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Epigenetic Regulation of APAF-1 Through DNA Methylation in Pancreatic Cancer

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Abstract. *Background/Aim: Apoptotic peptidase activating factor 1 (APAF-1) is essential regulator of apoptosis and inactivation by DNA methylation is common event in numerous cancer types. We investigated the regulation of APAF-1 through DNA methylation in pancreatic cancer. Materials and Methods: Datasets from 44 patients after pancreatoduodenectomy and the pancreatic adenocarcinoma (PDAC) cell lines Capan-2 and MIA PaCa-2 treated with decitabine were analyzed by RT-PCR, immunoblotting, methylation-specific PCR analysis, apoptosis and viability assays to identify effects of APAF-1 regulation. Results: APAF-1 mRNA and protein levels were significantly down-regulated, and APAF-1 methylation status was associated with perineural invasion in PDAC. Decitabine inhibited cell viability and increased apoptosis rates, however failed to restore APAF-1 mRNA and protein levels in cells. Conclusion: APAF-1 gene hypermethylation may contribute to the progression of PDAC through perineural invasion. Decitabine could sensitize pancreatic cancer cells to apoptosis and growth retardation, however, not directly through the APAF-1 demethylation process.*

The pancreatic ductal adenocarcinoma (PDAC) is characterized by its aggressive course, biodiversity and is often diagnosed late, leading to poor outcomes (1). Recent studies have revealed that defects in the apoptotic pathway may be related to cancer's development and resistance to chemo- and radio-therapy (2). Induction of apoptotic pathways in cancer therapy is often complicated because of molecular defects in apoptosis signaling pathways. One of these pathways is gene silencing through DNA methylation (3). It has already been shown that pancreatic cancer is an epigenetic disease, characterized by widespread and profound alterations in DNA methylation (4).

APAF-1 (apoptotic peptidase activating factor 1) is a central and integrant component of the apoptosome in the intrinsic pathway of apoptosis and is important for tumor suppression and drug resistance in DNA damage–induced apoptosis (3, 5). Aberration in apoptosome is associated with oncogenesis and chemo-resistance (6-8). Inactivation of *APAF-1* by DNA methylation may be a crucial factor disturbing the intrinsic pathway of apoptosis, leading to carcinogenesis in different cancer types including PDAC (7-11). Furthermore, it is known, that chemo-sensitivity may be increased by *APAF-1* gene demethylation. Decitabine (5-aza-2'-deoxycytidine), a hypomethylating agent, reverses methylation by inhibiting DNA methyltransferase, reconstitutes the expression of silenced genes and restores their anti-tumorigenic activities. Furthermore, it is known, that decitabine could increase susceptibility of tumor cells to the treatment and reconstitute the expression of silenced genes and restore their anti-tumorigenic activities (12-15). Based on this data the potential for decitabine in cancer therapy was suggested. Recent studies show, that restoring physiological levels of APAF-1 through decitabine treatment, markedly enhances chemo-sensitivity and rescues the apoptotic defects associated with APAF-1 loss in various cancers (8, 13, 16). As the ability of DNA hypermethylation to silence tumor suppressor genes in cancer is well established, however the mechanisms of the selection of genes targeted for this aberrant DNA methylation in

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pancreatic cancer are still unclear. Therefore, the present study aimed to clarify the role of *APAF-1* and its methylation pattern in pancreatic cancer and, also, to determine decitabine's effects on pancreatic cancer cells through the demethylation process in order to provide new information regarding pancreatic cancer's epigenetic regulation.

Materials and Methods

Human pancreatic cancer tissues and data collection. Pancreatic carcinoma tissues were obtained from 44 patients undergoing partial pancreatoduodenectomy between 2011-2016 at the Department of Surgery, Lithuanian University of Health Sciences. Adjacent normal pancreatic tissue samples obtained from the same patients and confirmed by histopathological examination as free of pancreatic cancer served as controls. Samples from PDAC tumors and adjacent pancreas were snap frozen in liquid nitrogen in the operating room upon surgical removal and maintained at –80˚C until use. All clinicopathological (patient's age, gender, tumor stage, lymph node status, differentiation grade, lymphatic, microvascular, peripancreatic and perineural invasion) data was retrieved from a prospectively maintained database. Cancer specific survival data was provided by Lithuanian National Health database (February, 2020). Ethical approval was issued by the Ethics Committee of the Lithuanian University of Health Sciences (Nr. BE-2-10).

Cell lines. Human pancreatic cancer cell lines Capan-2 and MIA PaCa-2 were obtained from ATCC and used for the analysis. Cells were grown in monolayers in sterile 25-cm^2 capacity flasks with 5-ml RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % FBS (Gibco) and 1% penicillin/streptomycin solution (Gibco). Cells were cultured at 37˚C temperature in a 5% CO_2–95% air atmosphere humidified incubator.

Decitabine (5-aza-2'-deoxycytidine) treatment of PDAC cells. Capan-2 and MIA PaCa-2 cells were cultured in 96-well cell culture plates (3×10^3 cells/ well) and treated with 0, 1, 5, 10 mM decitabine (Abcam, Cambridge, MA, USA) for 24, 48 and 72 h. Decitabine was refreshed every 24 h. Afterwards, *APAF-1* mRNA expression and protein levels, cell apoptosis and viability were evaluated.

Western blot analysis. The homogenates of the tissues or cultured cells were lysed using RIPA lysis buffer (Abcam) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) for 30 min on ice and centrifuged at 10000 × *g* for 10 min at 4˚C. The supernatants were assayed for protein concentration with a Pierce BCA protein assay kit (Pierce Biotechnology, Inc., Thermo Fisher Scientific, Rockford, IL, USA). Fifty μg of protein where suspended in Novex Tris-Glycine SDS sample Buffer (2x) (Life Technologies, Paisley, UK) with 3 μl reducing agent (Life Technologies, Paisley, UK) and H_2O (to a total volume of 30 μl). Prepared protein samples were heated at 97˚C for 5 min before loading and 50 μg of the samples were subjected to 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to poly-vinylidene fluoride (PVDF) membranes for 50 min at 20 V. The membranes were blocked with a blocking buffer (Invitrogen, Life Technologies, Carlsbad, CA, USA) for 30 min at room temperature and incubated overnight at 4˚C with primary antibodies. The following primary antibodies were used: 0.3 μg/ml mouse monoclonal anti-APAF-1 (MA5-15743, Thermo Fisher Scientific, Rockford, IL, USA) and 2.5 μg/ml mouse monoclonal anti-GAPDH (AM4300, Thermo Fisher Scientific, Rockford, IL, USA). The membranes were washed and incubated with the anti-mouse peroxidase-conjugated secondary antibody (Invitrogen) for 30 min, washed and incubated with a chemiluminescence substrate/detection kit (Invitrogen). Results were analyzed with ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., Watford, UK).

RNA extraction and real-time polymerase chain reaction. Total RNA extraction was performed from tissues using PureLink RNA Mini kit (Invitrogen) and TRI reagents (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocol without DNA'se treatment. Purified RNA was quantified and assessed for purity by UV spectrophotometry using NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was generated from 2 μg of RNA with High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The amplification of specific RNA was performed in a 20 μl reaction mixture containing 2 μl of cDNA template, 1×PCR master mix and the primers. The PCR primers used for the detection of *APAF-1* were from TaqMan, identification number ID: Hs00559441_m1. Quantitative RT-PCR (qRT-PCR) analysis was performed using ABI 7500 fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). For normalization, GAPDH housekeeping gene was used. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method.

Cell apoptosis. Capan-2 and MIA PaCa-2 cells were prepared according to the manufacturer's instructions. For caspases 3 and 7 activation analysis, DMEM FluoroBrite Media was prepared by adding 2 drops of CellEvent™ Caspase-3/7 Green ReadyProbes™ Reagent (Life Technologies, Carlsbad, CA, USA)/per 1 ml of media. Afterwards, 500 μl of prepared media was added to every cells' chamber and incubated for 30 min at 37˚C. Then cell apoptosis was analyzed with an Olympus IX71 fluorescent microscope.

Cell viability analysis. Cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay (Invitrogen). Cells were incubated with 1.2 mM MTT for 4 h at 37˚C in culture medium. After 4 h of incubation, all solutions were removed, and the formazan product was dissolved with 200-μl Dimethyl Sulfoxide (DMSO, Carl Roth GmbH Co KG, Karlsruhe, Germany). The absorbance was measured on a microplate Sunrise™ Absorbance Reader (Tecan Austria GmbH, Grödig, Austria) at a wavelength of 570 nm with a reference of 620 nm.

Bisulfite conversion. The extracted DNA underwent bisulfite conversion, which is based on the chemical conversion of unmethylated cytosine to uracil where methylated cytosine remains intact. Four hundred ng of genomic DNA were subjected to bisulfite conversion with the EZ DNA Methylation-Gold Kit (Zymo Research Corp.) according to manufacturer's recommendation. Modified DNA was suspended in elution buffer and was immediately used or stored at –20˚C.

Methylation-specific PCR (MSP). The promoter methylation status of *APAF-1* gene was examined in DNA samples of 44 PDAC tissues and adjacent normal pancreatic tissues from the same patients. *APAF-1* promoter methylation status was detected using the MSP method.

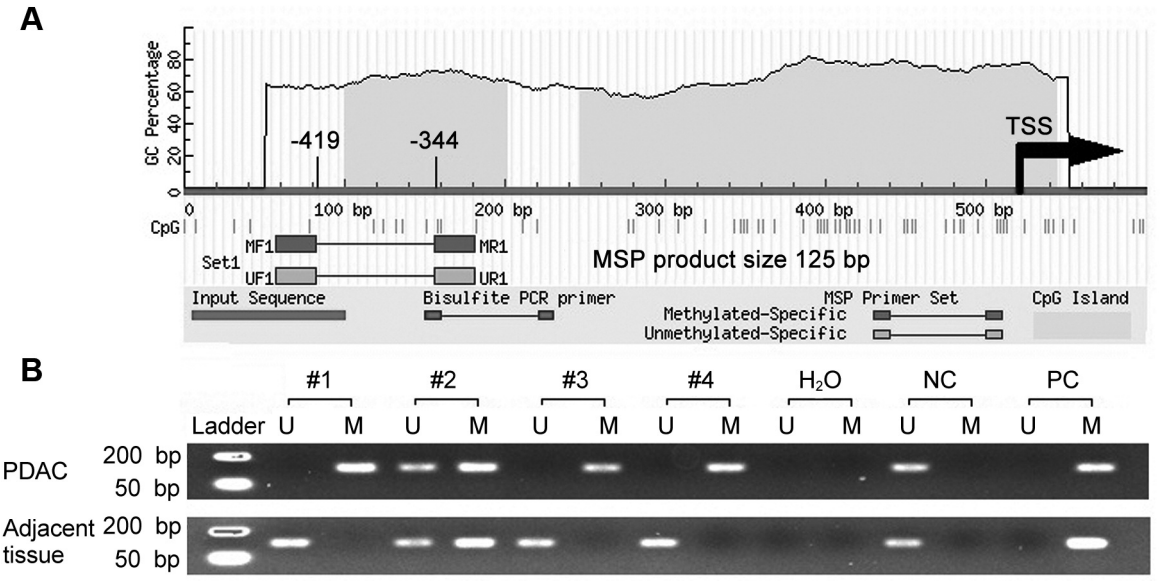

Figure 1. *Analysis of APAF-1 promoter methylation by MSP. (A) The CpG enrichment region of the Apaf-1 promoter was analyzed using Methprimer software. The arrow marker at position 520 bp indicates the transcription start site (TSS). (B) APAF-1 methylation analyses of PDAC patients (#) and adjacent tissues. APAF-1 primers designated as methylated (M) or unmethylated (U). Water was used as template in the 'no template control'. Human methylated and unmethylated DNA were used for negative (NC) and positive (PC) control.*

Bisulphite-treated DNA samples were subjected to PCR, which was performed with two primer sets for methylated (M) FW GATATGTTTGGAGATTTAGGACGA; REV TACCAAATCTATAA AAAAACGCGAT and unmethylated sequence (U) FW GATATGTTT GGAGATTTTAGGATGA; REV TACCAAATCTATAAAAAAACA CAAT. The primers were designed using MethPrimer software (Figure 1A). MSP was performed in 25 μl volume containing 10 pM of each forward and reverse primers, 1 μl of treated DNA, 200 μM of dNTPs, 1.5 mM of $MgCl_2$, 1.5 U of Taq polymerase, and 2.5 μl of 10X PCR buffer. MSP was performed in a thermal cycler using the following cycling profile: 94˚C for 2 min, the amplification was conducted in 35 cycles at 94˚C for 45 seconds, 58˚C for 45 s, and 72˚C for 30 s, followed by re-extension for 5 min at 72˚C. The 5 μl PCR products were loaded onto 2% agarose gel, electrophoresed and visualized under ultraviolet light subsequent to being stained with ethidium bromide using Gel Doc™ XR+ imaging system (Bio-Rad). For methylation positive and negative control Human Methylated & Non-methylated DNA Set (Zymo Research Corp.) was used.

The MSP analysis product was defined as methylation-positive when the methylated allele was present in the methylated DNA lane or both in the methylated and unmethylated DNA lanes, and the product was defined as methylation-negative when a band was present only in the unmethylated DNA lane (Figure 1B).

Statistical analysis. Statistical analysis was performed by a biomedical statistician by SPSS 23.0 software (SPSS Company, Chicago, IL, USA). As the hypothesis of 'normal distribution of data' was rejected by the Shapiro–Wilks test, nonparametric statistical tests were used. The comparisons were performed by the chi-square test, Fisher's exact test or the Mann–Whitney test. Survival rates were summarized using the Kaplan-Meier method and the log-rank test was performed to compare differences in survival between groups. For the survival analysis, patients were stratified into groups according to the mRNA expression of *APAF-1*. The "low" and "high" groups represent an expression lower and higher than the median value, respectively. Statistical significance was defined as $p<0.05$.

Results

Characteristics of the patients. The median patients' age was 68 (range 44-87). The male/female ratio was 0.83. Pathological evaluation classified the majority of analyzed tumors as T3 (93.2%) and moderately differentiated G2 (61.4%) tumors. Metastatic lymph-nodes (N1) and lymphatic invasion (L1) were detected in 90.9% of the cases. Additionally, microvascular invasion (V1) was detected in 81.8% and perineural invasion in 86.4% of cases. The median survival was 18.53 months. All descriptive data of the investigated group are summarized in Table I.

Methylation and expression status of APAF-1 in PDAC tissues. The RT-PCR analysis (Figure 2A) revealed that *APAF-1* mRNA expression was lower in the pancreatic ductal adenocarcinoma when compared to adjacent pancreatic tissue ($p=0.002$). Western blot analysis also confirmed lower protein levels of APAF-1 in the pancreatic cancer tissues, when compared to the adjacent normal pancreas (Figure 2B).

MSP analysis of the *APAF-1* promoter showed promoter methylation in 36 out of 44 (81.8%) PDAC samples and promoter methylation in 8 out of 23 (34.8%) adjacent

Table I. *Tumor characteristics of the patients after pancreatoduodenectomy for PDAC.*

Variable	No. of cases (n=44)
Gender [n (%)]	
Male	20 (45.4%)
Female	24 (54.6%)
Age, years (median)	68
T stage [n (%)]	
T1	2 (4.5%)
T2	1 (2.3%)
T3	41 (93.2%)
T4	0 (0%)
N status [n (%)]	
N0	4 (9.1%)
N1	40 (90.9%)
Lymphatic invasion [n (%)]	
L0	4 (9.1%)
L1	40 (90.9%)
Microvascular invasion [n (%)]	
V0	8 (18.2%)
V1	36 (81.8%)
Peripancreatic invasion [n (%)]	
No	10 (22.7%)
Yes	34 (77.3%)
Perineural invasion [n (%)]	
No	6 (13.6%)
Yes	38 (86.4%)
Differentiation grade [n (%)]	
G1	6 (13.6%)
G2	27 (61.4%)
G3	10 (22.7%)
G4	1 (2.3%)
Median survival (months)	18.53

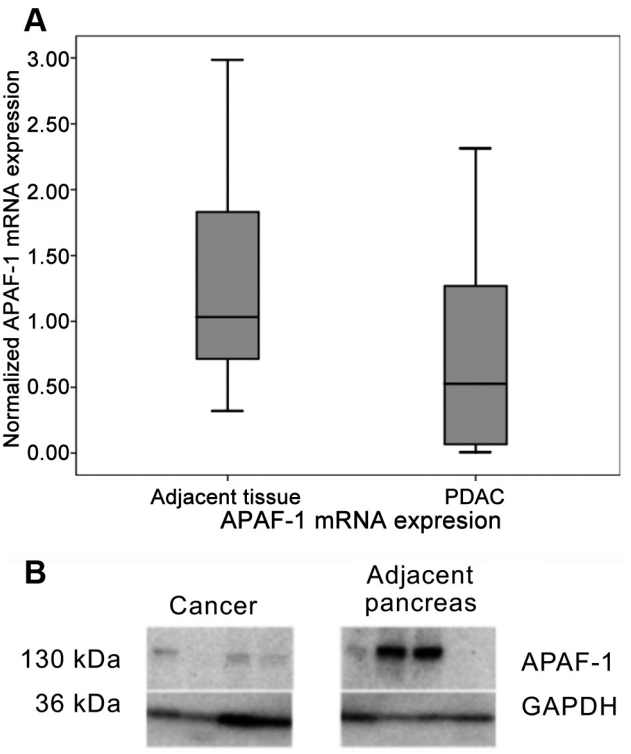

Figure 2. *PDAC specimens displayed increased APAF-1 expression in adjacent pancreas compared with pancreatic cancer. mRNA and protein expression of APAF-1 was evaluated by qRT-PCR (A) and western blot analysis (B) in PDAC and adjacent pancreatic tissue.*

pancreatic tissues $p<0.001$). *APAF-1* promoter methylation was found to be significantly ($p<0.001$) associated with lower *APAF-1* mRNA expression in PDAC tissues (Figure 3A). Median expression of *APAF-1* mRNA in methylated cases was 0.42 (IQR=0.77) and in non-methylated cases 1.62 (IQR=0.95). *APAF-1* mRNA expression differences between methylated and non-methylated cases within adjacent PDAC tissues were not observed (Figure 3B). Median expression of *APAF-1* mRNA in methylated cases was 0.95 (IQR=1.33) and non-methylated cases 1.02 (IQR=1.19).

MSP analysis showed that all (n=8) methylated cases of the adjacent normal pancreatic tissues were semi-methylated, meanwhile methylated cases (n=41) in PDAC tissues consisted of semi-methylated (n=17) and methylated (n=19) phenotypes.

Association of APAF-1 expression and methylation status with tumor characteristics and prognosis. We analyzed the relation between *APAF-1* mRNA expression, methylation status and tumor characteristics. We did not observe any relation between *APAF-1* mRNA expression and histopathological features. However, *APAF-1* mRNA methylation status was related to perineural invasion ($p<0.05$). Survival analysis revealed that neither *APAF-1* expression ($p=0.775$), nor methylation status ($p=0.631$) have influenced patient's survival. Meanwhile, univariate analysis of tumor factors that influence survival revealed that perineural invasion was the only prognostic factor for poor survival (Table II).

Methylation status of APAF-1 in pancreatic cancer cell lines after Decitabine treatment. Capan-2 and MIA PaCa-2 cell lines were treated with identical concentrations of decitabine, to compare promoter CpG methylation response. Methylation analysis was performed before and 72 h after decitabine treatment. Capan-2 cell lines exhibited semi-methylated phenotype before decitabine treatment, while MIA PaCa-2 was found as methylated profile. After treatment of the Capan-2 and MIA PaCa-2 cell lines with decitabine for 72 h, we observed a dose-dependent demethylating effect. Effective decitabine concentrations were 5 mM and 10 mM (Figure 4).

The effect of decitabine treatment on the APAF-1 expression in pancreatic cancer cells. Treatment of MIA PaCa-2 cells with 1 mM and 5 mM Decitabine for 24 h increased the

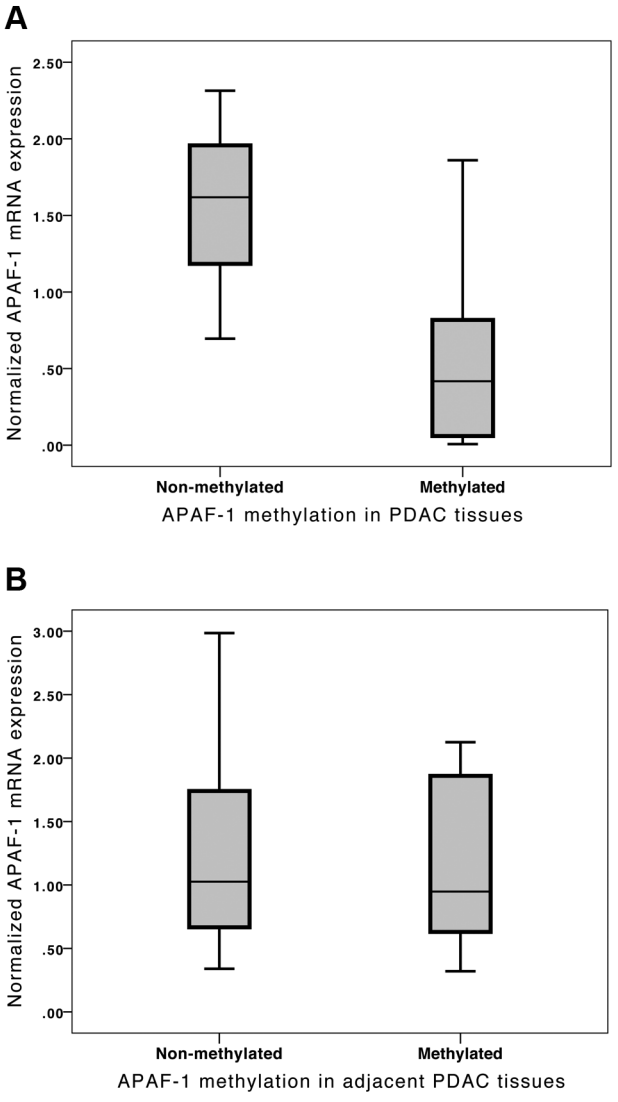

Figure 3. *The APAF-1 methylation and APAF-1 mRNA expression in PDAC tissues (A) and adjacent pancreatic tissues (B).*

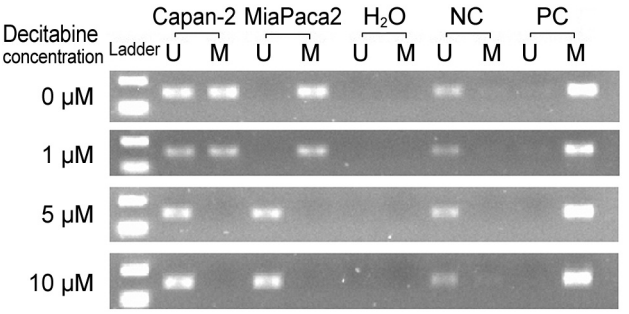

Figure 4. *APAF-1 promoter methylation analyses in the pancreatic cell lines Capan-2 and MIA Paca-2 after 72 h treatment with different decitabine concentrations. Water was used in 'no template' control. Human methylated and unmethylated DNA were used for negative (NC) and positive (PC) control. U-unmethylated band, M-methylated band.*

Table II. *Univariate analysis of histopathological features (log-rank).*

Variable		*p*-Value
T stage	T1-T2/T3-T4	0.210
Lymph-nodes	N0/N1	0.110
Tumor differentiation	G1-G2/G3-G4	0.655
Perineural invasion	Yes/No	0.017
Micro-vessel infiltration	Yes/No	0.380
Lymphatic invasion	Yes/No	0.081
Peripancreatic invasion	Yes/No	0.238
APAF-1 expression	High/Low	0.775
APAF-1 methylation	Yes/No	0.631

expression of *APAF-1* mRNA in the majority of cells. After 48 h, 1 mM treatment increased the expression levels, however, with the increase in the concentration of decitabine (5 and 10 mM) it started to decrease gradually and after 72 h of incubation it reached the lowest expression rates in all concentrations (Figure 5A). Treatment of Capan-2 cells line with 1, 5 and 10 mM decitabine for 24 h and 48 h increased the expression of APAF-1. After 72 h, expression was increased at 1 mM but started to decrease gradually at the other concentrations (Figure 5B). Additionally, decitabine-treated cells did not show an increase in APAF-1 protein expression. Western blot analysis revealed that APAF-1 protein expression was low in MIA PaCa-2 cells and barely detectable in Capan-2 cells (Figure 6).

The effect of decitabine treatment on the viability and apoptosis of pancreatic cancer cell lines. MIA Paca-2 cells' viability was slightly induced but only in the beginning (24 h) of treatment with 1 or 5 mM decitabine (Figure 7A). Higher dose (10 μM) of decitabine did not induce further growth increase. MIA PaCa-2 cells showed a slight decrease in viability after 48 h at all concentrations (Figure 7B). Maximum growth inhibition rates were reached after exposure for 72 h; viability decreased by more than 60.0% (with 5 and 10 μM decitabine) as compared to control (Figure 7C). Treatment of Capan-2 cell showed similar growth inhibition rates as MIA PaCa-2 cells. However, the viability of cells started to decrease in the earlier phase after 24 h incubation and decreased slightly in a dose-dependent manner (Figure 7A). After 72 h incubation, cell viability was decreased approximately by 20 % at all concentrations (Figure 7C).

MIA PaCa-2 cells showed the highest apoptotic activity starting from 24 h of incubation with decitabine that intensified progressively (Figure 8A) and after 48 h (Figure 8B) throughout all concentrations (1, 5, 10 mM) reaching the highest apoptotic rate after 72 h incubation with 10 mM of decitabine (Figure 8C). While, in Capan-2 cells the fluorescence was not very bright after 24 h treatment due to

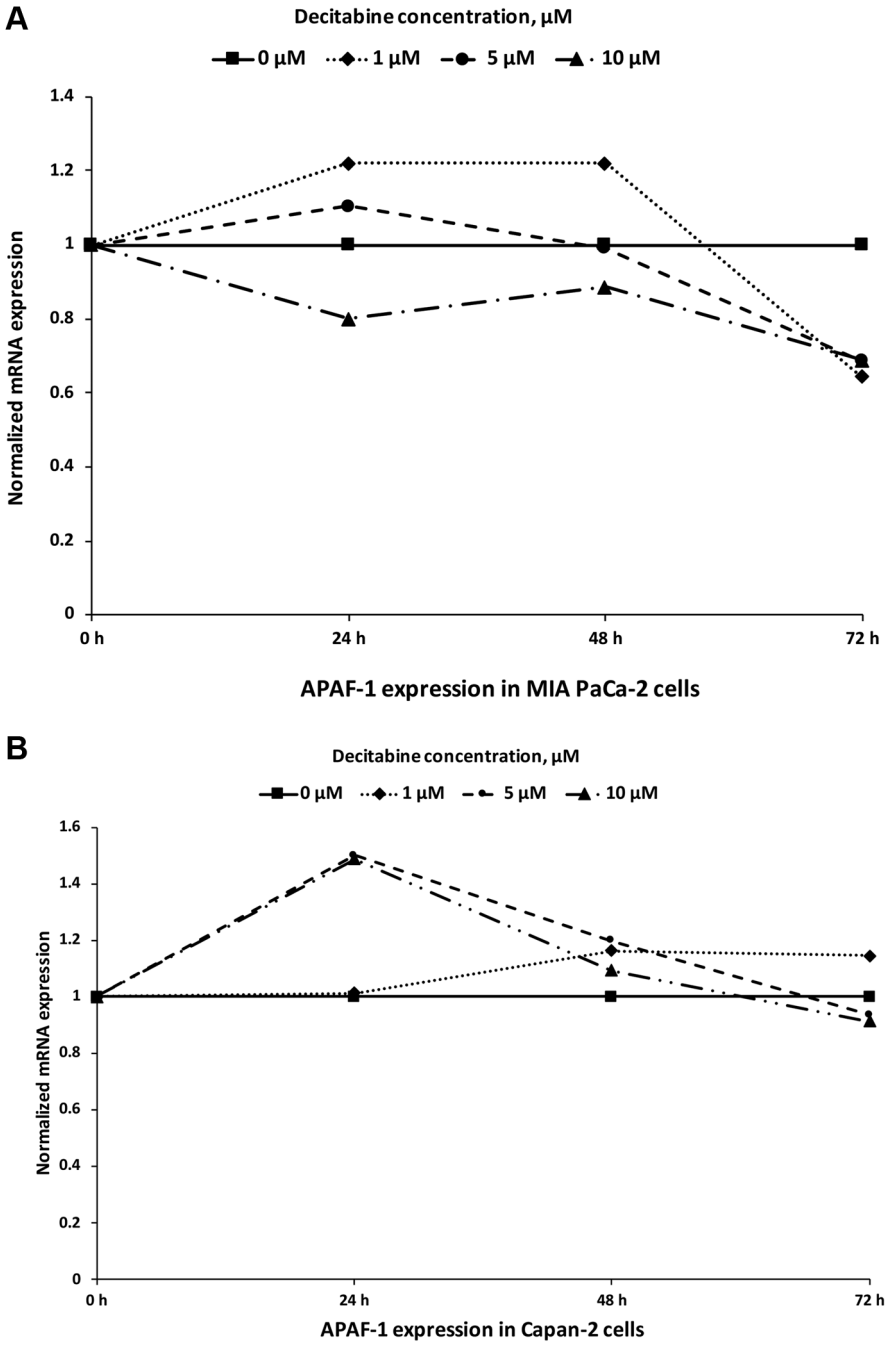

Figure 5. *The effect of decitabine on the expression of APAF-1 mRNA. The expression of APAF-1 mRNA was evaluated by qRT-PCR after exposure to at different concentrations (0, 1, 5, 10 µM) of decitabine for 24, 48, and 72 h in MIA PaCa-2 (A) and Capan-2 (B) cell lines.*

insufficient cell growth (Figure 9A), after 48 h incubation, the apoptosis rate of Capan-2 cells increased significantly at 1 µM decitabine and remained the same up to 10 µM of decitabine (Figure 9B). However, after 72 h exposure to the drug, fluorescence became very weak, possibly due to cell toxicity and the decreased number of cells (Figure 9C).

Discussion

It has been shown that epigenetic silencing of the *APAF-1* gene and abnormal expression of APAF-1 protein is a common event in various cancer tissues such as metastatic melanoma, leukemia, colon cancer, as well as cervical cancer cells *in vivo*

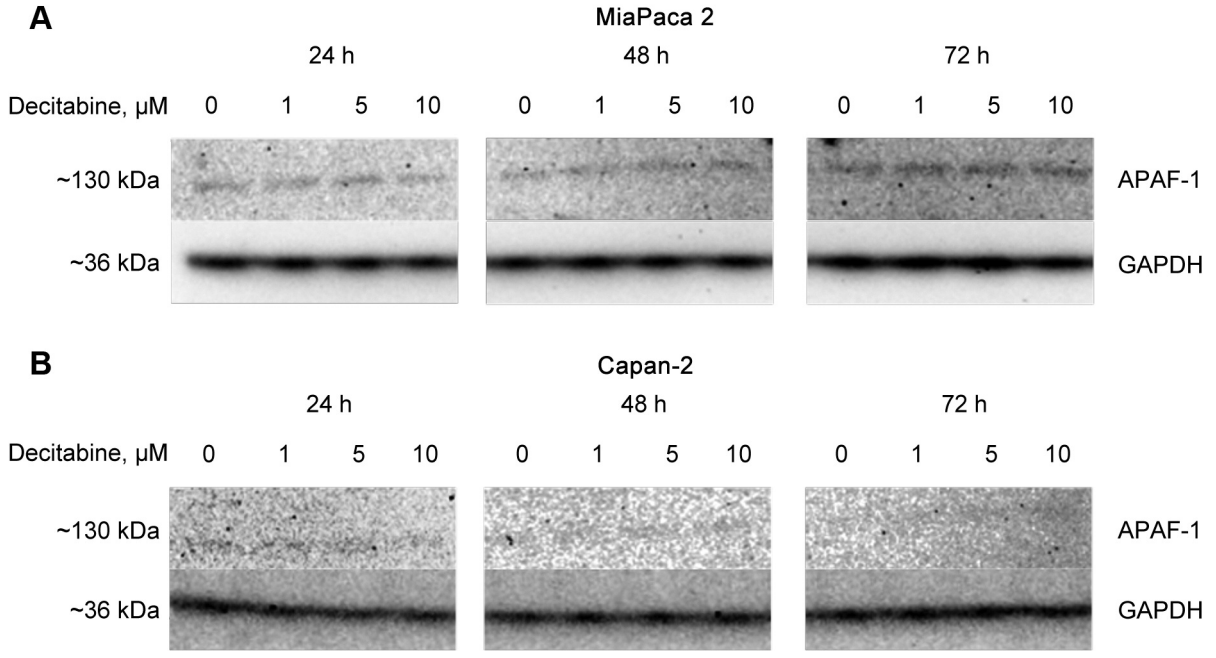

Figure 6. *The effect of decitabine on the APAF-1 protein expression. The expression of APAF-1 protein levels were evaluated by western blot analysis after treatment of cells with 0, 1, 5, or 10 μM decitabine for 24, 48, and 72 h in MIA PaCa-2 (A) and Capan-2 (B) cell lines.*

and *in vitro* (17-22). Additionally, Corvaro *et al*. have shown the dysregulation of *APAF-1* mRNA and protein was observed in a significant fraction of analyzed PDAC' samples (23). In this study we also confirmed down-regulation of *APAF-1* mRNA and protein levels in PDAC when compared to adjacent normal pancreatic tissue. Additionally, it's known that inactivation of *APAF-1* by DNA methylation plays a crucial role in the neoplastic process in different types of cancers (10, 19, 24). Furukawa and his study group have shown surprisingly high methylation of *APAF-1* gene during acute leukemia (25). Similarly, we detected a high frequency of *APAF-1* promoter's methylation in the majority of pancreatic cancer samples. *APAF-1* promoter methylation was found significantly associated with lower *APAF-1* mRNA expression in PDAC tissues. However, methylation was also observed in normal tissue, but at a lower frequency and was not related to lower *APAF-1* mRNA.

There is still an ongoing controversy whether *APAF-1* silencing is essential for tumor oncogenesis and/or progression. Even though we did not observe any relation between APAF-1 mRNA expression and histopathological features, *APAF-1* mRNA methylation status was related to perineural invasion. Our results support earlier reports disclosing associations of APAF-1 expression with cancer progression (18). Furthermore, based on data from the R2 Genomics Analysis and Visualization Platform, the expression of APAF-1 was highly associated with the poor prognosis of patients with PDAC (26). However, we cannot corroborate this association in our study where neither APAF-1 expression, nor methylation status have influenced patient's survival. However, we found that perineural invasion was the only prognostic factor influencing survival. Based on our findings indicating that *APAF-1*'s hypermethylation was related to perineural invasion, we believe that we could not show the association between hypermethylation of *APAF-1* and poor prognosis possibly due to the insufficient number of patients.

Soengas *et al*. have suggested that the inactivated *APAF-1* gene in malignant melanoma is switched off, instead of being completely lost or mutated and physiological levels of APAF-1 could be restored through decitabine treatment (10). Yao *et al*. have reported a tumor-suppressive effect of decitabine resulting from the reactivation of silenced *APAF-1* through demethylation in cervical cancer (8). Therefore, in order to determine if decitabine could restore APAF-1 levels and decrease cell viability and activate apoptosis, MIA PaCa-2 and Capan-2 cells were treated with different concentrations of decitabine for different periods of time. Capan-2 cell lines exhibited a semi-methylated phenotype before decitabine treatment, while MIA PaCa-2 exhibited a methylated profile. Albeit we observed a dose-dependent demethylating effect reaching a peak at 72 h, *APAF-1* mRNA expression increased only slightly at the beginning of treatment. Paradoxically, after 72 hours of incubation, decitabine failed to promote *APAF-1* mRNA expression and showed the opposite effect - *APAF-1* mRNA levels reached the lowest expression rates at all

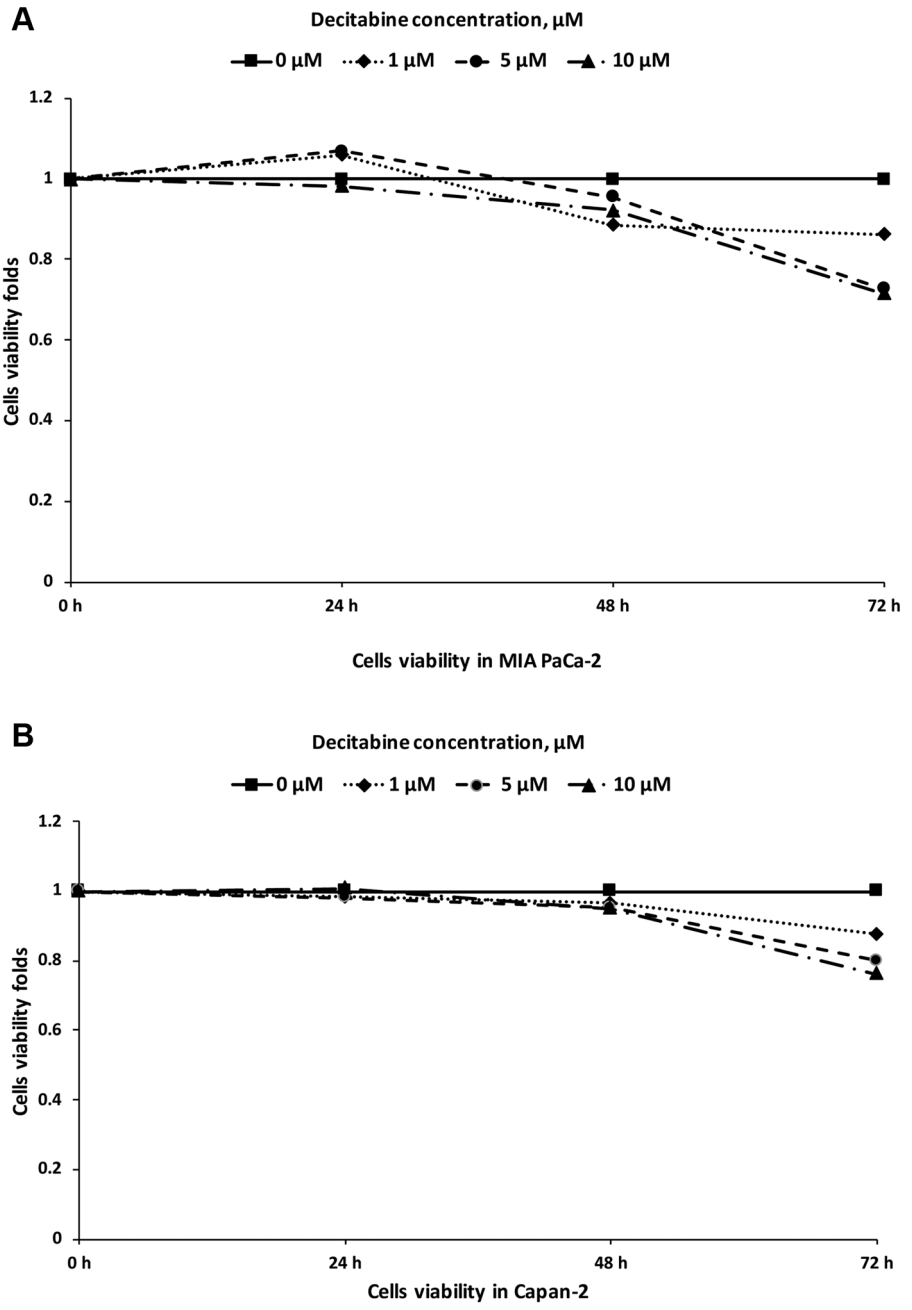

Figure 7. *Cell viability after exposure to decitabine for different incubation times. Cell viability was evaluated by MTT test after treatment of MIA PaCa-2 (A) and Capan-2 (B) cell lines with 0, 1, 5, or 10 µM decitabine for 24, 48, 72 h.*

concentrations, especially in MIA PaCa-2 cells. It is possible that methylation-related alterations of an enhancer element of *APAF-1* lead to disrupted APAF-1 expression (16). Likewise, we hypothesize that during the demethylation process some genes, responsible for regulation of the external apoptotic pathway, may be upregulated as well. The activation of the external apoptotic pathway and decreased apoptotic signaling by mutated TP53 may silence the internal apoptotic pathway and APAF-1 expression (16, 17). Subsequently, our western blot analysis revealed no difference in APAF-1 protein expression in both cell lines treated with decitabine. Some studies have postulated that experimental conditions may not allow the evaluation of APAF-1 expression by western blotting (23, 27). Since there is evidence that disruption of apoptotic

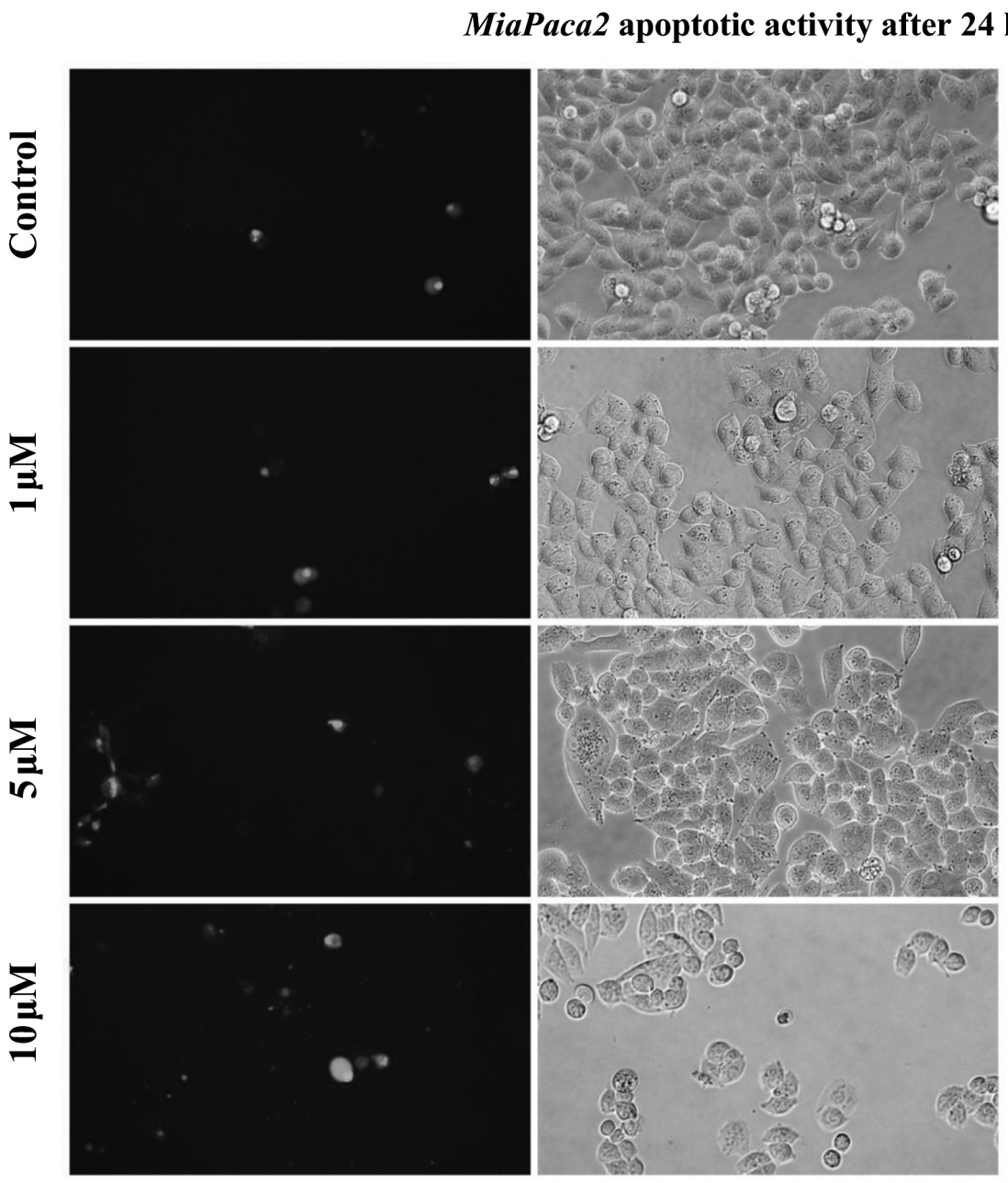

Figure 8. *Continued*

pathways is pertinent to drug resistance in melanoma, at least *in vitro*, it is important to examine whether the same is true *in vivo*, given that cytotoxic drugs are rarely effective in this context (27). Corvaro *et al*. have analyzed APAF-1 protein levels in 5 pancreatic cancer cell lines using 4 different antibodies. However, APAF-1 showed very weak expression in most of them, and MIA PaCa-2 cells showed the lowest expression (23). Similar results have been reported in the human leukemia cell lines studied by Fu *et al*. (2003) where some of them expressed high levels of APAF-1 mRNA but low levels of APAF-1 protein (28). This study points to the possible post-transcriptional regulation of APAF-1 protein expression (28). Other studies have reported that decitabine may change the gene expression not only through, but also independently from DNA demethylation (29). In this way, our data indirectly supports the concept that the effects of decitabine are not limited to direct hypomethylation but through converging pathways may affect other epigenetic factors.

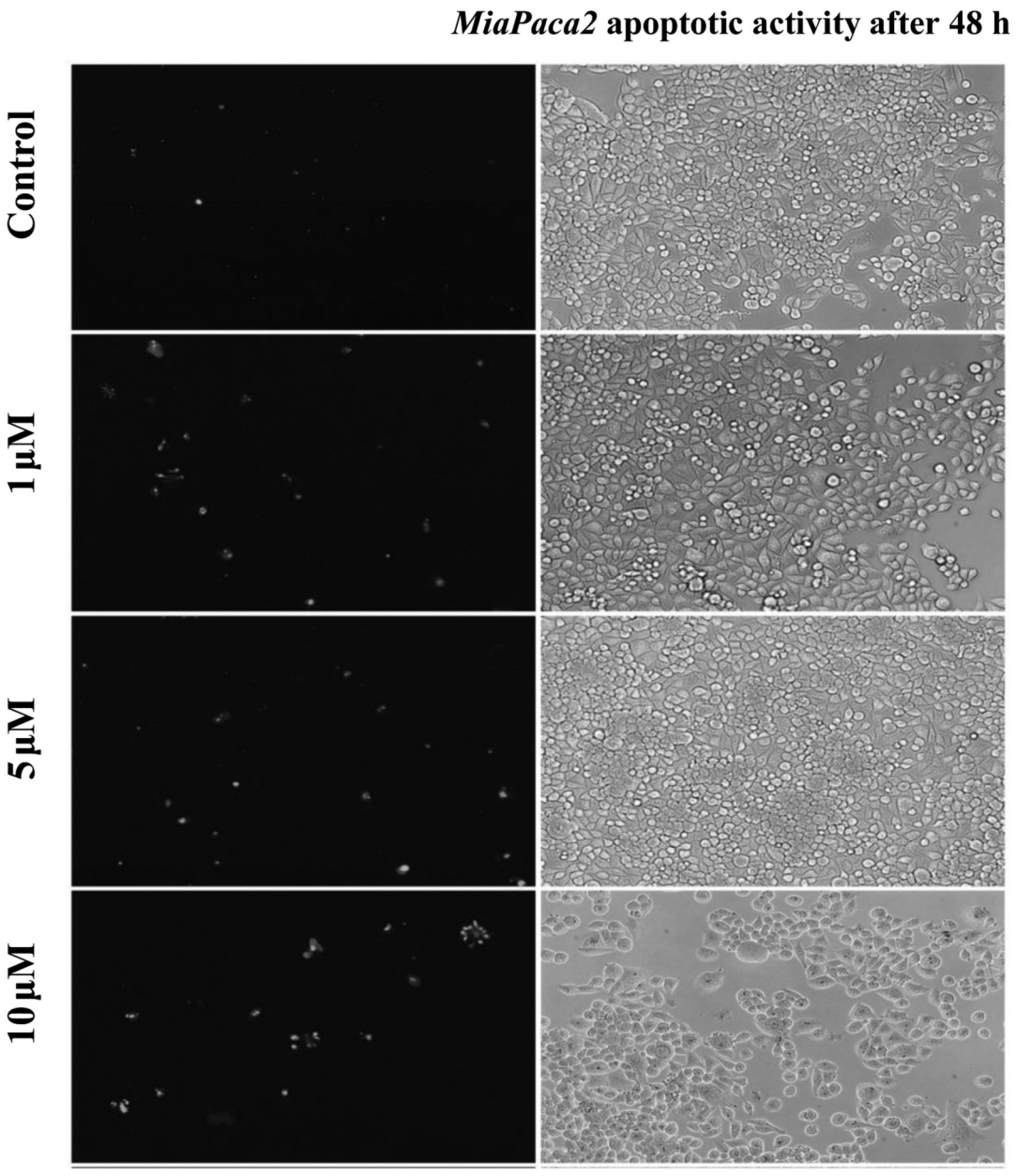

Figure 8. *Continued*

To determine if decitabine could affect PDAC progression, we analyzed cell viability and proliferation after decitabine treatment. Consistent inhibition of Capan-2 and MIA PaCa-2 cell viability and proliferation were apparent in a time-dependent manner. The growth inhibition peaked after incubation for 72 h, while increasing concentrations of decitabine resulted only in slight further growth inhibition. This could be explained by the Gomyo's study showing that decitabine induced TP53 expression, and activated p53 damage-response pathway at higher concentrations (16).

Inactivation of APAF-1 by promoter methylation has been suggested as a factor responsible for the inability of a cell to undergo apoptosis (10, 30). We report that decitabine treatment induced apoptosis from the start of the treatment reaching the highest apoptotic activity after 72 h, especially in MIA PaCa-2 cells. Yao and his group have shown increased apoptosis in HeLa and SiHa cell lines of cervical cancer after restoring APAF-1 expression by decitabine (8). Gomyo *et al*. have suggested that sensitization of lung cancer cells to apoptosis may be based on the activation of the mitochondria pathway

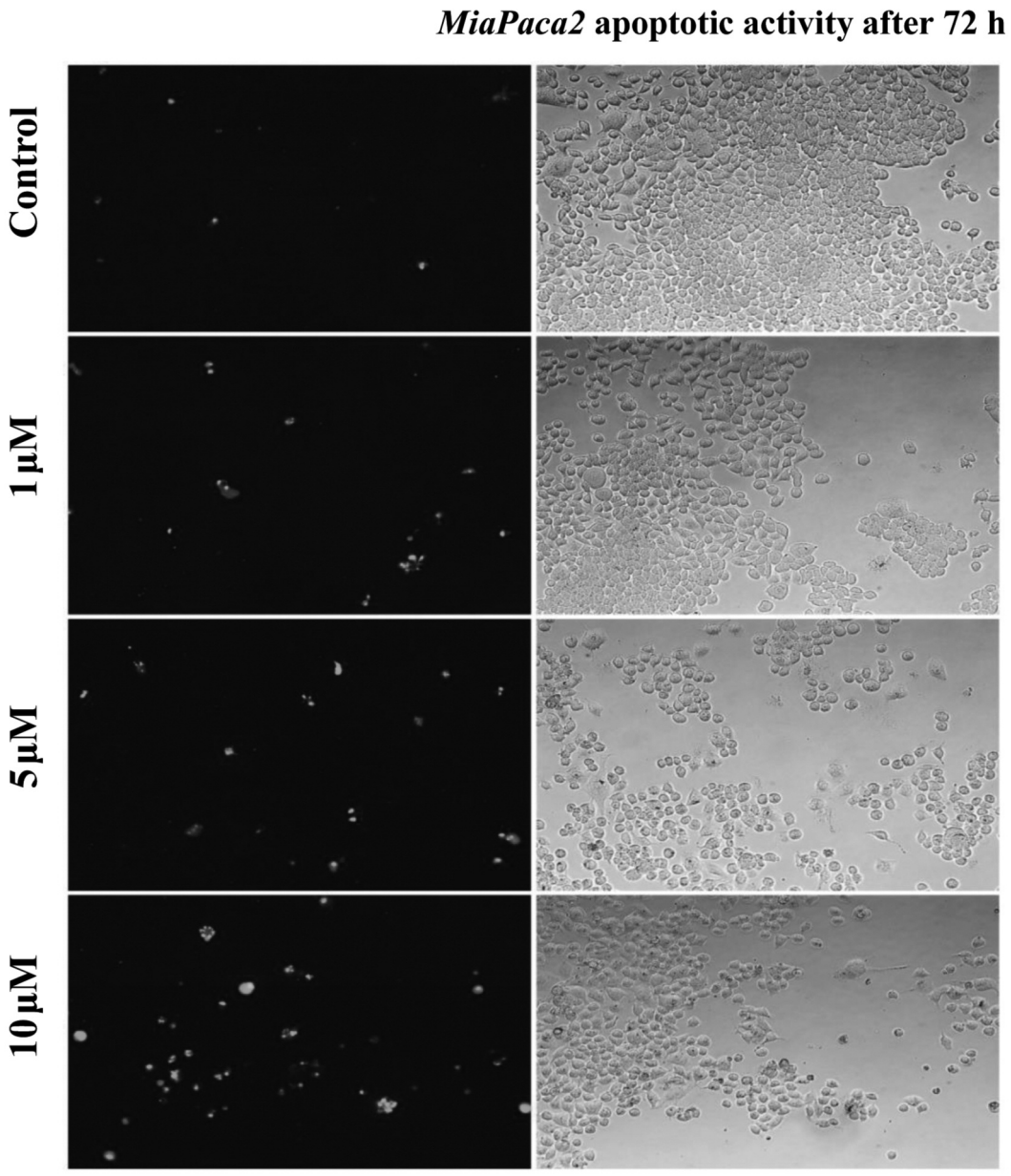

Figure 8. *Cell apoptosis after exposure of MIA PaCa-2 cells to decitabine. Cell apoptosis was evaluated by fluorescent emission after exposure of MIA PaCa-2 cells to decitabine (0, 1, 5, 10 μM) for 24 h (A), 48 h (B) and 72 h (C).*

through upregulation of caspase-9 and APAF-1 expression (16). Contrary, in our study, decitabine could induce apoptosis, however not through APAF-1 demethylation, as after 72 h of treatment, we observed the lowest APAF-1 mRNA levels. Uneven fluorescence activity in Capan-2 cells may be explained by the regulation of transcriptional genes or decreased viability and number of cells. Others have suggested that alternative signaling pathways may be activated when APAF-1 is not functional or when the APAF-1-dependent apoptotic pathway is completely impaired (23). Furthermore, we hypothesize that cellular toxicity, associated with decitabine, is undesirable as may result in secondary effects related to drug induced apoptosis interfering with gene and protein expression.

Conclusion

Our study shows that *APAF-1* gene hypermethylation may contribute to the prognosis and progression of pancreatic adenocarcinoma through perineural invasion. Additionally, decitabine treatment could sensitize pancreatic cancer cells

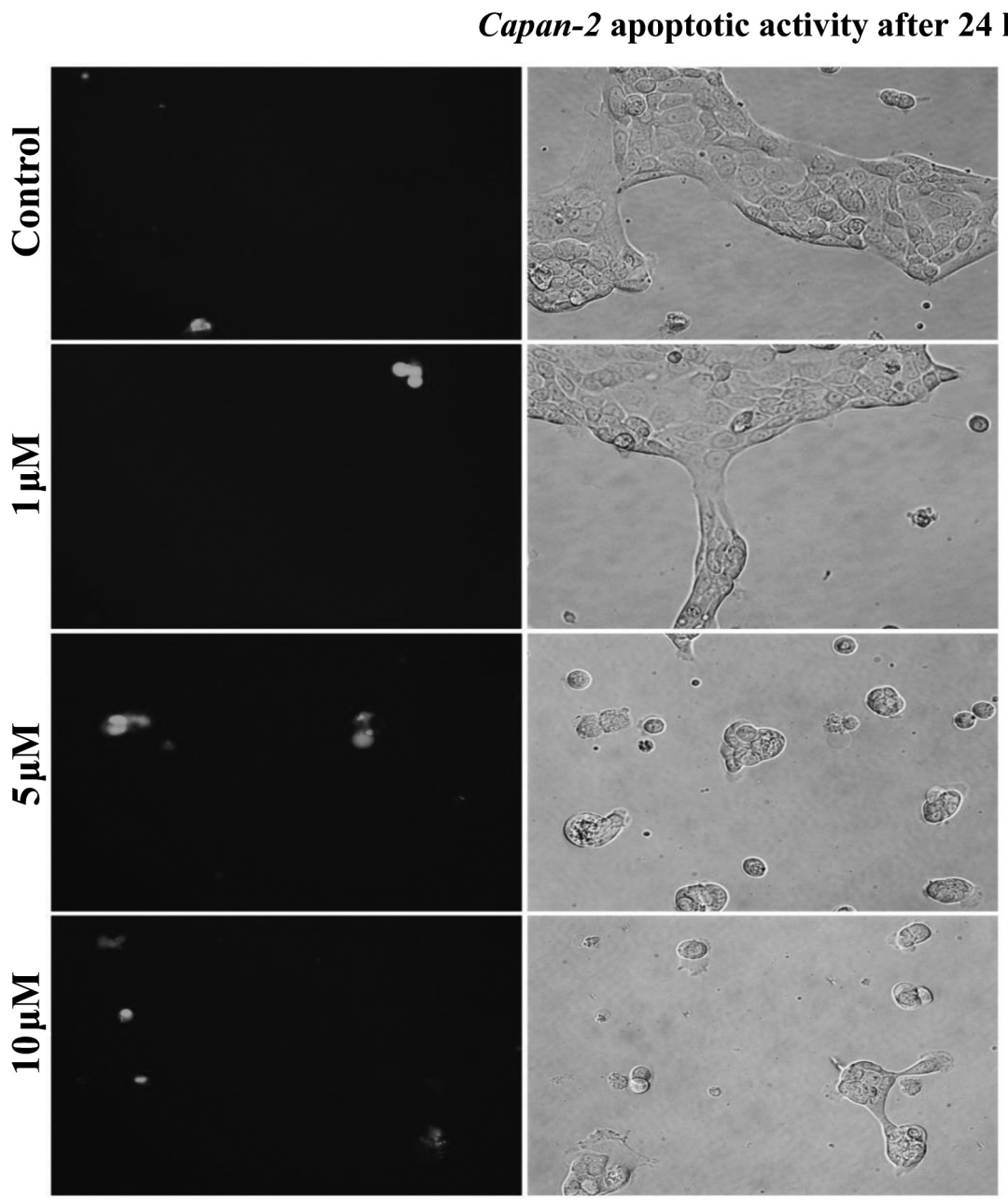

Figure 9. *Continued*

to apoptosis and growth retardation. However, the decitabine effect is not exerted only through direct hypomethylation of APAF-1, but also through other converging pathways.

Limitations of the Study

Even though, as a part of a retrospective analysis of patient's data, we were able to collect survival rates over 5 years, the relatively small number of patients is a limitation of the study. The majority of patients had advanced tumors; therefore, we could not evaluate the exact relation and role of tumor characteristics to the APAF-1 expression, methylation status and survival. In our experimental setting the effects of demethylation were not examined in combination with the common chemotherapy drugs (*e.g.*, gemcitabine, platinum agents).

Conflicts of Interest

All Authors have no conflicts of interest to disclose regarding this study.

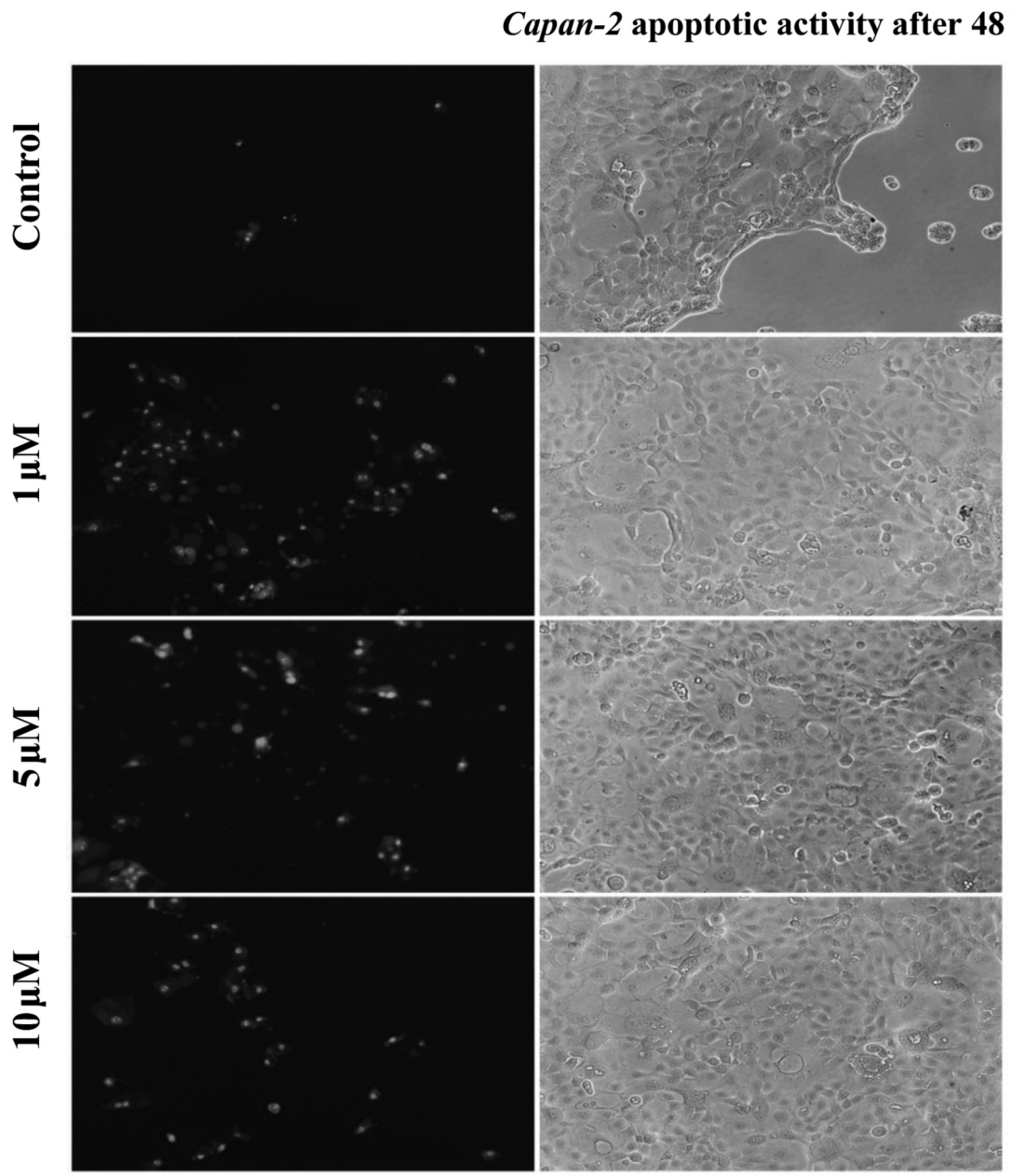

Figure 9. *Continued*

Authors' Contributions

Ausra Lukosiute-Urboniene carried out the RT-PCR studies, analyzed the data and drafted the manuscript; Augustina Mazeike maintained cell cultures experiments; Mintaute Kazokaite and Albertas Dauksa executed and analyzed Methylation-Specific PCR; Giedre Silkuniene and Mantas Silkunas performed the western blot analysis, Vidmantas Barauskas, Giedrius Barauskas and Antanas Gulbinas helped to draft the manuscript; Zilvinas Dambrauskas designed and coordinated the study; All Authors have read and approved the final manuscript.

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References

1 Malvezzi M, Bertuccio P, Levi F, La Vecchia C and Negri E: European Cancer Mortality Predictions for the Year 2014. Ann Oncol *25(8)*: 1650-1656, 2014. PMID: 24759568. DOI: 10.1093/annonc/mdu138

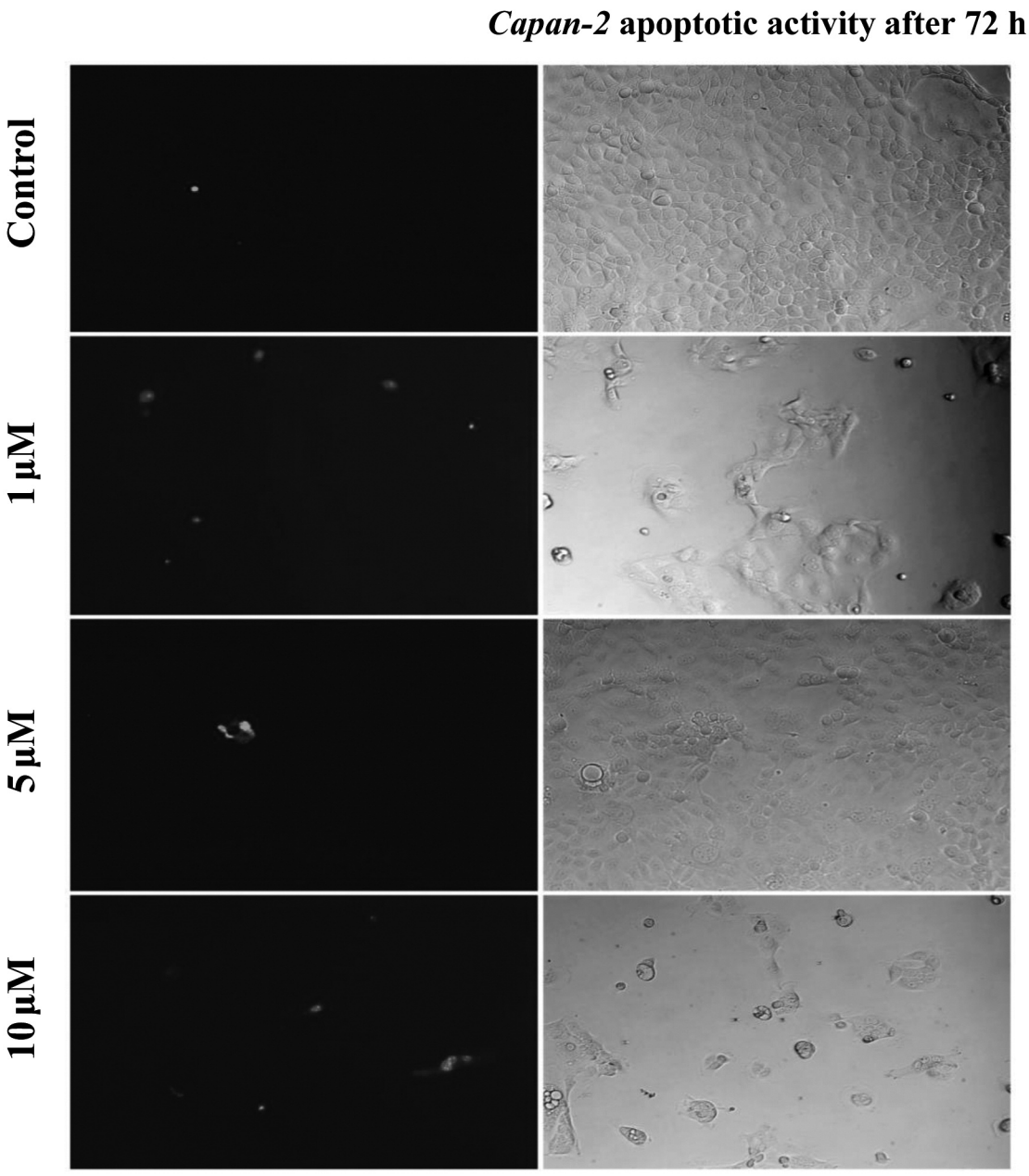

Figure 9. *Cell apoptosis activity after exposure of Capan-2 cells to decitabine. Cell apoptosis was evaluated by fluorescent emission after exposure of Capan-2 cells to decitabine (0, 1, 5, 10 µM) for 24 h (A), 48 h (B) and 72 h (C).*

2 Mohamed MS, Bishr MK, Almutairi FM and Ali AG: Inhibitors of apoptosis: Clinical implications in cancer. Apoptosis *22(12)*: 1487-1509, 2017. PMID: 29067538. DOI: 10.1007/s10495-017-1429-4

3 Hervouet E, Cheray M, Vallette FM and Cartron P-f: DNA methylation and apoptosis resistance in cancer cells. Cells *2*: 545-573, 2013. PMID: 24709797. DOI: 10.3390/cells2030545

4 Sato N and Goggins M: The role of epigenetic alterations in pancreatic cancer. J Hepatobiliary Pancreat Surg *13*: 286-295, 2006. PMID: 16858539. DOI: 10.1007/s00534-005-1057-1

5 Davis CD and Uthus EO: DNA methylation, cancer susceptibility, and nutrient interactions. Exp Biol Med (Maywood) *229(10)*: 988-995, 2004. PMID: 15522834.

6 Thompson MJ, Rubbi L, Dawson DW, Donahue TR and Pellegrini M: Pancreatic cancer patient survival correlates with DNA methylation of pancreas development genes. PLoS One *10(6)*: 1-18, 2015. PMID: 26039411. DOI: 10.1371/journal.pone.0128814

7 Rostami S, Nadali F, Alibakhshi R, Zaker F, Nasiri N, Payandeh M, Chahardouli B and Maleki A: Aberrant methylation of APAF-

1 gene in acute myeloid leukemia patients. Int J Hematol Oncol Stem Cell Res *11(3)*: 225-231, 2017. PMID: 28989589.

8 Yao TT, Mo SM, Liu LY, Lu HW, Huang ML and Lin ZQ: 5-aza-2'-deoxycytidine may influence the proliferation and apoptosis of cervical cancer cells via demethylation in a dose- and time-dependent manner. Genet Mol Res *12(1)*: 312-318, 2013. PMID: 23408418. DOI: 10.4238/2013.February.4.5

9 Daukša A, Jakštaitė A, Gasianec A and Dambrauskas Ž: Loss of proapoptotic gene apaf-1 expression in pancreatic cancerproapoptozinio geno apaf-1 raiškos sumažėjimas kasos vėžio audinyje. Sveikatos Mokslai *25(2)*: 9-12, 2015. DOI: 10.5200/sm-hs.2015.022

10 Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordón-Cardó C and Lowe SW: Inactivation of the apoptosis effector apaf-1 in malignant melanoma. Nature *409(6817)*: 207-211, 2001. PMID: 11196646. DOI: 10.1038/35051606

11 Ashraf QM, Mishra OP and Delivoria-Papadopoulos M: Mechanisms of expression of apoptotic protease activating factor-1 (apaf-1) in nuclear, mitochondrial and cytosolic fractions of the cerebral cortex of newborn piglets. Neurosci Lett *415(3):* 253-258, 2007. PMID: 17275190. DOI: 10.1016/j.neulet.2007.01.023

12 Esteller M, Corn PG, Baylin SB and Herman JG: A gene hypermethylation profile of human cancer. Cancer Res *61(8)*: 3225-3229, 2001. PMID: 11309270.

13 Hassler MR, Klisaroska A, Kollmann K, Steiner I, Bilban M, Schiefer AI, Sexl V and Egger G: Antineoplastic activity of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine in anaplastic large cell lymphoma. Biochimie *94(11)*: 2297-2307, 2012. PMID: 22687603. DOI: 10.1016/j.biochi.2012.05.029

14 Chu SH, Ma YB, Feng DFU, Zhang H, Qiu JH and Zhu ZAN: Effect of 5-aza-2'-deoxycytidine on slc22a18 in glioma u251 cells. Mol Med Rep *5(1)*: 138-141, 2012. PMID: 21993522. DOI: 10.3892/mmr.2011.620

15 Christman JK: 5-azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: Mechanistic studies and their implications for cancer therapy. Oncogene *21(35)*: 5483-5495, 2002. PMID: 12154409. DOI: 10.1038/sj.onc.1205699

16 Gomyo Y, Sasaki JI, Branch C, Roth JA and Mukhopadhyay T: 5-aza-2'-deoxycytidine upregulates caspase-9 expression cooperating with p53-induced apoptosis in human lung cancer cells. Oncogene *23(40)*: 6779-6787, 2004. PMID: 15273730. DOI: 10.1038/sj.onc.1207381

17 Dai DL, Martinka M, Bush JA and Li G: Reduced apaf-1 expression in human cutaneous melanomas. Br J Cancer *91(6)*: 1089-1095, 2004. PMID: 15305193. DOI: 10.1038/sj.bjc.6602092

18 Mustika R, Budiyanto A, Nishigori C, Ichihashi M and Ueda M: Decreased expression of apaf-1 with progression of melanoma. Pigment Cell Res *18(1)*: 59-62, 2005. PMID: 15649154. DOI: 10.1111/j.1600-0749.2004.00205.x

19 Ashur-Fabian O, Adamsky K, Trakhtenbrot L, Cohen Y, Raanani P, Hardan I, Nagler A, Rechavi G and Amariglio N: Apaf1 in chronic myelogenous leukemia (cml) progression: Reduced apaf1 expression is correlated with a h179r p53 mutation during clinical blast crisis. Cell Cycle *6(5)*: 589-594, 2007. PMID: 17361096. DOI: 10.4161/cc.6.5.3900

20 Ahn BK, Kim SH, Paik SS and Lee KH: Loss of apaf-1 expression is associated with early recurrence in stage I, II, and III colorectal cancer. Langenbeck's Arch Surg *401(8)*: 1203-1210, 2016. PMID: 27480415. DOI: 10.1007/s00423-016-1483-3

21 Leo C, Richter C, Horn LC, Schütz A, Pilch H and Höckel M: Expression of apaf-1 in cervical cancer correlates with lymph node metastasis but not with intratumoral hypoxia. Gynecol Oncol *97(2)*: 602-606, 2005. PMID: 15863166. DOI: 10.1016/j.ygyno.2005.01.044

22 Li J, Li Q, Huang H, Li Y, Li L, Hou W and You Z: Overexpression of mirna-221 promotes cell proliferation by targeting the apoptotic protease activating factor-1 and indicates a poor prognosis in ovarian cancer. Int J Oncol *50(4)*: 1087-1096, 2017. PMID: 28350128. DOI: 10.3892/ijo.2017.3898

23 Corvaro M, Fuoco C, Wagner M and Cecconi F: Analysis of apoptosome dysregulation in pancreatic cancer and of its role in chemoresistance. Cancer Biol Ther *6(2)*: 209-217, 2007. PMID: 17224646. DOI: 10.4161/cbt.6.2.3622

24 Sato N, Maitra A, Fukushima N, Van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, Rosty C and Goggins M: Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. Cancer Res *63(14)*: 4158-4166, 2003. PMID: 12874021.

25 Furukawa Y: Methylation silencing of the apaf-1 gene in acute leukemia. Mol Cancer Res *3(6)*: 325-334, 2005. PMID: 15972851. DOI: 10.1158/1541-7786.mcr-04-0105

26 R2 database: Genomic Analysis and visualization platform. Dep Hum Genet Amsterdam Med Cent, 2019.

27 Allen JD, Zhang XD, Scott CL, Boyle GM, Hersey P and Strasser A: Is apaf-1 expression frequently abrogated in melanoma? Cell Death Differ *12(6)*: 680-681, 2005. PMID: 15832174. DOI: 10.1038/sj.cdd.4401634

28 Fu WN, Bertoni F, Kelsey SM, McElwaine SM, Cotter FE, Newland AC and Jia L: Role of DNA methylation in the suppression of APAF-1 protein in human leukaemia. Oncogene *22(3)*: 451-455, 2003. PMID: 12545166. DOI: 10.1038/sj.onc.1206147

29 Schaefer M, Hagemann S, Hanna K and Lyko F: Azacytidine inhibits rna methylation at dnmt2 target sites in human cancer cell lines. Cancer Res *69(20)*: 8127-8132, 2009. PMID: 19808971. DOI: 10.1158/0008-5472.CAN-09-0458

30 Christoph F, Kempkensteffen C, Weikert S, Köllermann J, Krause H, Miller K, Schostak M and Schrader M: Methylation of tumour suppressor genes apaf-1 and dapk-1 and *in vitro* effects of demethylating agents in bladder and kidney cancer. Br J Cancer *95(12)*: 1701-1707, 2006. PMID: 17133271. DOI: 10.1038/sj.bjc.6603482

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