Silencing of X-linked Inhibitor of Apoptosis (XIAP) Decreases Gemcitabine Resistance of Pancreatic Cancer Cells

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Abstract. *Background: The X-linked inhibitor of apoptosis (XIAP) belongs to a family of proteins that suppresses apoptosis by inhibition of caspases in some cancers. It confers resistance to apoptosis induction by chemotherapeutic agents. The aim of this study was to evaluate the influence of XIAP in pancreatic cancer. Patients and Methods: Tissue samples from 43 patients with pancreatic adenocarcinoma (median age of 67 years, range from 39-81 years) were analyzed. Pancreatic samples from healthy organ donors (10) and chronic pancreatitis patients (10) served as controls. XIAP expression and localization analysis was carried out by quantitative RT-PCR and immunohistochemistry (IHC). XIAP silencing was achieved by transfection of specifically designed siRNA oligonucleotides. Proliferation and chemotherapy experiments were performed by MTT cell growth assays. Results: There was a 2.1-fold increase of median XIAP mRNA levels in pancreatic cancers compared to controls. Kaplan-Meier analysis indicated a tendency for reduced patient survival with increasing levels of XIAP mRNA (higher levels: 13.4 months; lower levels: 16.1 months). IHC revealed strong XIAP staining in tubular complexes and pancreatic cancer cells. XIAP silencing resulted in a slight reduction of the proliferation of Capan-1 and T3M4 pancreatic cancer cells. In addition, XIAP silencing resulted in increased sensitivity of both cell lines to gemcitabine. Conclusion: XIAP is overexpressed in pancreatic cancer and contributes to chemoresistance. Interfering with this pathway may have potential therapeutic role in the treatment of this disease.*

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with an overall 5-year survival rate of less than 5% when all stages are combined. One of the most important reasons for this dismal prognosis is the resistance of pancreatic cancer to chemotherapeutic agents making it an essentially chemo-refractory disease (1-3).

On the molecular level, a number of aberrations have been identified that contribute to this chemoresistance. De-regulated expression of members of the Bcl-2 family such as Bcl-2, Bax, Bcl-xl (4-9), have been shown to influence the sensitivity towards chemotherapeutic agents. Additional factors that influence the apoptotic pathway in pancreatic cancer are, for example, Cyclin D1 (10), BNIP3 (11), p8 (12) and SODD (13) among a pool of many others (14).

XIAP belongs to the inhibitor of apoptosis proteins (IAP) that represent a family of endogenous caspase inhibitors that share a conserved structure known as the BIR domain (15). Amongst others, the caspase inhibitory mechanism is best characterized for the X-linked inhibitor of apoptosis (XIAP). The XIAP protein contains three BIR domains. These are responsible for binding and inhibiting active caspases 3, 7 and 9 and, thus, inhibit apoptosis. Eight IAP-encoding genes are found in the human genome and some of these are over-expressed in different cancers, such as non-small cell lung cancer, breast cancer, ovarian cancer and acute myeloid leukemia (16-18). Various studies using antisense oligonucleotides or chemical inhibitors of IAP have suggested that these proteins are important for maintaining tumor cell survival or for conferring resistance to apoptosis induction by chemotherapeutic agents in transitional cell carcinoma, neuroblastoma, gliobastoma, ovarian carcinoma and breast cancer (19-23).

This study was undertaken to evaluate the expression and localization of XIAP in PDAC and to analyze its functional role, especially with respect to chemoresistance.

Patients and Methods

Tissue sampling. Pancreatic tissue specimens were obtained from 43 patients (22 male and 21 female) of PDAC with a median age of 67 years (range 39-81 years) in whom pancreatic resections were performed. Pancreatic tissue specimens were also obtained from 10 patients (9 male and 1 female) of chronic pancreatitis (CP) with a median age of 36.5 years (range 16-64 years). Normal human pancreatic tissue samples were obtained through an organ donor program from 10 previously healthy individuals (7 male and 3 female) (median age 40 years; range 16-70 years). All samples were confirmed histologically. Freshly removed tissues were fixed in para-formaldehyde solution for 12 to 24 h and were then paraffin-embedded for histological analysis. In addition, a portion of the tissue samples was kept in RNAlater (Ambion Ltd., Huntingdon, Cambridgeshire, UK) or snap-frozen in liquid nitrogen immediately (within 5 min) upon surgical removal and maintained at –80˚C until use. The Human Subjects Committees of the University of Bern, Switzerland, and the University of Heidelberg, Germany, approved all studies and written informed consent was obtained from all patients.

Real-time quantitative polymerase chain reaction (QRT-PCR). All reagents and equipment for mRNA/cDNA preparation were supplied by Roche Applied Science (Mannheim, Germany). mRNA was prepared by automated isolation using the MagNA pure LC instrument and isolation kit I (for cells) and kit II (for tissues). cDNA was prepared using the first strand cDNA synthesis kit for RT-PCR (AMV) according to the manufacturer's instructions. Target sequences were amplified using the specific primers with the LightCycler FastStart DNA SYBR Green I kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's protocol. The primer pair sequences used for XIAP were obtained from Search-LC (Heidelberg, Germany). RNA input was normalized to the average expression of the two housekeeping genes HPRT and cyclophilin B. The data of the two independent analyses for each sample and parameter were averaged and presented as adjusted transcripts/μl cDNA, as described previously (24, 25).

Immunohistochemistry. Paraffin-embedded tissue sections (3- to 5-μm thick) were subjected to immunostaining. The tissue sections were deparaffinized in xylene and rehydrated in progressively decreasing concentrations of ethanol. Slides were placed in washing buffer (10 mM Tris-HCl, 0.85% NaCl, 0.1% BSA, pH 7.4) and subjected to immunostaining. After antigen retrieval by boiling the tissue sections in 10 mM citrate buffer for 10 min in the microwave oven, the sections were incubated with normal goat serum (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) for 45 min, to block non-specific binding sites. Sections were incubated with rabbit polyclonal XIAP antibody (Abcam Ltd., Cambridge, MA, USA) or normal rabbit IgG as a negative control at 4˚C overnight. The slides were rinsed with washing buffer and incubated with biotinylated goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, Inc.) for 45 min at room temperature. Tissue sections were then washed in washing buffer and incubated with streptavidin peroxidase (Kirkegaard & Perry Laboratories, Inc.) for 35 min at room temperature. Each section was subjected to DAB-chromogen substrate mixture (DAKO Corporation, Carpinteria, CA, USA), and was then counterstained with Mayer's hematoxylin. The sections were washed, dehydrated in progressively increasing concentrations of ethanol and mounted with xylene-based mounting medium. Slides were visualized using the Axioplan 2 imaging microscope (Carl Zeiss Lichtmicroskopie, Göttingen, Germany).

Immunoblot analysis. Cells were washed in PBS pH 7.4, lysed and homogenized in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% SDS) supplemented with protease inhibitor cocktail tablet (Roche Diagnostics GmbH). The homogenized material was collected and centrifuged at 4˚C for 30 min at 14,000 rpm to remove the insoluble material. The protein concentration of the supernatant was measured spectrophotometrically using the BCA protein assay method (Pierce, Rockford, IL, USA). A total of 20 μg of protein/lane was separated by 4-12% SDS-PAGE electrophoresis (Invitrogen Corporation, Carlsbad, CA, USA). After transfer to nitrocellulose membranes, blots were blocked, incubated with a rabbit polyclonal XIAP antibody (Abcam Ltd.), and exposed to donkey anti-rabbit HRP-labeled IgG (Amersham Biosciences Europe GmbH, Freiburg, Germany). Visualization was performed by the enhanced chemiluminescence method (Amersham). Equal loading and transfer were confirmed using an anti-γ-tubulin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Cell culture. Aspc-1, BxPc-3, Capan-1, Colo-357, Mia-PaCa-2, Panc-1, SU-8686 and T3M4 pancreatic cancer cells were routinely grown in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany), supplemented with 10% FCS (Sigma-Aldrich, St. Louis, MO, USA) and 100 U/ml penicillin/streptomycin (Life Technologies).

siRNA transfection. For evaluating the effect of gene silencing on cell proliferation, pancreatic cancer cells (400,000/well) were grown in 6-well plates until 50% confluence. Next, cells were transfected at 0 h and again at 48 h with 50 nM XIAP siRNA (sense - UAG UGC CAC GCA GUC UAC A; antisense – UGU AGA CUG CGU GGC ACU A) duplex oligonucleotides (Qiagen Gmbh, Hilden, Germany), or 50 nM control (non-silencing) RNA using the RNAifect transfection reagent (Qiagen GmbH) in medium according to the manufacturer's instructions. The target sequence was AAT AGT GCC ACG CAG TCT ACA corresponding to residues 331 to 351 of human XIAP cDNA (U45880). Protein was then extracted after 96 h (*i.e.,* 48 h after second transfection) and used for immunoblot analysis.

MTT assay. Pancreatic cancer cells were plated in 24-well plates at a density of 20,000 cells/well. Transfection with XIAP siRNA was performed 24 h after seeding of cells. The siRNA was allowed to act only for 4 h after which it was replaced by cell culture medium. The transfection was repeated 20 h later. Seventy-two h after seeding of cells (*i.e.,* 24 h after the second transfection), gemcitabine (Gemzar, Eli Lilly and Company, IN, USA) was added at the indicated concentrations and allowed to act for an additional 72 h. Then, 50 μl/well of MTT were added for 4 h at 37˚C. Formazan products were solubilized with 0.04 mM HCl-isopropanol. The optical density was measured at 570 nm with an ELISA plate reader (Opsys MR, ThermoLabsystems, Frankfurt, Germany). All experiments were performed in triplicate.

Statistical analysis. All data are presented as mean±SEM (standard error of mean) unless indicated otherwise. For statistical analysis, the Student's *t*-test was used. The Kaplan-Meier method and log-rank test were used for survival analysis. Statistical analysis and graph presentation were made using Graph Pad Prism 2 Software (GraphPad, San Diego, CA, USA), $p<0.05$ was taken as the level of significance.

Results

Expression and localization of XIAP

a) XIAP mRNA is overexpressed in pancreatic cancer. XIAP mRNA levels in pancreatic tissues were first analyzed by real-time quantitative RT-PCR in a panel of 43 pancreatic cancer samples and 10 samples each of chronic pancreatitis and normal pancreas. This analysis revealed that while the median (range) XIAP mRNA levels in the normal pancreas and chronic pancreatitis were 150.5 (62-269) and 200.5 (35-471), respectively, the levels in pancreatic cancer were 372 (75-1270). Thus, XIAP mRNA levels were 2.1-fold increased ($p<0.0001$) in human PDAC compared to controls (2.5-fold compared to healthy controls and 1.9-fold compared to CP controls, $p<0.01$) (Figure 1A). To evaluate the clinical relevance of this increased expression of XIAP mRNA in PDAC tissues, the mRNA levels of 42 patients with PDAC (1 patient lost to follow up) were then correlated with their survival data. At a cut-off level of 400 copies of cDNA/µl, these 42 patients could be split into two equal groups of 21 each. While no significant difference ($p=0.41$) was noted between the groups, there was a tendency to longer survival in the group of patients with <400 copies of cDNA/µl (median survival 16.1 months) compared to the median survival of 13.4 months in the group of patients with >400 copies of cDNA/µl. Thus, higher levels of XIAP mRNA in PDAC were associated with a tendency towards poorer survival (Figure 1B).

b) Immunohistochemical localization of XIAP in pancreatic cancer. XIAP was subsequently localized in different pancreatic tissues by immunohistochemistry using a XIAP-specific antibody. This analysis revealed only weak expression of XIAP in the acini of 10 normal pancreatic tissues (Figure 2A). The expression was moderate to strong in tubular complexes in 37.2% (n=16) from a total of 43 slides of PDAC that were evaluated (Figure 2B). Consistent and strong expression of XIAP in the cytoplasm of pancreatic cancer cells was observed in 69.8% (n=30) of the tissues evaluated (Figure 2C and D). Evaluation of lymph node (n=5) and liver metastases (n=5) also revealed strong and consistent XIAP immunostaining in the cancer cells in all sections evaluated (Figure 2E and F). No specific immunostaining was present in the consecutive sections evaluated (n=62), using rabbit IgG as a negative control (Figure 2C and 2F, inset).

Functional analysis of XIAP in pancreatic cancer

a) XIAP mRNA is expressed in cultured pancreatic cancer cells. In view of the expression and localization of XIAP, we next sought to evaluate the functional role of XIAP in PDAC. Therefore, in order to select pancreatic cancer cell lines with detectable XIAP levels for functional analysis, qRT-PCR analysis was performed in 8 pancreatic cancer cell lines. This analysis revealed relatively high levels of XIAP mRNA in Aspc-1, Capan-1, T3M4 and BxPc-3 cell lines and relatively low levels in the remaining cell lines (Figure 3A). Immunoblot analysis demonstrated a good correlation between XIAP mRNA and protein levels. Thus, a specific band of 57 kDa corresponding to the known size of XIAP was detected at relatively strong intensity in the same cell lines that exhibited high XIAP mRNA levels (Figure 3B).

b) XIAP silencing in pancreatic cancer cells. The Aspc-1 and Capan-1 cell lines were selected for gene silencing since they displayed relatively high XIAP mRNA and protein levels. XIAP down-regulation was performed by transfecting the cells with custom designed siRNA oligonucleotides. This analysis revealed consistent gene silencing in repeated transfection experiments in both Capan-1 and Aspc-1 cell lines. Thus, XIAP mRNA levels in Aspc-1 cells decreased by 54% (24 h) and 69% (48 h) (data not shown), while they decreased by 33% (24 h), 43% (48 h), and by 54% (72 h) in Capan-1 cells respectively, compared to control samples transfected with control siRNA (Figure 4A).

However, consistent gene silencing at the protein level was difficult to demonstrate especially in the Aspc-1 cells. In the Capan-1 cells, consistent gene silencing was demonstrated on the protein level with a reduction of 46% (48 h) compared to control samples as shown by immunoblot analysis and densitometry (Figure 4B).

c) XIAP gene silencing reduces proliferation of pancreatic cancer cells. To assess the role of XIAP in pancreatic cancer growth, the influence of siRNA transfection on the proliferative activity of Capan-1 and T3M4 cells was evaluated. Compared to the negative control siRNA transfected cells (71.1±3.1%), XIAP siRNA-transfected Capan-1 cells showed a further reduction to 56.8±1.9%. This difference was significant ($p=0.03$) (Figure 5A). Also, the XIAP siRNA-transfected T3M4 cells showed an additional reduction to 65.3±3.4% compared to 68.5±3.7% reduction in negative control siRNA transfected cells. This difference, compared to negative control transfected cells, was not significant ($p=0.059$) (Figure 5B).

d) XIAP gene silencing reduces chemoresistance of pancreatic cancer cells. Since XIAP plays a role in chemoresistance of different cancers and since a reduction in proliferation in XIAP silenced Capan-1 and T3M4 cell lines was observed, we next analyzed the influence of XIAP silencing on chemoresistance. siRNA- and control-transfected cells were exposed to graded concentrations of gemcitabine. In both cell lines there was an increase in the sensitivity to gemcitabine following XIAP silencing. Thus, at a dose of 50 ng/ml of gemcitabine (Figure 6A), there was a 45±1.9% growth reduction in XIAP-silenced Capan-1 cells compared to a growth reduction of 27±10% in control siRNA (negative control) transfected cells ($p=0.02$). Similarly, in the case of the T3M4 cells, at a dose of 50 ng/ml of gemcitabine (Figure 6B),

there was a 68±4.3 % growth reduction in XIAP-silenced T3M4 cells compared to a growth reduction of 44±1.5% in the control siRNA (negative control) transfected cells ($p=0.01$).

Discussion

Apoptosis is a complex and multifaceted process that requires the activation of a family of cysteine aspartyl proteases termed caspases (26). Adaptor proteins promote the autocleavage and activation of initiator caspases (*e.g.*, caspase-8 and caspase-9), initiator caspases cleave and activate effector caspases (*e.g.*, caspase-3) and effector caspases induce a multiplicity of events that ultimately result in cell death (27, 28). There are two apoptosis signaling pathways: the death receptor pathway and the mitochondrial pathway. When active, death receptors interact with adaptor proteins such as FADD and TRADD and activate caspase-8. When damaged, mitochondria release cytochrome c, which associates with the adaptor protein Apaf-1 and activates caspase-9. Most drugs signal apoptosis through the mitochondrial pathway (29).

Proteins that modulate caspase activity and, thus, determine whether cells live or die, include the inhibitor of apoptosis proteins (IAPs) and the Bcl-2 family of proteins. Of these proteins, XIAP is the most potent one and it inhibits the activity of processed caspases; thus, it functions as a "brake" that restricts the apoptotic process once it is initiated (30). XIAP was previously demonstrated to be overexpressed in some human cancers such as in non-small cell lung cancer (16, 17), myeloid leukaemias (18) and prostate cancer (31) and in pancreatic cancer cell lines (23, 32, 33).

The results of our study now demonstrate that, apart from its overexpression in pancreatic cancer cell lines, XIAP is overexpressed in pancreatic cancer tissues as well. Given its mechanism of action in the apoptotic pathway, low XIAP expression levels should translate into better survival. However, in non-small cell lung cancer (NSCLC) where XIAP expression was evaluated in relationship with chemosensitivity, it was observed that there were differences in the level of expression and in the subcellular distribution of XIAP in tumors derived from NSCLC patients and that XIAP expression could not predict response to chemotherapy in patients with advanced NSCLC (17). In contrast to these findings, our clinical correlation analysis appears to suggest that higher levels of XIAP are indeed associated with reduced survival in pancreatic cancer, although our results did not reach statistical significance. These observations are similar to studies that reported higher XIAP levels having an adverse prognostic significance for patients with acute myeloid leukemia (AML) and in transitional cell carcinoma compared to those with lower levels of XIAP (18, 19).

Our IHC findings of cytoplasmic XIAP in pancreatic cancer cells are in line with observations in NSCLC (17). In addition, moderate to strong XIAP immunostaining in the tubular complexes of nearly one-third of the pancreatic cancer tissues evaluated reinforces the belief, as previously suggested in transitional cell cancer (19), that XIAP plays a role in carcinogenesis right from the initial stages.

At the other end of the spectrum, XIAP immunostaining in common metastatic sites of pancreatic cancer, *i.e.,* lymph nodes and the liver, raises the possibility that XIAP is up-regulated in response to cancer cells being detached from the primary tumor during the process of metastasis, thus, ensuring favorable conditions for the continuing progression and spread of this cancer. Lending further credence to this concept, higher XIAP mRNA levels in patients with metastatic lymph nodes were associated with a tendency for reduced survival (>400 copies of cDNA/μl: median survival 13.2 months *versus* <400 copies of cDNA/μl: median survival 22.9 months; $p=0.1$; data not shown). Since XIAP is involved in the process of anoikis in prostate cancer (34), our data provide indirect evidence that XIAP might also be involved in anoikis in pancreatic cancer. Thus, it appears that XIAP plays an influential role from the early to the end stages of pancreatic carcinogenesis.

Pancreatic cancer is one of the most common malignancies in Europe and North America. In the United States, more than 30,000 people develop pancreatic cancer each year (2). The best treatment for pancreatic cancer is radical resection. However, only a limited number of patients are amenable to surgery due to the advanced stage of the disease at the time of presentation. Patients with advanced pancreatic cancer are subjected to chemotherapy with only modest success (gemcitabine is considered the standard of care since its approval in 1997) because pancreatic cancer cells are largely chemo- and radio-resistant (3, 35).

As mentioned before, XIAP has been implicated in chemoresistance in other cancers (19-23). In order to restrict tumor growth and overcome chemoresistance, a number of different approaches (*e.g.,* full length antisense viral expression vectors, antisense or RNAi oligonucleotides, small molecule inhibitors, *etc.*) (36-42) to down-regulate or inhibit XIAP have been reported to be successful in different tumors such as breast cancer, hepatocellular carcinoma, and prostate cancer (34, 43, 44). The recent initiation of phase I studies evaluating XIAP inhibition in cancer cells are further testimony of the importance of this gene in oncology (45).

In conclusion, XIAP is overexpressed in pancreatic cancer and possibly contributes to chemoresistance. XIAP silencing with specific siRNA molecules or chemical compounds blocking XIAP might have a potential therapeutic role in the treatment of this dismal disease.

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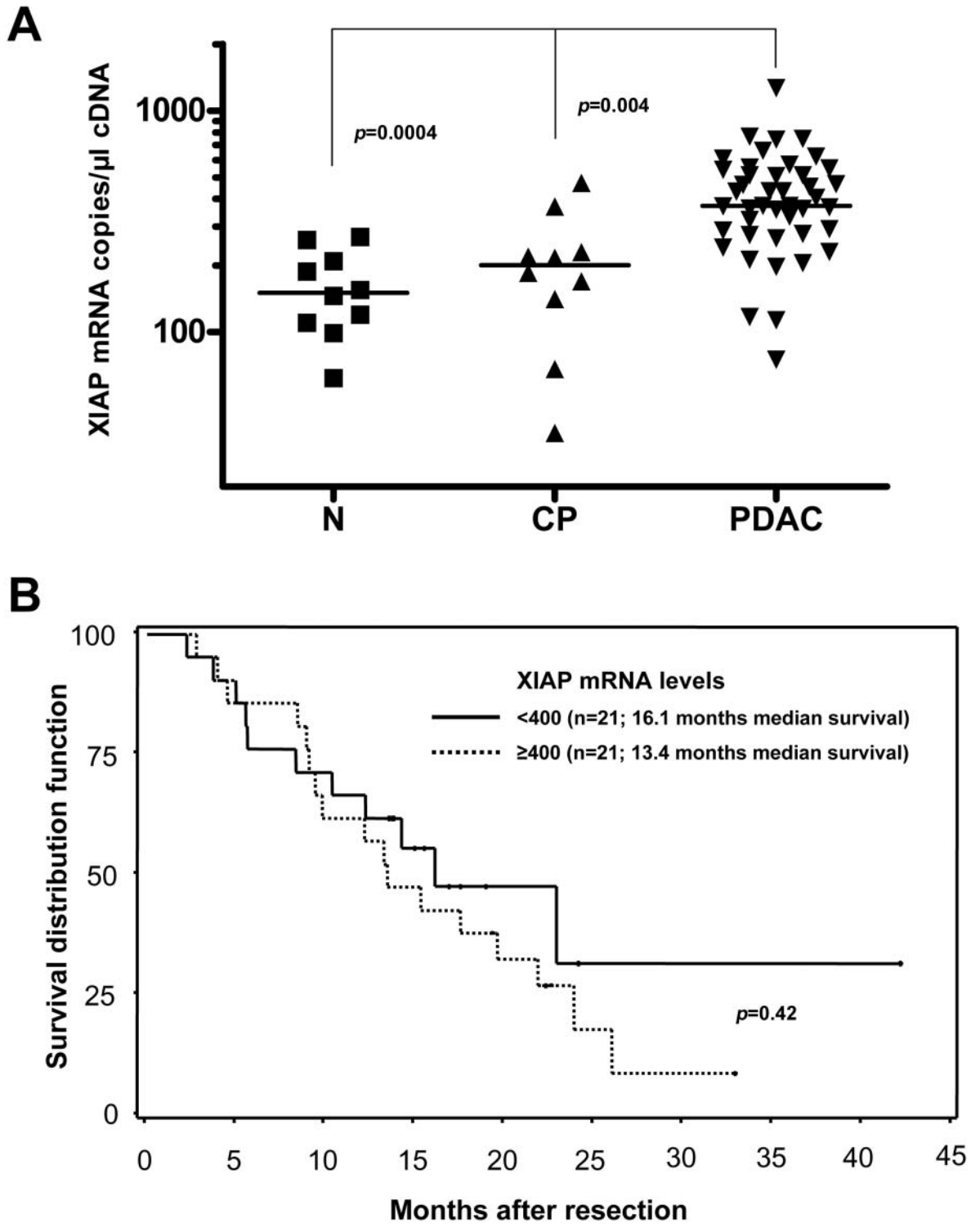

Figure 1. *A) Expression analysis of XIAP mRNA in pancreatic tissues QRT-PCR was carried out as described in the Patients and Methods section. Individual samples are expressed on a logarithmic scale as copies/µl cDNA normalized to housekeeping genes. Horizontal bars show the median values. N=normal pancreas (represented by squares); CP=chronic pancreatitis (represented by triangles); PDAC=pancreatic cancer (represented by inverted triangles). B) XIAP mRNA levels and correlation with patient survival XIAP mRNA levels of 42 patients evaluated were correlated with patient survival using Kaplan-Meier analysis. Patients alive at the last follow-up are censored and marked (|). Median survival in the group of patients with <400 copies of cDNA/µl was 16.1 months and was 13.4 months in the group of patients with >400 copies of cDNA/µl.*

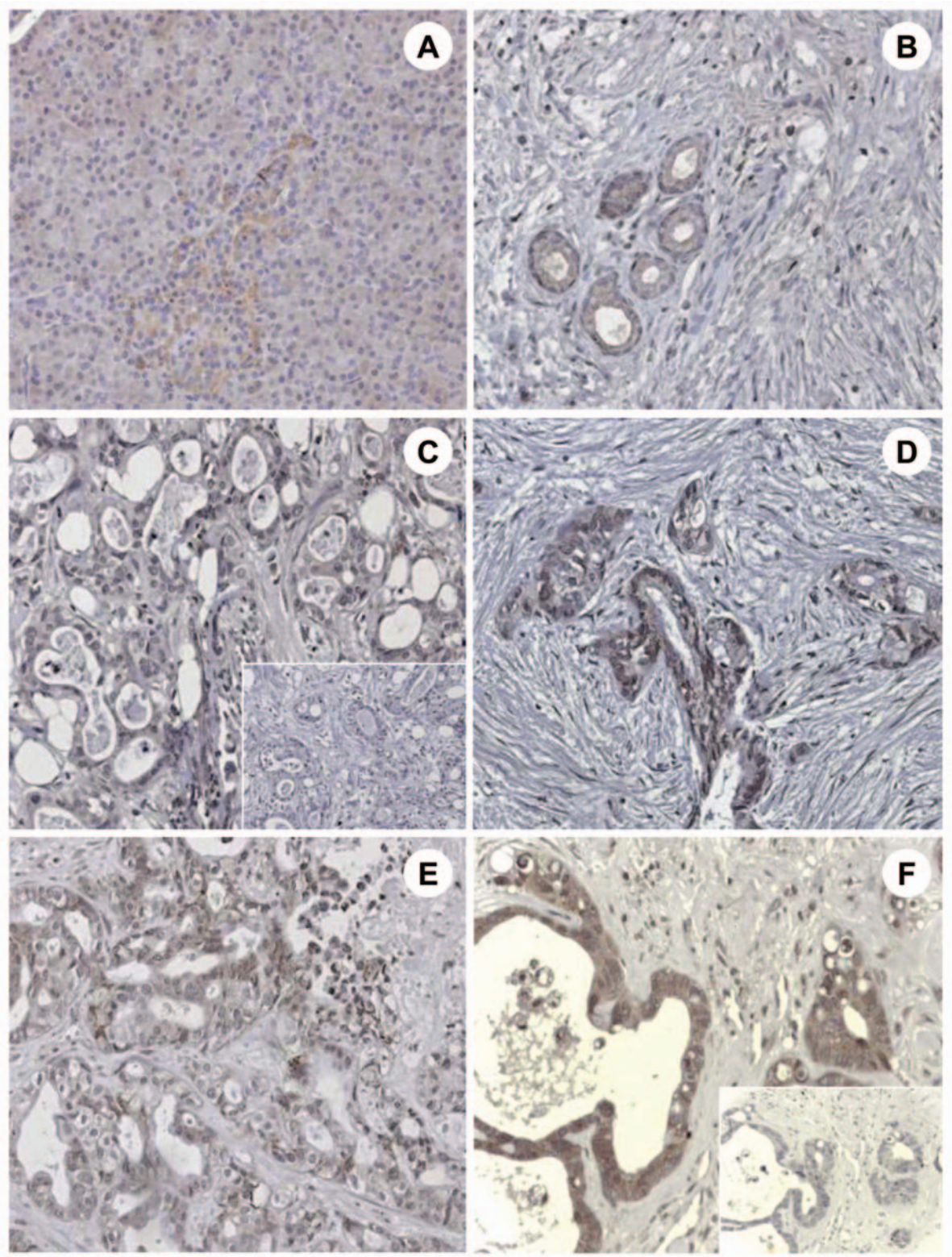

Figure 2. *Localization of XIAP in normal pancreas and PDAC. Immunohistochemistry on paraffin sections was carried out using a polyclonal rabbit XIAP antibody or rabbit IgG as a negative control as described in the Patients and Methods section. A) Weak to moderate immunoreactivity in the acinar cells of the normal pancreas. B) Moderate to strong immunoreactivity in the tubular complexes in PDAC. C) Strong immunoreactivity in the cytoplasm of pancreatic cancer cells. Inset: Negative control. D) Strong immunoreactivity in cancer cells in PDAC. E) Liver metastasis of PDAC. F) Lymph node metastasis of PDAC. Inset: Negative control.*

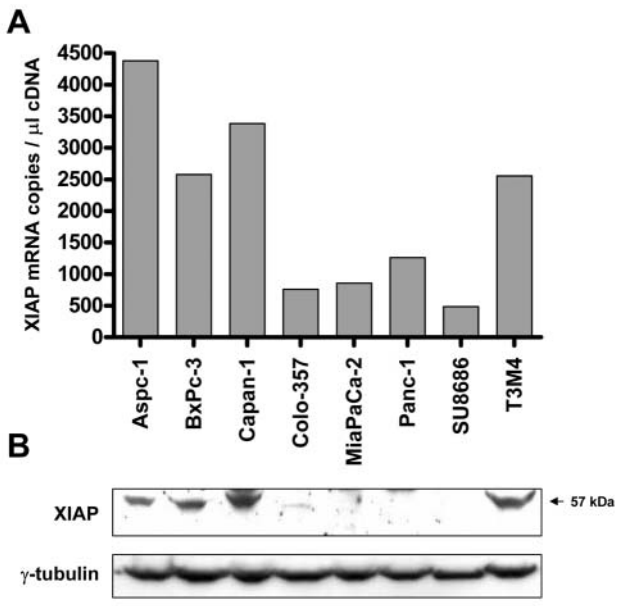

Figure 3. *Expression analysis of XIAP mRNA in pancreatic cancer cell lines. A) QRT–PCR was carried out as described for different pancreatic cancer cell lines as indicated. RNA input was normalized to the average expression of the housekeeping genes and is presented as copy number/µl cDNA. Vertical bars represent the mRNA expression values for each cell line. B) Cell lysate from pancreatic cancer cell lines were subjected to immunoblot analysis as described in the Patients and Methods section. XIAP was detected as a 57-kDa band (upper panel). Equal loading and transfer was confirmed by reblotting the membrane with an anti-γ-tubulin antibody (lower panel).*

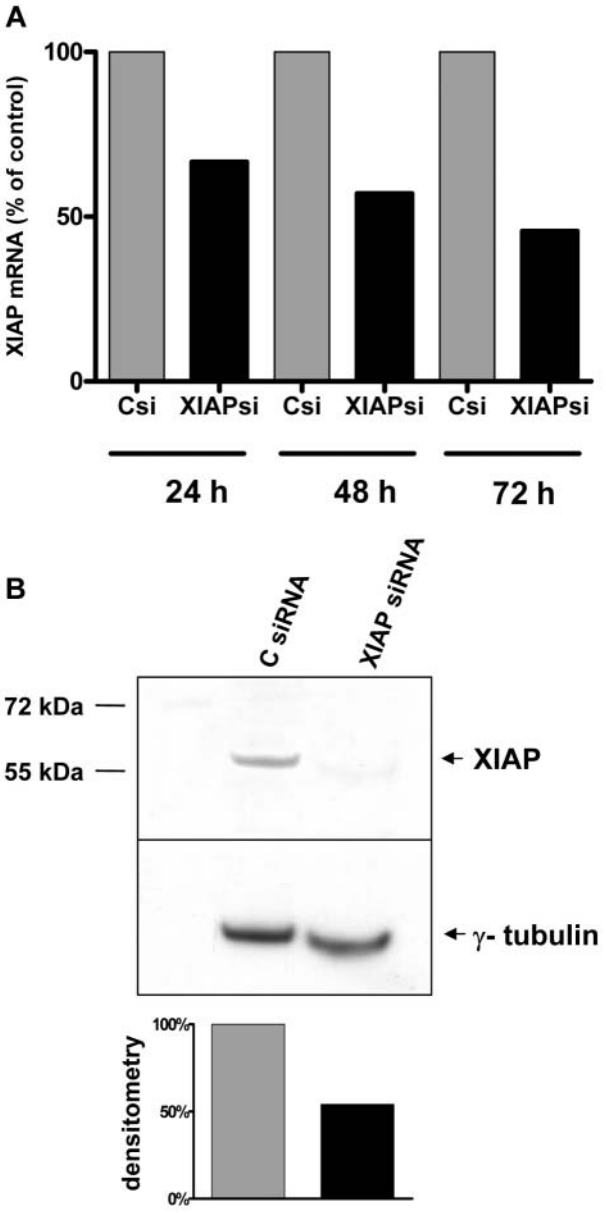

Figure 4. *XIAP silencing using siRNA oligonucleotides. A) Capan-1 pancreatic cancer cells were transfected with XIAP siRNA, and control siRNA at the indicated time points (i.e., 24, 48 and 72 h) and were subjected to QRT-PCR analysis as described in the Patients and Methods section. Data are presented as percentage of control. B) Cell lysates from negative control siRNA and XIAP siRNA transfected cells were subjected to immunoblot analysis as described. Equal loading and transfer was confirmed by reblotting the membrane with an anti-γ-tubulin antibody. Densitometric analysis (lower panel) is presented as percent change of XIAP protein levels in XIAP siRNA transfected cells compared to control siRNA transfected cells. XIAP si=XIAP siRNA transfected cells; Csi=Control siRNA transfected cells.*

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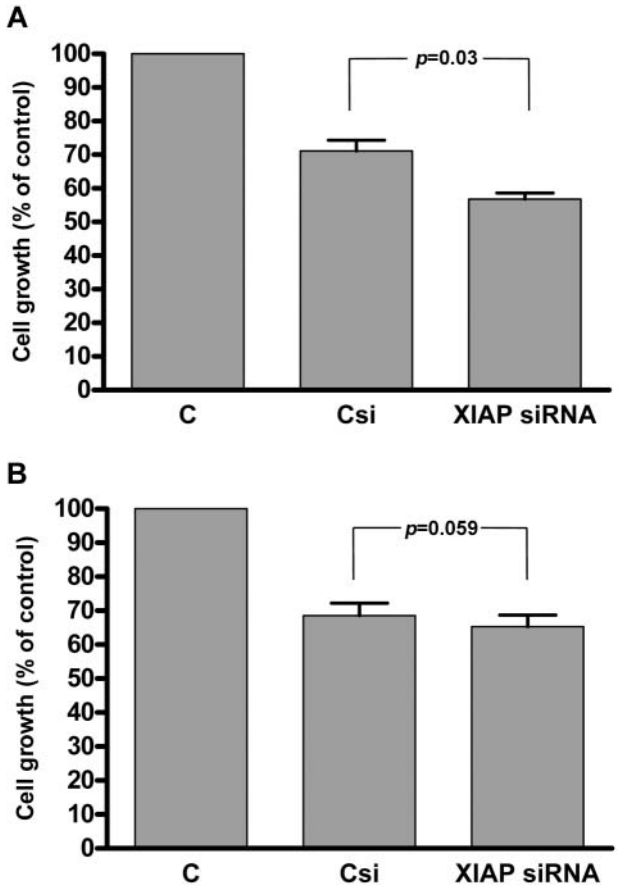

Figure 5. *The influence of XIAP silencing on cell growth. Influence of XIAP siRNA transfection on proliferation of Capan-1 (A) and T3M4 (B) cells by MTT assay as described in the Patients and Methods section. The vertical bars indicate cell growth in percent and values are expressed as percentage of control from three independent experiments. C= untreated control cells; Csi=negative control siRNA transfected cells; XIAP siRNA=XIAP siRNA transfected cells.*

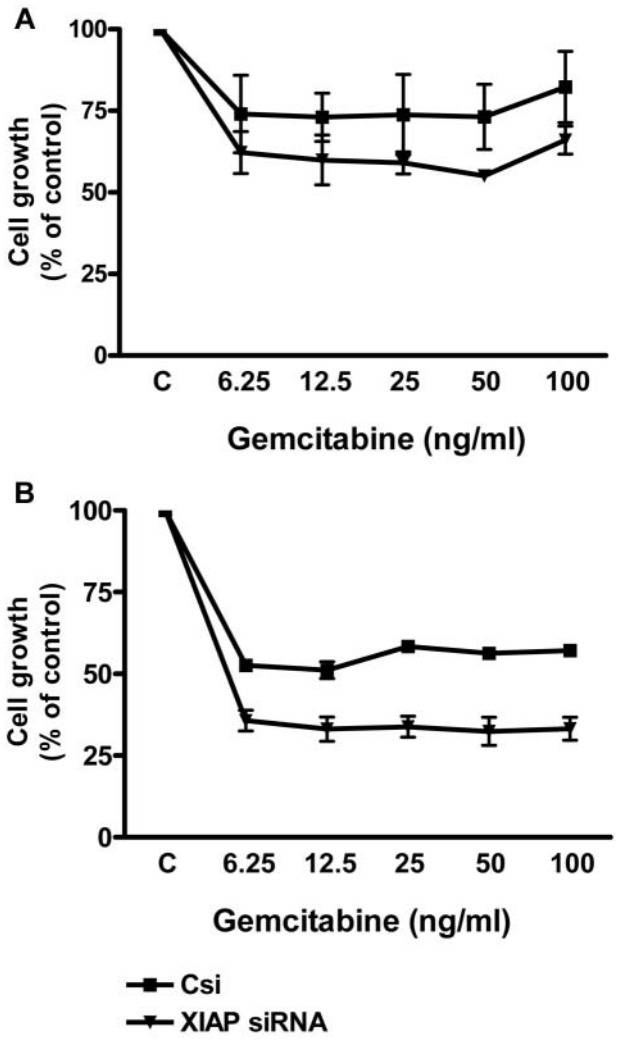

Figure 6. *XIAP silencing and chemosensitivity of pancreatic cancer cells. Effects of XIAP siRNA transfection and response to gemcitabine of Capan-1 (A) and T3M4 (B) pancreatic cancer cells. Negative control siRNA transfected cells (Csi) and XIAP siRNA transfected cells (XIAP siRNA) were exposed to increasing concentrations of gemcitabine as indicated for 72 h after transfection. The decrease in cell growth is expressed as percentage of negative control siRNA-transfected cells. Results are presented as mean±SEM (standard error of mean) of three independent experiments.*

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