_2 with digitoxin, 20 µl MTS tetrazolium were added to each well and incubated for 1 h at 37˚C with 5% CO_2. Viable cells metabolize tetrazolium to formazan, which was measured at 490 nm (FLUOstar Omega, BMG Labtech). The obtained value is directly proportional to the number of viable cells in the cell culture.

Intracellular Ca^{2+} assay. Fluo-4 assay (Ca^{2+}; Abcam) was used to determine the intracellular Ca^{2+}concentrations following treatment with digitoxin. The cells were seeded and treated for 48 h with digitoxin at 37˚C with 5% CO_2 in triplicate. Following treatment, 100 µl of Fluo-4 AM dye-loading solution were added to each well and incubated for 1 h. The fluorescence intensity was measured at Ex/Em 485/520 (FLUOstar Omega, BMG Labtech).

RNA-extraction and reverse transcription-quantitative PCR. The cells were seeded at a density of $1.5x10^5$ in 2.4 ml medium in six-well plates and incubated at 37˚C in 5% CO_2 to a sub-confluent monolayer. After 20 h, the old medium was removed and new medium with digitoxin was added. The control cells only received new media. Following 48 h of treatment, RNA was extracted using a RNeasy Mini kit (cat. no. 74104, Qiagen GmbH), according to the supplier's protocol. A total 1 µg RNA from each sample was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit Reagent (25˚C for 10 min, 37˚C for 120 min, 85˚C for 5 min) according to the manufacturer's manual (Thermo Fisher Scientific, Inc.).

Complementary DNA (cDNA) corresponding to 5 ng RNA was used in each qPCR reaction performed in duplicate for the following TaqMan target transcripts: *ATP1A1* (Hs00167556_m1), *ATP1A2* (Hs00265131_m1) and *ATP1A3* (Hs00958036_m1) using TaqMan™ Gene Expression Master Mix (4369016, Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Quantitative gene expression data were normalized to the expression level of the human reference gene, phosphomannomutase 1 (*PMM1*; Hs00963626_m1). The ΔCq values was used to analyze the relative expression between the genes *ATP1A1* and *ATP1A3* for each cell line (23). All qPCR reactions were performed on a Pikoreal qPCR System (Thermo Fisher Scientific, Inc.).

Protein extraction and western blot analysis. The cells were seeded at a quantity of $5x10^5$ cells per 75 cm^2 flask and incubated for 20 h in 37˚C in 5% CO_2. Thereafter, the old medium was removed and new medium with digitoxin was added. The controls only received new, complete medium. Following 48 h of treatment, protein extraction was performed and the cells were lysed in lysis buffer (cat. no. FNN0011, Thermo Fisher Scientific, Inc.) supplemented with phenylmethylsulphonyl fluoride (PMSF; cat. no. 36978, Thermo Fisher Scientific, Inc.) and protein inhibitor cocktail (cat. no. P2714, MilliporeSigma). The protein concentration was determined using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.).

From each sample, 10 µg of total protein were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis in 8-16%, stain free gel (Bio-Rad Laboratories Inc.), Proteins were later blotted onto PVDF membranes, blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) with 5% milk in 20˚C for 1 h, incubated with either ATP1A1 and ATP1A3 primary antibodies (1:1,000; cat. nos. MA1-16731 and MA3-915, Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h in room temperature. After washing with TBST three times, the membranes were further incubated with the secondary antibody, Alexa Fluor

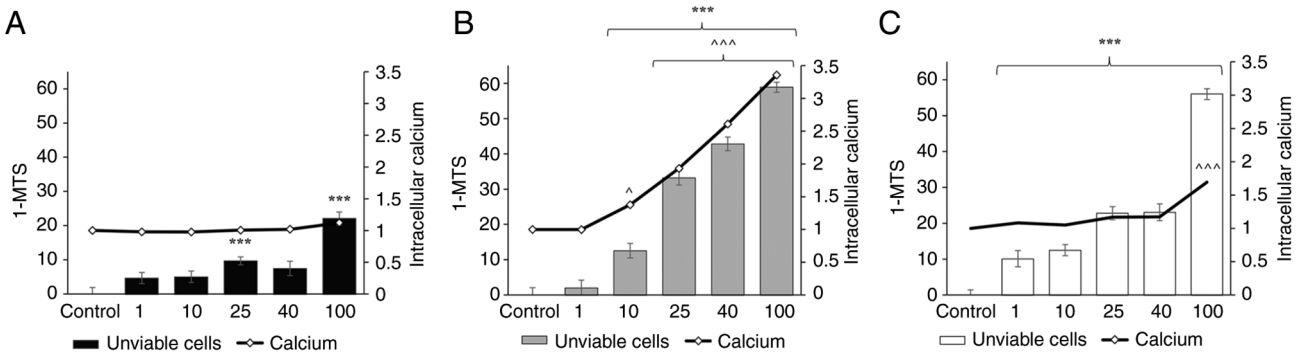

Figure 1. Cell viability and intracellular Ca^{2+} levels following treatment with digitoxin in (A) AsPC-1, (B) Panc-1, and (C) CFPAC-1 cells. The cells were incubated for 48 h with digitoxin at concentrations of 1-100 nM; the control cells only received complete cell medium. The number of viable cells was measured using MTS assay. The decrease in viable cells 'unviable cells', was normalized to the control (left axis) and intracellular Ca^{2+} values (right axis) control. Error bars represent the means ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) followed by the Bonferroni correction, to confirm significant differences between treated vs. untreated cells (i.e., control). ***P<0.001, significant differences in cell viability; $^{\wedge}$P<0.05; and $^{\wedge\wedge\wedge}$P<0.001, significant differences in intracellular calcium levels.

Plus 555 (1:2,500; cat. no. A32727, Invitrogen; Thermo Fisher Scientific, Inc.). The protein expression was measured using the ChemiDoc System (Bio-Rad Laboratories Inc.), densitometric analysis of single and total protein expression was performed using Image Lab Software ver. 6.1 (Bio-Rad Laboratories, Inc.). Protein expression was normalized to the total protein for each sample, total protein normalization (Figs. S1-S4).

Statistical analysis. The used assays are based on absorbance and fluorescence in microplate format and RT-qPCR. Biological and technical replicates were three or more, (two technical replicates for RT-qPCR). Statistical analysis was performed using IBM SPSS Statistics 27 software (IBM Corp.) and one-way analysis of variance (ANOVA) followed by the Bonferroni correction, to confirm significant differences between treated vs. untreated cells (i.e., control) in each assay. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Digitoxin within the therapeutic range exerts an effect on cell viability and intracellular Ca^{2+} concentrations in Panc-1 and CFPAC-1 cells, but not in AsPC-1 cells. The AsPC-1 cell line was less affected by digitoxin compared to the Panc-1 and CFPAC-1 cells, with only a significant effect observed on cell viability in the AsPC-1 treated with 25 and 100 nM digitoxin (Fig. 1A). Digitoxin at concentrations ranging from 10-100 nM decreased the viability of the Panc-1 cells following 48 h of treatment and also that of the CFPAC-1 cells at concentrations ranging from 1-100 nM. For the Panc-1 cells treated with digitoxin at the concentration of 10 nM, the number of viable cells decreased (number of unviable cells, 12.6±1.15%, P<0.001) compared to the control cells. Within the therapeutic range in humans (25-40 nM), the viable cell number was decreased even further (number of unviable cells at 40 nM: Panc-1 cells, 43.8±1.15%, P<0.001; CFPAC-1 cells, 23.0±1.16%; P<0.001) compared to the controls (Fig. 1B and C).

The intracellular Ca^{2+} concentrations increased in a concentration-dependent manner following 48 h of treatment with digitoxin in the Panc-1 cells. Digitoxin at a concentration of 25 nM led to a 2-fold increase (±0.04, P<0.001) in intracellular Ca^{2+} levels in the Panc-1 cells. The digitoxin concentrations of 40 and 100 nM increased the intracellular Ca^{2+} levels 2.6-fold (±0.04, P<0.001) and 3.4-fold (±0.04, P<0.001), respectively in Panc-1 cells (Fig. 1B). In the Panc-1 cells, a marked increase in intracellular Ca^{2+} levels were observed, while the CFPAC-1 cells only exhibited a significant increase in intracellular Ca^{2+} levels following treatment with digitoxin at 100 nM (±0.03, P<0.001) (Fig. 1C). In the AsPC-1 cells, the concentration of intracellular Ca^{2+} was not markedly altered following treatment with digitoxin (Fig. 1A).

Expression of ATP1A3 is high in Panc-1 and CFPAC-1 cells, but not in AsPC-1 cells. The basal transcriptional expression of *ATP1A1* was low in AsPC-1 and Panc-1 cells, and 6-fold higher in the CFPAC-1 cells compared to the AsPC-1 cells (Fig. 2A). The *ATP1A3* subunit in Panc-1 cells was transcriptionally expressed >50-fold higher (P<0.001) compared to the AsPC-1 cells, and 4-fold times higher than the CFPAC-1 cells (Fig. 2B). At the protein level, the AsPC-1 cells exhibited a significant ~7-fold higher expression of ATP1A1 compared to the Panc-1 cells (±0.01, P=0.002, AsPC-1 cells) (Fig. 2C). The Panc-1 cells exhibited a significantly higher expression (4-fold) of ATP1A3 compared to the AsPC-1 cells (±0.01, P=0.022), while the CFPAC-1 cells had a 2-fold higher protein expression of ATP1A3 compared to the AsPC-1 cells (Fig. 2D).

Digitoxin treatment affects the gene and protein expression of ATP1A1 and ATP1A3 in Panc-1 cells. A one-way between-groups ANOVA was conducted to explore the effects of digitoxin treatment on *ATP1A1* and *ATP1A3* gene expression levels. Significant differences were found for *ATP1A* expression in the AsPC-1 cells [F(5, 12)=14.71, P<0.001] and CFPAC-1 cells [F(5, 11)=7.16, P=0.003]. No effects on *ATP1A1* gene expression were observed in the Panc-1 cells; instead a change in the expression of the *ATP1A3* gene was observed with digitoxin treatment. A decrease in *ATP1A3* expression with the increasing digitoxin concentration was confirmed in the Panc-1 cells [F(5, 12)=5.51, P=0.007]. In order to analyze the effects of each digitoxin concentration on *ATP1A1* and *ATP1A3* expression separately, post hoc comparisons using

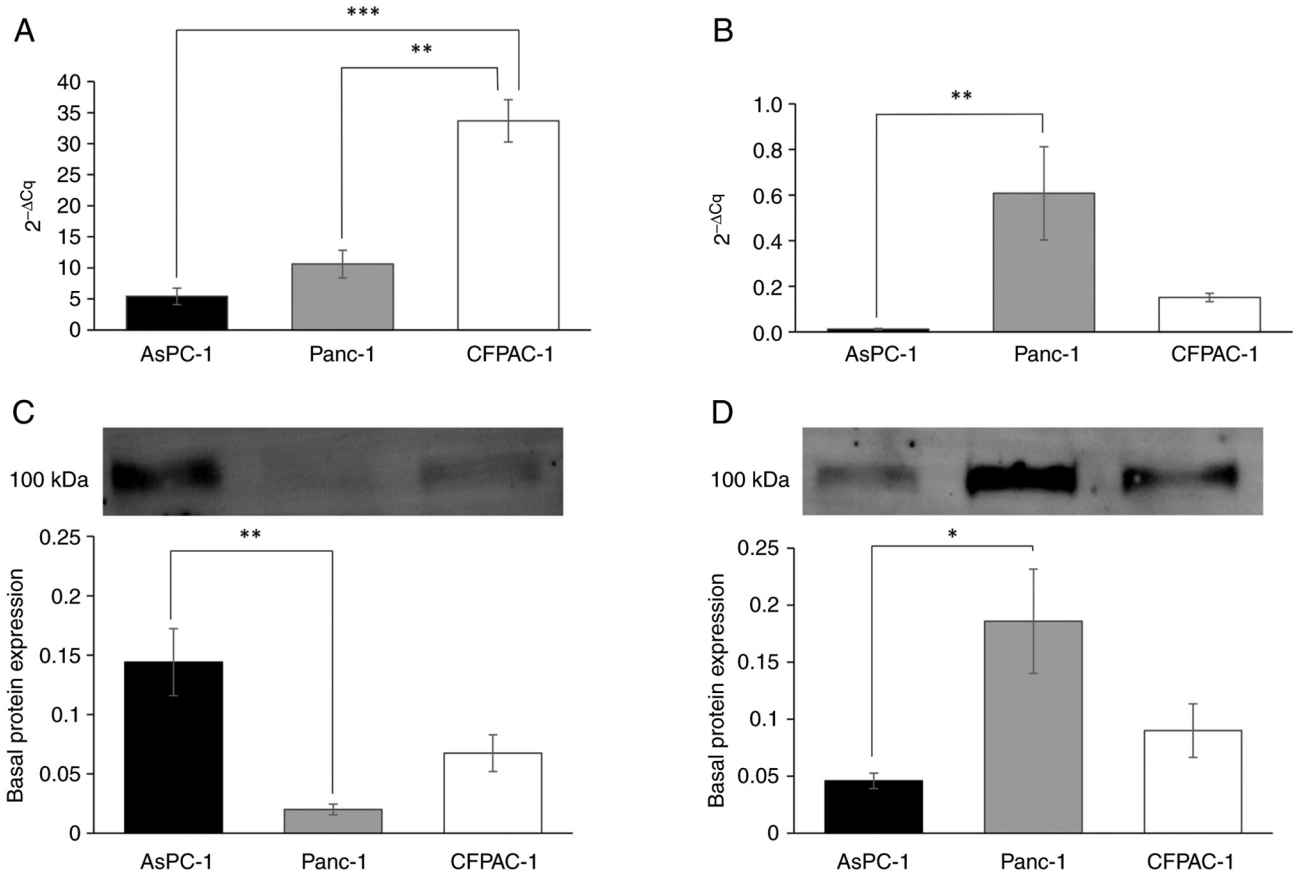

Figure 2. Gene expression for the control (basal) samples, $2^{-\Delta Cq}$ for (A) *ATP1A1* and (B) *ATP1A3*. Protein expression of (C) ATP1A1 and (D) ATP1A3, control (basal) protein expression of the subunits ATP1A1 and ATP1A3 normalized to total protein in each sample. Error bars represent the means ± SEM. Data were analyzed using one-way between-groups ANOVA. *P<0.05; **P<0.01 and ***P<0.001. Protein expression was normalized to the total protein for each sample (Fig. S1). ATP1A1 and ATP1A3, Na⁺/K⁺-ATPase alpha subunits 1 and 3.

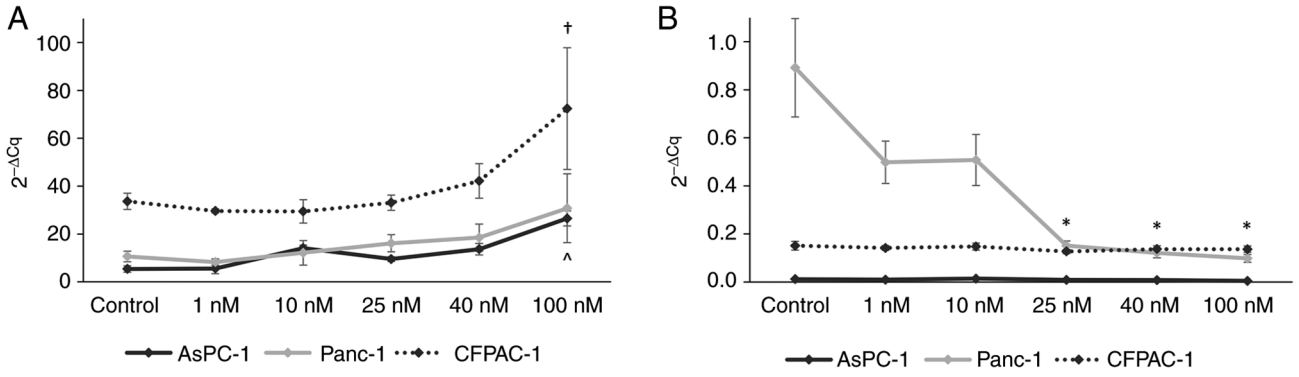

Figure 3. Gene expression, $2^{-\Delta Cq}$ for (A) *ATP1A1* and (B) *ATP1A3* in untreated cells (control) compared to cells treated with digitoxin 1-100 nM for 48 h. Error bars represent the means ± SEM. Data were analyzed using one-way between-groups ANOVA. $^{*,\wedge,\dagger}$<0.05, significant differences for the Panc-1 (*), AsPC-1 (^) and CFPAC-1 (†) cells. ATP1A1 and ATP1A3, Na⁺/K⁺-ATPase alpha subunits 1 and 3.

the Bonferroni correction were made. The analysis revealed a significant difference in *ATP1A1* expression in the AsPC-1 cells with digitoxin treatment at 100 nM (±0.172, P<0.001) (Fig. 3A). In the CFPAC-1 cells, treatment with digitoxin at 100 nM led to a significant increase in *ATP1A1* gene expression (±3.474, P=0.011) (Fig. 3A). In the Panc-1 cells, no significant effects on *ATP1A1* gene expression were observed for the individual digitoxin concentrations; however, there was a significant decrease in *ATP1A3* gene expression with various digitoxin concentrations (25 nM: ±0.059, P=0.034; 40 nM: ±0.059, P=0.024; 100 nM: ±0.059, P=0.018) (Fig. 3B). *ATP1A3* expression was not markedly altered by digitoxin either in the AsPC-1 or in the CFPAC-1 cells (Fig. 3B).

Western blot analysis of ATP1A1 and ATP1A3 was performed following treatment with digitoxin to evaluate the effects at the protein level (Fig. 4). A one-way between-groups ANOVA was conducted to explore the effects of digitoxin treatment on ATP1A1 and ATP1A3 protein expression. The

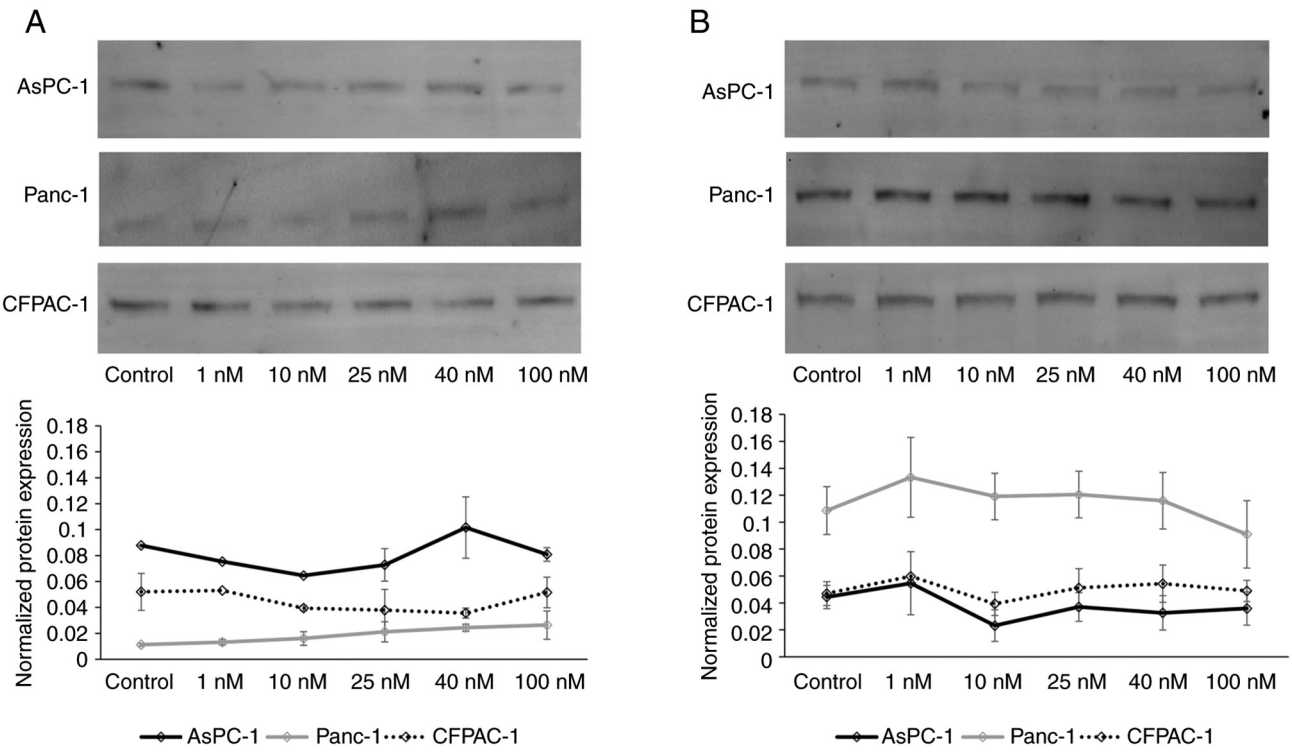

Figure 4. Protein expression of (A) ATP1A1 and (B) ATP1A3, normalized to total protein for each sample, in AsPC-1, Panc-1 and CFPAC-1 cells. Untreated cells (Control) compared to cells treated with digitoxin 1-100 nM for 48 h. Error bars represent the means ± SEM. Data were analyzed using one-way between-groups ANOVA. No significant differences between controls and treatments were found. Protein expression was normalized to the total protein for each sample (Figs. S2-S4). ATP1A1 and ATP1A3, Na^+/K^+-ATPase alpha subunits 1 and 3.

results revealed slightly increased protein levels of ATP1A1, and decreased protein levels of ATP1A3 in the Panc-1 cells following treatment, although with no significant differences (Fig. 4).

Discussion

The cardiac glycoside, digitoxin, is an extensively used drug in the treatment of cardiovascular diseases (15), and when used within the therapeutic range of 25-40 nM, no major side-effects have been observed in normal cells (24,25). The present study investigated a potentially novel use of this already established drug for the treatment of pancreatic cancer, a disease which remains very difficult to cure with current treatments (26,27). Previous studies have demonstrated a reduction in cell viability in several cancer cell lines following treatment with cardiac glycosides (20,27), and the results from the present study with digitoxin treatment in the pancreatic cancer cell lines, AsPC-1, Panc-1 and CFPAC-1, confirm that digitoxin may be useful as a potential treatment for subgroups of patients with pancreatic cancer.

The hypothesis behind the present study relies on the fact that the membrane receptor Na^+/K^+-ATPase isoform ATP1A3 has a higher affinity for digitoxin than the ATP1A1 isoform, which suggests that a higher expression of ATP1A3 compared to ATP1A1 would provide a more effective blockage of the Na^+/K^+-ATPase by digitoxin (14). A change in expression of the α-subunits ATP1A1 and ATP1A3 is observed in certain tumors compared to normal tissue. In a study on human colorectal carcinoma, colorectal cancer tissue was compared with normal mucosa, and it was found that *ATP1A1* was transcriptionally downregulated in cancer tissues and that *ATP1A3* expression was upregulated (13). Banerjee *et al* (28) also demonstrated a decreasing expression of ATP1A1 in primary tumors and metastases compared to the normal tissue. The altered expression of these two isoforms is probably the reason for an increased sensitivity to digitoxin treatment, considering the higher affinity of digitoxin to the ATP1A3 isoform (9). In another study, an overall downregulation in Na^+/K^+-ATPase activity was observed in colorectal cancer cells following digitoxin treatment (13), and an unregulated increase in intracellular Ca^{2+} levels induced the apoptosis of HeLa cells (29).

In the present study, the data suggested a close association between the fraction of unviable cells and intracellular Ca^{2+} concentrations in all three pancreatic cancer cell lines. However, there was a major difference in the response to digitoxin within the therapeutic range (25-40 nM) between the different cell lines, where the Panc-1 cells exhibited a marked response, with a notable decrease in viability and a marked increase in the intracellular Ca^{2+} levels, while AsPC-1 cell viability was only slightly affected by digitoxin at these concentrations.

There is a notable difference in characteristics between the cell lines in the present study with regards to their basal levels of ATP1A1 and ATP1A3 gene and protein expression. Since the Panc-1 cells had a very high basal expression of ATP1A3, and a lower expression of ATP1A1 compared to the other two cell lines, it was hypothesized that the blockage of Na^+/K^+-ATPase by digitoxin in this cell line was dependent on

the relative high expression of ATP1A3. The more effective blockage of ATP1A3 by digitoxin led to a notable increase of the intracellular Ca^{2+}concentrations in the Panc-1 cells. The CFPAC-1 cells with a relatively higher expression of ATP1A1 and a lower expression of ATP1A3 compared to the Panc-1 cells exhibited only a significant increase in intracellular Ca^{2+} levels with the supratherapeutic concentration of digitoxin (100 nM). No change in the intracellular Ca^{2+}concentrations was found in the AsPC-1 cells. The AsPC-1 cell line had a high ATP1A1 expression and a low ATP1A3 expression, which further underlines the importance of the expression levels of these subunits.

The Panc-1 cells exhibited a response to digitoxin affecting the gene expression of the Na^+/K^+-ATPase α-subunit *ATP1A3*, which decreased in the cells treated with 25-100 nM digitoxin. This effect is possibly a protective cell survival mechanism. In the AsPC-1 and CFPAC-1 cells, only the gene expression of *ATP1A1* was affected (increased expression) by treatment with 100 nM digitoxin. Cells are heavily dependent on the function of Na^+/K^+-ATPases for the intracellular ion homeostasis, blocking these pumps with drugs or siRNA trigger the cells to produce more Na^+/K^+-ATPases to compensate (28).

The sensitivity to digitoxin may be explained by this difference in the expression of the Na^+/K^+-ATPase α-subunit isoforms between the cell lines. A high ATP1A3 and low ATP1A1 expression corresponded with a high sensitivity to digitoxin treatment, considering the decrease in cell viability. An increase in ATP1A1 expression and/or a decrease in ATP1A3 expression may be a way for the cells to rescue the blockage of the ATP1A3 subunits in the Na^+/K^+-ATPase by digitoxin. These results are correlative and further studies on the mechanisms behind the difference in responses of the different ATP1A isoforms are warranted.

In conclusion, the present study demonstrated the potential of digitoxin as an anticancer agent for a subset of pancreatic cancers. A very high anticancer efficacy of digitoxin was observed in pancreatic cancer cells with a high expression of the ATP1A3 subunit of the Na^+/K^+-ATPase, compared to the cells that had a low expression of the ATP1A3 Na^+/K^+-ATPase subunit. Thus, this may be useful as a marker for effective digitoxin treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL has performed the experiments, statistical analysis and drafted the manuscript. HL and FS designed the study and performed the data interpretation. FS contributed with manuscript writing and critical editing. KE contributed to the study methodology, manuscript writing and critical editing. HL and FS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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