22

Human Telomerase Reverse Transcriptase Gene Antisense Oligonucleotide Increases the Sensitivity of Pancreatic Cancer Cells to Gemcitabine *In Vitro*

Yong-ping Liu, Yang Ling, Yue-di Hu, Ying-ze Kong,
Feng Wang and Peng Li
Clinical Oncology Laboratory, Changzhou Tumor Hospital Affiliated to Medical School of Suzhou University, Changzhou, China

1. Introduction

Resistance to gemcitabine is the major problem in pancreatic cancer chemotherapy, and recent evidence suggests that down-regulation of hTERT mRNA could enhance the antitumor efficacy of other well known chemotherapy agents targeting DNA. The aim of this study was to evaluate the combined antitumor efficacy of antisense oligonucleotides (AS-ODN) targeting hTERT mRNA and gemcitabine in human pancreatic cancer cells. Our results showed that transient transfection in clones of the human pancreatic cancer cell lines BxPC-3 and Panc-1 with 0.2μM hTERT AS-ODN for 24 h diminished the abundance of hTERT mRNA and inhibited telomerase activity, but only resulted in a slightly attenuated ability of proliferation. While pretreatment with 0.2μM AS-ODN for 24 h followed by gemcitabine in BxPC-3 or Panc-1 cells led to tumor cell growth suppression more significantly than gemcitabine alone in MTT, and the IC50 of gemcitabine was reduced to about 8.7 times in Panc-1 cells, and 4.2 times in BxPC-3 cells. Likewise, after treatment with gemcitabine for 48 h, the AS-ODN-transfected cells exhibited significantly decreased colony formation ability relative to the parental cells. Apoptosis analysis indicated that hTERT AS-ODN increased the gemcitabine-induced apoptosis in both cell lines. All together, these findings implied that hTERT AS-ODN could increase the chemosensitivity of gemcitabine through down-regulation of hTERT mRNA expression and inhibition of telomerase activity, which may make it an attractive agent for the sensitization of pancreatic cancer cells to gemcitabine.

Pancreatic cancer is one of the most common causes of cancer death in the world. Surgery is the only chance for cure, unfortunately, late diagnosis often results in less than 20% of patients for tumor resection [1,2]. Gemcitabine, a novel pyrimidine nucleoside analogue, has become the standard first-line chemotherapeutic agent used in patients with pancreatic cancer [1]. It is, however, moderately effective, showing a tumor response rate of only 12% [3] and a median survival time of 5 months [4]. Increasing the susceptibility of pancreatic

cancer cells to gemcitabine, therefore, is of importance to the outcome of therapy. In order to investigate the mechanisms of gemcitabine -resistance, multiple mechanisms have been proposed, including enhanced NF-kB activation [5], increased activity of Src tyrosine kinase and expression of the M2 subunit of ribonucleotide reductase (RRM2) [6], deficiency in deoxycytidine kinase (dCK) [7], and altered transport over the cell membrane [8]. Recent studies indicate that acquired gemcitabine resistance in pancreatic cancer cells may be mainly attributed to an altered apoptotic threshold [9].

Telomerase is an RNA-dependent DNA polymerase that is rarely present in normal somatic cells but is observed in 85% of all cancer cells tested, making the telomerase enzyme an attractive target for anticancer therapeutics [10,11]. The human telomerase is composed of a constitutively expressed RNA subunit (hTR), human telomerase-associated protein (TEP1) and a catalytic protein subunit (hTERT). The protein subunit hTERT is a reverse transcriptase, and hTERT expression is the rate-limiting component of the telomerase complex and therefore determines telomerase activity [12]. The main function of the telomerase is the lengthening and capping of the ends of linear chromosomes, the telomeres [13-15].Uncapped or critically shortened telomeres cause cell apoptosis (15). Many labs have reported that telomerase may play an active role in the response to DNA damaging agents [16-18], and could been implicated in suppression of apoptosis [19]. It has also been demonstrated recently that antisense-mediated down-regulation of hTERT quickly induced programmed cell death in human tumour cells [20-23] and sensitized cancer cells to DNA damaging agents through the activation of the apoptotic program [24-26]. For pancreatic cancer, it was not known so far whether hTERT mRNA silencing leads to sensitization to gemcitabine as the standard of care for pancreatic cancer.

In this study, sequence-specific antisense oligonucleotides targeting the coding region of the protein component of human telomerase were designed to examine whether hTERT mRNA and telomerase activity could be inhibited and chemosensitivity to gemcitabine could be increased in pancreatic cancer cells.

2. Materials and methods

2.1 Oligonucleotides and drug

Based on the hTERT gene cDNA sequence (4015 nt; accession no. AF015950), the antisense oligonucleotide was designed to be complementary to the translation initiation region of hTERT mRNA; the antisense oligodeoxynucleotide sequence (AS-ODN) is 5′-GGAGCGCGCGGCATCGCGGG-3′; Non-specific oligodeoxynucleotide sequence (NS-ODN) is 5′-CATTTCTTGCTCTCCACGCG-3′as a control, having the same base number as the antisense oligonucleotide but with different sequence. All oligodeoxynucleotides were fully phosphorothioate, and were synthesized by Invotrogen (Carlsbad, CA, USA). Their lack of significant interfering homology was validated using BLAST analysis. Gemcitabine was obtained from Eli Lilly, and the dilutions of gemcitabine were freshly prepared before each experiment.

2.2 Cell culture and transfection

Pancreatic cancer cell lines BxPC-3 and Panc-1 were kindly provided by the center laboratory of the Second Hospital of ChangZhou in China, and were routinely incubated in

DMEM (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL), 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were grown at 37℃ in a humidified chamber of 95% air containing 5% CO2. The transfection procedure of oligonucleotides(ODN) was performed according to the user manual of Oligofectamine TM Reagent(Invitrogen, Carlsbad, CA, USA). Briefly, The cells were seeded the day before the experiment in different culture plates at different density per well at 30% to 50% confluence on the day of the experiment, and then were transfected with 0.2μM of Oligofectamine and 0.2μM of oligonucleotides (ODN) in the serum-free DMEM, incubated at 37℃ for 4 hr, and then added different volume of growth medium containing 3× the normal concentration of serum according to the different culture plates without removing the transfection mixture. To assess ODN uptake, pancreatic cancer cells were transfected with the FITC-labeled ODN, and then Flow cytofluorometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA) was used to quantify FITC -positive cells at defined times after transfection.

2.3 Quantitative RT-PCR

Pancreatic cancer cell lines BxPC-3 and Panc-1 were harvested with trypsin, washed with PBS, and collected by centrifugation at 1,000 rpm for 5 min. Total RNA was extracted using SV Total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer's protocol. And its purity and quality were measured by Bio-visible spectrophotometer (Eppendorf, Germany); 1% agarose gel electrophoresis was used to assess the integrity of the obtained RNA. cDNA with a total volume of 20μl was synthesized using the reverse transcription system containing reverse transcriptase (Promega, Madison, WI, USA) according to the recommended protocol by the manufacturer. Real-time quantitative PCR of the target hTERT gene and β-actin as internal control was carried out with icycler iQ Multicolor Real-time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The 20 μl PCR reaction mixture contained 1× primers and probe mixture [Applied Biosystems, Foster city , CA. Assay IDs: Hs99999022_m1 (hTERT); Hs99999903 _m1 (β-actin)], 1× Absolute QPCR Mix (ABgene, Surrey, UK). The PCR conditions were 50°C for 2 min, 95°C for 15 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Relative gene expression quantifications were calculated according to the comparative Ct method using β-actin as an endogenous control and commercial human total RNA (BD Clontech, CA, USA) as calibrators. Final results were determined by the formula $2^{-\Delta\Delta CT}$ method[27].

2.4 Telomerase activity assay

A commercial telomerase PCR ELISA kit (Roche Diagnostics. Scandinavia AB, Stockholm, Sweden) was used to determine telomerase activity in cells according to the manufacturer's instructions. Briefly, 5μl amplification product which had been denaturated at room temperature for 10 min with 20μl denaturation reagent was hybridized with a digoxigenin-labeled probe specific for human telomeric repeats. The probe bound to the strand with the labeled biotin at the 5' end. The hybrid was immobilized to a streptavidin-coated microtiter plate via the biotin-labeled primer at 37 °C on a shaker for 2 h, and washed 3 times. The reaction product was detected with 100μl anti-digoxigeninperoxidase and 100μl peroxidase substrate TMB. Color intensities were measured with a model 450 microplate reader (BIO-RAD) at 450 nm.

2.5 Cell viability assay

Cytotoxicity was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Briefly, ODN-transfected and Oligofactamine- transfected cancer cells growing in log-phase were trypsinized and seed at 2×10^3 cells per well into 96-well plates and allowed to attach overnight. Medium in each well was replaced with fresh medium or medium with various concentrations of drug in at least 6 replicate wells and left contact for 48 h. One-fifth volume of CellTiter 96 AQueous One Solution was added to each well and incubated for an additional 3 h, Absorbance was determined with a microplate reader (BIO-RAD) at 490 nm. The blank control wells were used for zeroing absorbance. Each experiment was allocated ten wells containing drug-free medium for the control. The inhibition rate (I %) was calculated using the background-corrected absorbance by the following equation: $I\% = 100\times (A_{\text{untreated control well}} - A_{\text{experimental well}}) / A_{\text{untreated control well}}$. The IC50 was defined as the concentration required for 50% inhibition of cell growth. Each experiment was performed in triplicate, with representative data presented.

2.6 Colony-forming cell assay

Pancreatic tumor cells were transfected with 0.2μM AS-ODN or NS-ODN for 24 h, and then the transfected cells were treated with gemcitabine at 0.05μM in BxPC-3 cells and 0.8μM in Panc-1cells for 48 h. Subsequently, gemcitabine-treated cells and parental cells (300 cells /well) were plated in triplicate in 60-mm Petri dishes. On day 7, the plates were fixed in 70% methanol and treated with Giemsa stain. Clonogenic survival was determined by counting the macroscopically visible colonies.

2.7 Apoptosis assay

Cells quantification of apoptosis cells was performed using an Annexin-V- FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were plateded in a 60-mm Petri disk and treated with drugs for 48h .Then cells were collected and resuspended in 500μl of binding buffer, and 5μl of Annexin- V-fluorescein isothiocyanate (FITC) and 5μl of propidium iodide (PI) were added. Analyses were performed with a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

2.8 Statistical methods

Values were expressed as means ±standard deviations. Statistical comparison was performed using Student's ***t***-test, and a ***p*** value of less than 0.05 was considered statistically significant.

3. Results

3.1 Assessment of AS-ODN uptake by pancreatic cancer cells

The use of 0.2μM Oligofectamine allowed a very efficient internalization of ODNs already after 4 h of transfection (> 20% FITC-positive cells), and at 24 h, the fluorescence intensity in both cell lines reached the strongest (> 30% FITC-positive cells), and then gradually decreased (data not shown). In contrast, transfection without Oligofectamine resulted in

<10% FITC-positive cells. After a 24-h transfection, the percentages of FITC-positive cells could attain to 33.6% in BxPC-3 cells and 41.8% in Panc-1 cells.

3.2 hTERT antisense oligodeoxynucleotide(AS-ODN) down-regulates hTERT mRNA expression and telomerase activity of pancreatic cancer cells

We first examined the mRNA expression of hTERT mRNA in BxPC-3 and Panc-1 using quantitative RT-PCR. The expression levels of hTERT mRNA in Panc-1 cells was higher than that in BxPC-3 cells ($p < 0.001$) (Fig. 1). We further examined whether hTERT AS-ODN could downregulate the expression levels of hTERT mRNA in both cell lines. As shown in Fig. 1, treatment with 0.2μM hTERT AS-ODN for 24 h down-regulated the levels of hTERT mRNA in BxPC-3 to 29 % and in Panc-1 cells to 35 %, relative to the Oligofectamine- treated control. While the same concentration of NS-ODN sequence did not down-regulate the levels of hTERT mRNA expression in both cell lines. We also examined the effects of gemcitabine on the levels of hTERT mRNA expression, and the results showed that gemcitabine alone at IC50 for 24 h only led to moderate down-regulation of hTERT mRNA in BxPC-3 cell lines and slight up-regulation of that in Panc-1 cell lines. Additionally, we examined the effect of suppressing hTERT mRNA on telomerase activity. We found that NS-ODN control clones showed significant telomerase activity, equal to parental cells, whereas 0.2μM AS-ODN

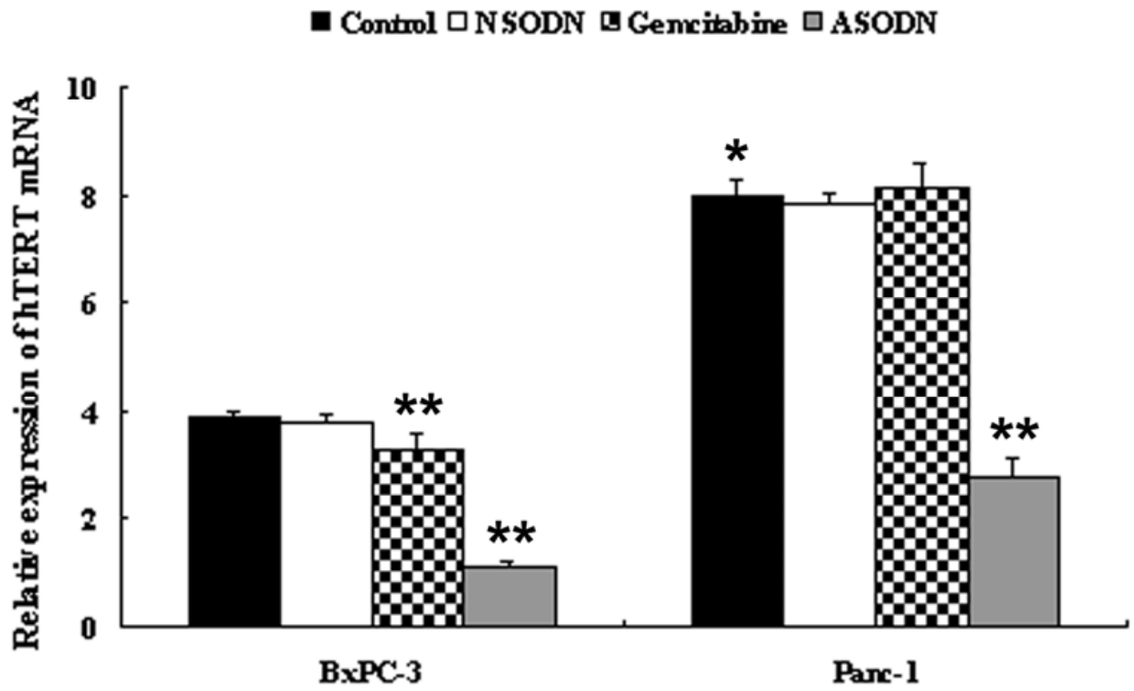


Fig. 1. Sequence-specific suppression of hTERT mRNA by hTERT antisense oligonucleotide in BxPC-3 cells (A) and Panc-1 cells (B). Both cell lines were treated with 0.2μM ODN or gemcitabine at IC50 for 24 h. Cells were harvested for RNA analysis after 24 h incubation. Relative gene expression quantifications were calculated according to the comparative Ct method. Final results were determined by the formula $2^{-\Delta\Delta CT}$ method. Values represent means ± SD, from three independent experiments; *$p < 0.001$ vs oligofectamine transfected BxPC-3 control cells. **$p < 0.05$ vs oligofectamine transfected control group.

clones expressed significantly decreased levels of telomerase activity in both cell lines (Fig. 2) at 24 h as assessed by TRAP-ELISA Assay. At the same time, we found the level of telomerase activity in Panc-1 parental cells was higher than that in BxPC-3 parental cells (p=0.003), and gemcitabine at IC50 for 24 h moderately down-regulated the telomerase activity in both cell lines(data not shown).

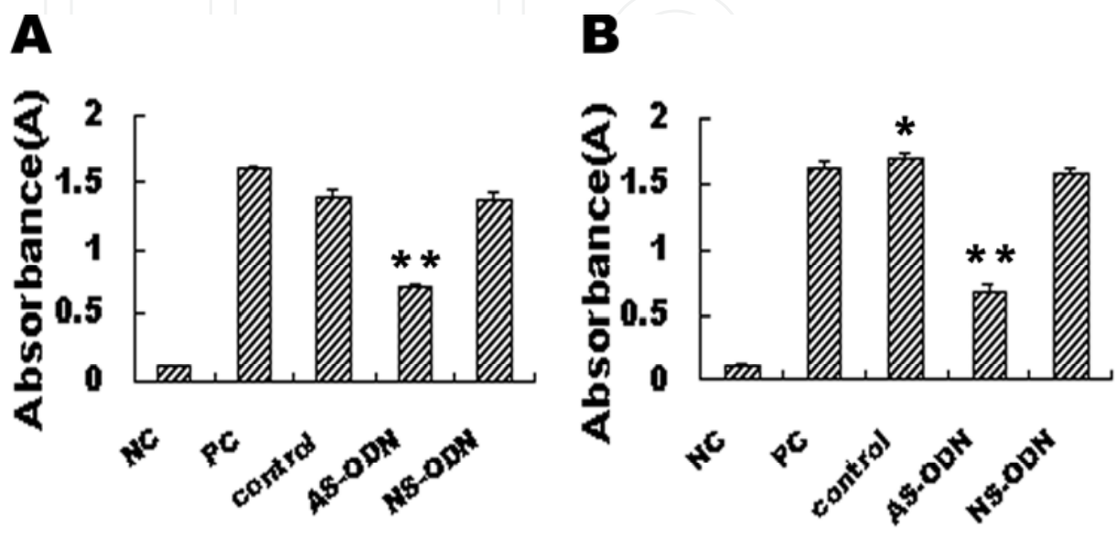


Fig. 2. Telomerase activity in transfected cells of BxPC-3 (A) and Panc-1 (B). Telomerase PCR ELISA was performed 24 h after transfection in triplicates. Values represent the mean Absorbance ± SD, from three independent experiments; *p < 0.001 vs oligofectamine transfected BxPC-3 control cells. **p < 0.001 vs oligofectamine transfected control group.

3.3 hTERT antisense oligodeoxynucleotide (AS-ODN) increases gemcitabine-induced cytotoxicity in pancreatic cancer cells

We next investigated whether the addition of hTERT AS-ODN could indeed increase gemcitabine sensitivity. BxPC-3 and Panc-1 cells were treated with gemcitabine in the presence of 0.2μM hTERT AS-ODN at different concentrations. Fig. 3 shows the IC_{50} value of gemcitabine in BxPC-3 and Panc-1 cells were 0.23μM and 7.13μM, respectively. That is to say Panc-1 cells were 31-fold more resistant to gemcitabine than BxPC-3 cells, suggesting that the more higher expression of hTERT mRNA or telomerase activity, the more resistant of cancer cells to gemcitabine. hTERT AS-ODN was able to reduce the IC50 of gemcitabine to about 8.7 times in Panc-1 cells, and only about 4.2 times in BxPC-3 cells, suggesting hTERT antisense oligodeoxynucleotide could increase gemcitabine- induced cytotoxicity in both cell lines, and sensitize the gemcitabine -resistant cells. But the same concentration NS-ODN control sequence could not increase gemcitabine-induced cytotoxicity in both cells (data not shown). At the same time, we found AS-ODN treatment at 0.2μM for 24 h resulted in a slightly attenuated ability of proliferation in both cell lines (data not shown), suggesting that a lag phase between telomerase inhibition and growth inhibition and/or cell death may limited the application of telomerase inhibition therapy alone in solid cancer treatment.

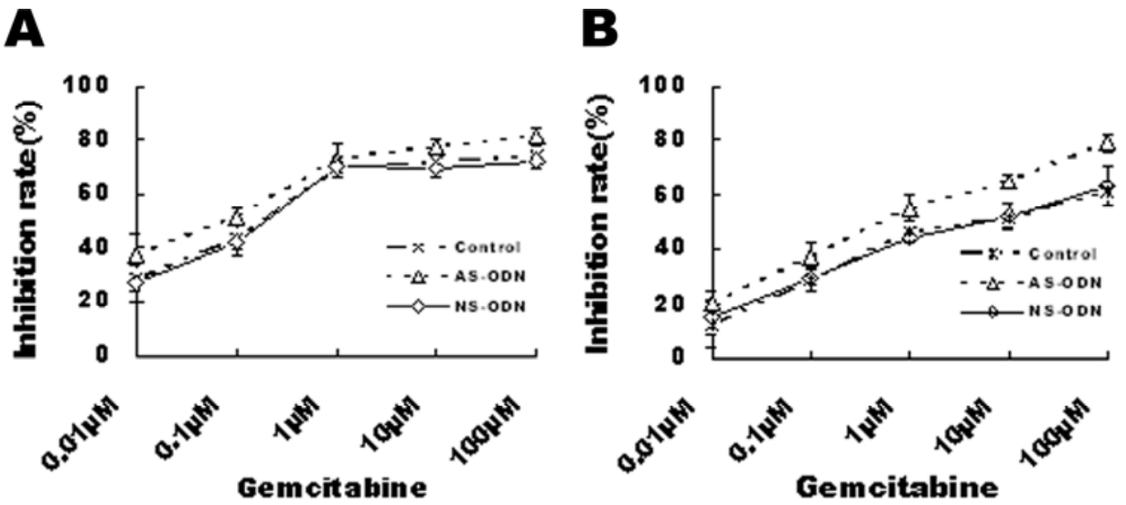


Fig. 3. hTERT antisense oligodeoxynucleotide(AS-ODN) increases gemcitabine-induced cytotoxicity in BxPC-3(A) and Panc-1(B) cells. Briefly, ODN transfected cells and parental cells were treated with gemcitabine at different concentrations. The inhibition rate (I %) was calculated using the background-corrected absorbance by the following equation: I% = 100× ($A_{\text{untreated control well}} - A_{\text{experimental well}}$) / $A_{\text{untreated control well}}$. Values represent the mean inhibition rates ± SD, from three independent experiments, compared to an untreated control cells.

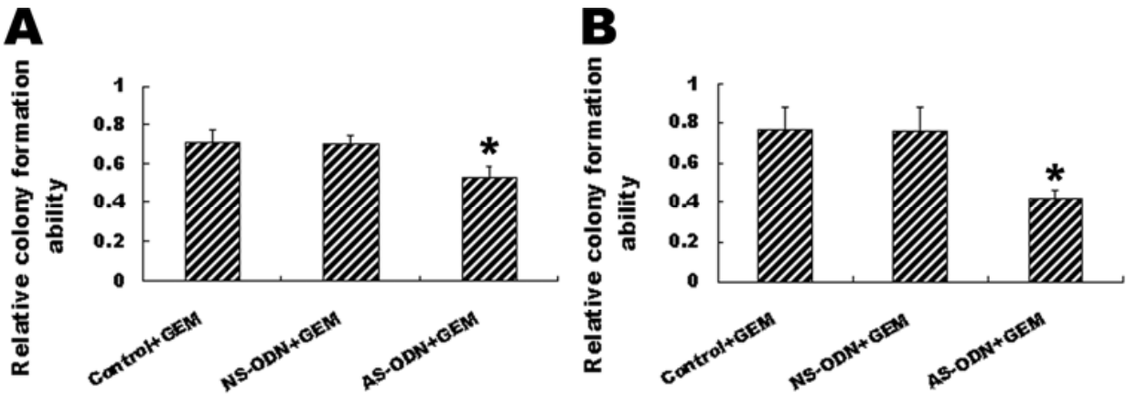


Fig. 4. Cells treatment with gemcitabine (GEM) in presence of AS-ODN exhibited significantly decreased colony formation ability in BxPC-3 (A) and Panc-1(B) cells. Briefly, cells were transfected with 0.2μM AS-ODN or NS-ODN for 24 h, and then the transfected cells and parental cells were treated with gemcitabine at 0.05μM in BxPC-3 cells and 0.8μM in panc-1cells for 48 h. Subsequently, gemcitabine-treated cells and parental cells (300 cells/ well) were plated in triplicate in 60-mm Petri dishes. On day 7, the plates were fixed in 70% methanol and were treated with Giemsa stain. Clonogenic survival was determined by counting the macroscopically visible colonies. The relative colony formation ability normalized to the untreated parental control is displayed. Data represent the mean values ± SD, from three independent experiments. *P<0.05 vs. the group of (control +GEM).

3.4 Cells treatment with gemcitabine in presence of AS-ODN exhibited significantly decreased colony formation ability

To further investigate the combined antitumor efficacy of hTERT AS-ODN and gemcitabine in human pancreatic cancer cells, colony formation ability was evaluated by colony formation assays. The ability of isolated cells to proliferate and generate colonies was clearly reduced in the cells treatment with gemcitabine in presence of AS-ODN, but not in presence of NS-ODN (Fig. 4). In particular, treatment with 0.8μM gemcitabine for 48 h, the AS-ODN-transfected Panc-1 cells showed a remarkable decrease of ≥50% in the relative colony number. Furthermore, after treatment with gemcitabine for 48 h, colonies arising from AS-ODN- transfected cells were smaller than colonies originating from NS-ODN-treated cells or parental control cells.

3.5 hTERT antisense oligodeoxynucleotide increases gemcitabine- induced apoptosis in both cell lines

We further examined whether down-regulation of hTERT mRNA and telomerase activity could increase cytotoxicity of gemcitabine by induction of apoptosis. Gemcitabine single agent treatment at 0.2μM for 48 h resulted in 30.5% of early apoptosis in BxPC-3 cells, and at 7μM for 48 h resulted in 15.8% of early apoptosis in Panc-1 cells, but when 0.2μM AS-ODN was previously added to both cell lines for 24 h, the effects were dramatically increased to 58.5% and 29.2%, respectively. At the same time, the percentages of late apoptosis were increased to 21.3% in BxPC-3 cells and 18.5% in Panc-1 cells. While when AS-ODN was added alone at 0.2μM for 24 h, the percentages of early apoptosis in BxPC-3 and Panc-1 cells were only 8.4% and 5.2%, respectively, and the same concentration of NS-ODN control sequence resulted in the similar percentage of early apoptosis as parental cells (data not shown). Thus, it appears that hTERT suppressing might increase gemcitabine -induced apoptosis in both cell lines and subsequently lead to an increased cytotoxicity of gemcitabine (see Fig. 5).

4. Discussion

Pancreatic cancer has a poor prognosis, even after curative resection. Gemcitabine is established as the reference treatment for pancreatic cancer patients [28]. However, clinical efficacy with gemcitabine as a single agent remains poor. Gemcitabine-based combinations are needed to improve outcomes. In the present study, we evaluated the effect of a combined gemcitabine and antisense hTERT gene therapy on tumor growth in human BxPC-3 and Panc-1 pancreatic cancer cell lines in vitro. We initially demonstrated that an AS-ODN complementary to the translation region of hTERT mRNA inhibited the expression of hTERT mRNA and telomerase activity in both cell lines, while gemcitabine alone resulted in only moderate down-regulation of hTERT expression in BxPC-3 cells and slight up-regulation of hTERT expression in Panc-1 cell lines. Then we demonstrated that down-regulation of the human telomerase reverse transcriptase mRNA and inhibition of telomerase activity by AS-ODN could sensitize both cell lines to gemcitabine, leading to enhanced cytotoxicity in vitro. These consequences suggest that the anti-proliferative effect of the combination gemcitabine and antisense hTERT therapy in human pancreatic cancer are mediated through the down-regulation of hTERT mRNA and inhibition of telomerase activity. These findings also make an antisense technology for hTERT inhibition therapy an attractive approach for the sensitization of pancreatic cancer cells to gemcitabine.

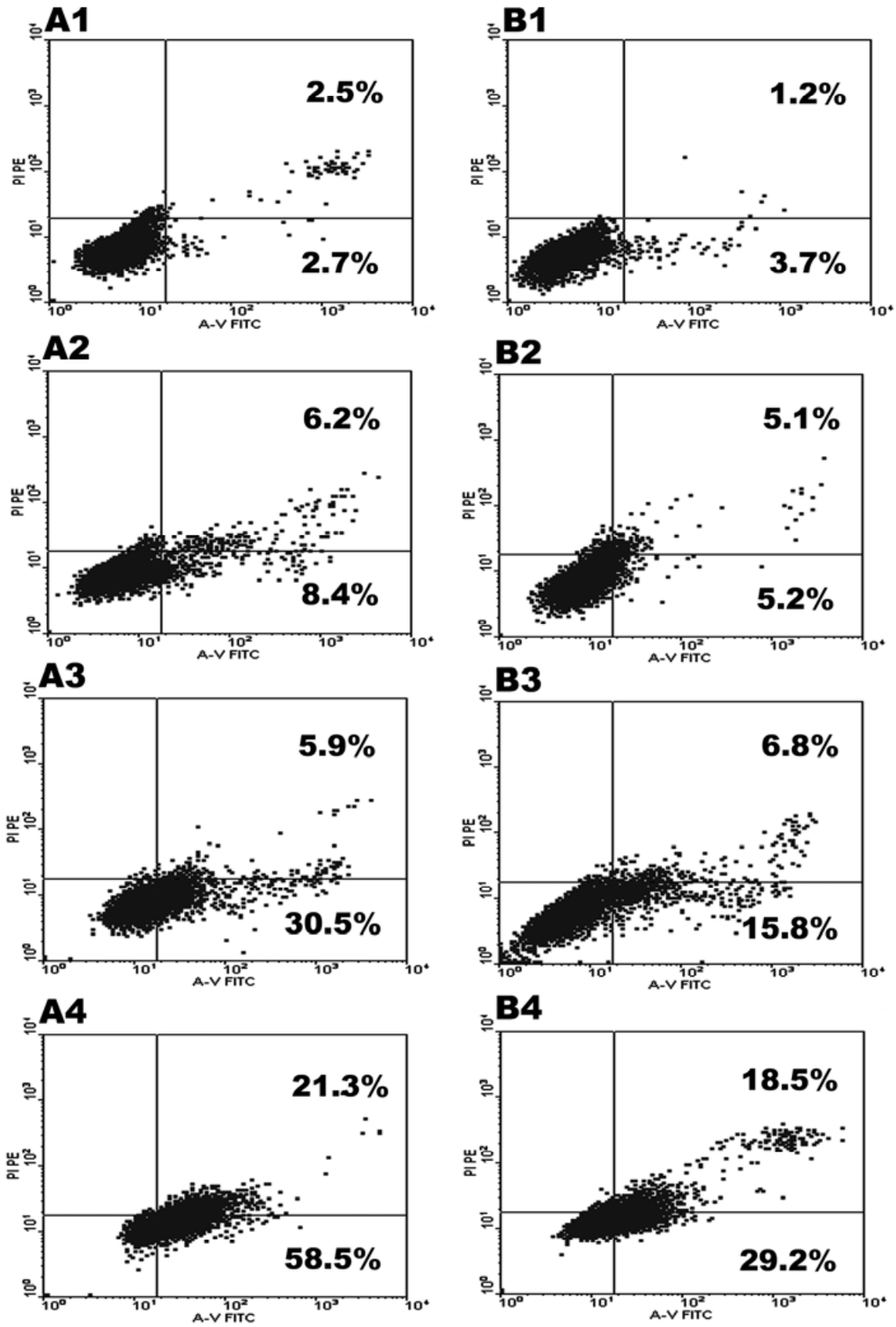


Fig. 5. hTERT Antisense oligodeoxynucleotide (AS-ODN) increases gemcitabine-induced apoptosis in BxPC-3 (A) and Panc-1(B) cells. Cells quantification of apoptosis cells was

performed using an Annexin-V- FITC Apoptosis Detection Kit according to the manufacturer's instructions. Briefly, cells were plateded in a 60-mm Petri disk and treated with drugs for 48 h .Then cells were collected and resuspended in 500μl of binding buffer, and 5μl of Annexin-V-fluoresce in isothiocyanate (FITC) and 5μl of propidium iodide (PI) were added. Analyses were performed with a flow cytometer. A1, B1: both cell lines were treated without drug.A2, B2: both cell lines were treated with AS-ODN alone.A3, B3: both cell lines were treated with gemcitabine alone.A4, B4: both cell lines were treated with AS-ODN for 24 h followed by gemcitabine treatment for 48 h. Early apoptotic cells are defined as Annexin V-positive, PI- negative cells, late apoptotic cells are defined as Annexin V-positive, PI- positive cells.

Telomerase is a ribonucleoprotein enzyme responsible for lengthening and capping the ends of linear chromosomes, the telomeres [13-15]. Telomerase activation is required for the survival and proliferation of the large majority of tumor cells. Uncapped or critically shortened telomeres cause cellular responses such as inhibition of cell proliferation and apoptosis. It is currently unclear how telomerase is regulated in human cancer cells. Previous studies indicated that telomerase activity is strongly correlated with the abundance of hTERT mRNA but not the hTER[29-31], and ectopic expression of hTERT in somatic cells is sufficient to restore telomerase activity[32-35].Thus, strategies targeting hTERT may be a new approach for inhibition of telomerase activity and gene therapy of cancer. Recent studies indicate that down-regulation of hTERT expression or expression of dominant -negative hTERT could inhibit telomerase activity and prevent the malignant proliferation of tumor cells after considerable passages in culture [21,36-38]. In our experiments, we have demonstrated that treatment of pancreatic cancer cells with hTERT AS-ODN could down-regulate the levels of hTERT mRNA expression, inhibit the telomerase activity, but result in a slightly attenuated ability of proliferation in both cell lines. In fact, as human cells reduce their telomere length by 50-100 base pairs per cell division, a long lag phase is required before growth arrest can be obtained, even in cancer cells with relatively short telomeres [10,39]. Thus , in present study, the moderate anticancer efficacy of hTERT AS-ODN in both cell lines may be independent of telomere shortening ,but partially dependent of the loss of the hTERT-mediated capping function of telomerase [40].

It was obvious that anti-telomerase therapy alone was not the best selection of cancer treatment for its requiring long time to reduce the telomere length [10,39].However, transiently transfection of hTERT AS-ODN may enhance the anticancer efficacy of other well known chemotherapy agents targeting DNA [25,26]. Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a synthetic pyrimidine nucleoside analogue, the diphosphate (dFdCDP) and triphosphate (dFdCTP) forms of the drug play an important role in the cytotoxic effect: dFdCDP is an inhibitor of ribonucleotide reductase, while dFdCTP is incorporated into DNA, both leading to the inhibition of DNA synthesis and making genomic instability[41]. Thus, we speculate that hTERT mRNA silencing may lead to sensitization of pancreatic cancer cells to gemcitabine. Our study showed that hTERT AS-ODN significantly increased the gemcitabine- induced cytotoxicity in both cell lines, especially sensitize the gemcitabine -resistant cells. Apoptosis test further demonstrated that hTERT suppressing could increase gemcitabine-induced apoptosis in both cell lines, but the same concentration NS-ODN control sequence could not increase gemcitabine-induced cytotoxicity in any of pancreatic cancer cell lines. The similar results were also acquired by

other report in the bladder cancer cell lines [26]. Assessment of AS-ODN uptake showed that Panc-1 cells are more easily to be transfected with Oligofectamine than BxPC-3 cells, which might partly explain why the sensitizing effects of hTERT AS-ODN on Panc-1 cells are more obvious than on BxPC-3 cells.

It is not clear of the relationship between the expression levels of telomerase activity or hTERT mRNA and chemotherapy resistanse. In our study, we initially found that the expression levels of hTERT mRNA and telomerase activity in Panc-1 cells were higher than those in BxPC-3 cells. Then we found Panc-1 cells were 31-fold more resistant to gemcitabine than BxPC-3 cells. It seems that the more higher expression of hTERT mRNA or telomerase activity, the more resistant of cancer cells to gemcitabine. Our following study showed that down- regulation of hTERT mRNA and telomerase activity could increase the sensitivity of cancer cells to gemcitabine, especially could restore the sensitivity of gemcitabine-resistant cells to gemcitabine, which indirectly denmonstrated hTERT mRNA or telomerase may be implicated in gemcitabine resistance. Xi and his associates introduced vectors encoding dominate negative (DN)-hTERT, wild-type (WT)-hTERT, or a control vector expressing only a drug-resistance marker into HeLa cells. Results showed that DN-hTERT transfected HeLa cells with shortened telomeres were more susceptible to multiple chemotherapeutic agents and radiation. WT-hTERT transfected HeLa cells with longer telomeres exhibited resistance to radiation and chemotherapeutic agents [42]. Our results showed that at least in part, gemcitabine resistance was associated with the high expression of hTERT mRNA or high telomerase activity.

In conclusion, our results demonstrate that down-regulation of hTERT mRNA and inhibition of telomerase activity by hTERT AS-ODN could increase the sensitivity of pancreatic cancer cells to gemcitabine and especially sensitize the gemcitabine -resistant cells. These findings should further be explored in vivo.

5. Acknowledgements

This study was supported by Science and Technology Planning Project of Changzhou, Jiangsu Province, China (CS2004214 and CS20092025) and the key medical innovation talents training project of Changzhou, Jiangsu Province, China

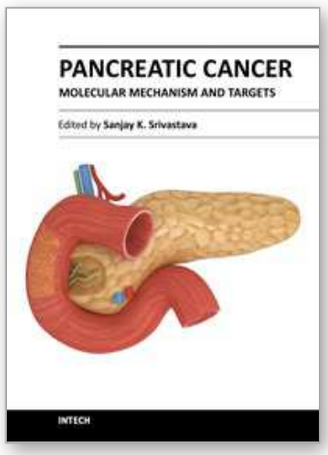
6. References

[1] Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. Lancet 2004; 363: 1049-57.
[2] Bray F, Sankila R, Ferlay J, Parkin DM. Estimates of cancer incidence and mortality in europe in 1995. Eur J Cancer 2002; 38: 99-166.
[3] Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML, Schilsky R. An investigational new drug treatment program for patients with gemcitabine: Results for over 3000 patients with pancreatic carcinoma. Cancer 1999;85: 1261-68.
[4] Rocha Lima CM, Green MR, Rotche R, Miller WH, Jr., Jeffrey GM, Cisar LA, et al. Irinotecan plus gemcitabine results in no survival advantage compared with gemcitabine monotherapy in patients with locally advanced or metastatic pancreatic cancer despite increased tumor response rate. J Clin Oncol 2004; 22: 3776-83.

[5] Hering J, Garrean S, Dekoj TR, Razzak A, Saied A, Trevino J, et al. Inhibition of proliferation by omega-3 fatty acids in chemoresistant pancreatic cancer cells. Ann Surg Oncol 2007;14: 3620-28.
[6] Duxbury MS, Ito H, Benoit E, Zinner MJ, Ashley SW, Whang EE. Retrovirally mediated rna interference targeting the m2 subunit of ribonucleotide reductase: A novel therapeutic strategy in pancreatic cancer. Surgery 2004;136: 261-69.
[7] Sebastiani V, Ricci F, Rubio-Viqueira B, Kulesza P, Yeo CJ, Hidalgo M, et al. Immunohistochemical and genetic evaluation of deoxycytidine kinase in pancreatic cancer: Relationship to molecular mechanisms of gemcitabine resistance and survival. Clin Cancer Res 2006;12: 2492-97.
[8] Bergman AM, Kuiper CM, Noordhuis P, Smid K, Voorn DA, Comijn EM, et al. Antiproliferative activity and mechanism of action of fatty acid derivatives of gemcitabine in leukemia and solid tumor cell lines and in human xenografts. Nucleosides Nucleotides Nucleic Acids 2004;23: 1329-33.
[9] Schniewind B, Christgen M, Kurdow R, Haye S, Kremer B, Kalthoff H, et al. Resistance of pancreatic cancer to gemcitabine treatment is dependent on mitochondria-mediated apoptosis. Int J Cancer 2004;109: 182-88.
[10] Corey DR. Telomerase inhibition, oligonucleotides, and clinical trials. Oncogene 2002;21: 631-637.
[11] Cech TR. Beginning to understand the end of the chromosome. Cell 2004;116: 273-79.
[12] Yokoyama Y, Takahashi Y, Shinohara A, Wan X, Takahashi S, Niwa K, et al. The 5'-end of htert mrna is a good target for hammerhead ribozyme to suppress telomerase activity. Biochem Biophys Res Commun 2000;273: 316-21.
[13] Lingner J, Cech TR. Telomerase and chromosome end maintenance. Curr Opin Genet Dev 1998;8:226- 32.
[14] Zhu J, Wang H, Bishop JM, Blackburn EH. Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. Proc Natl Acad Sci U S A 1999;96: 3723-28.
[15] Chan SW, Blackburn EH. New ways not to make ends meet: Telomerase, DNA damage proteins and heterochromatin. Oncogene 2002;21: 553-63.
[16] Moriarty TJ, Dupuis S, Autexier C. Rapid upregulation of telomerase activity in human leukemia hl-60 cells treated with clinical doses of the DNA-damaging drug etoposide. Leukemia 2002;16: 1112-20.
[17] Klapper W, Qian W, Schulte C, Parwaresch R. DNA damage transiently increases trf2 mrna expression and telomerase activity. Leukemia 2003;17: 2007-15.
[18] Jeyapalan J, Leake A, Ahmed S, Saretzki G, Tilby M, von Zglinicki T. The role of telomeres in etoposide induced tumor cell death. Cell Cycle 2004;3: 1169-76.
[19] Wu P, Meng L, Wang H, Zhou J, Xu G, Wang S, et al. Role of htert in apoptosis of cervical cancer induced by histone deacetylase inhibitor. Biochem Biophys Res Commun 2005;335: 36-44.
[20] Saretzki G, Ludwig A, von Zglinicki T, Runnebaum IB. Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells. Cancer Gene Ther 2001;8: 827-834.
[21] Kraemer K, Fuessel S, Schmidt U, Kotzsch M, Schwenzer B, Wirth MP, et al. Antisense-mediated htert inhibition specifically reduces the growth of human bladder cancer cells. Clin Cancer Res 2003;9: 3794-800.

[22] Tao Z, Chen S, Wu Z, Xiao B, Liu J, Hou W. Targeted therapy of human laryngeal squamous cell carcinoma in vitro by antisense oligonucleotides directed against telomerase reverse transcriptase mrna. J Laryngol Otol 2005;119: 92-96.
[23] Folini M, Brambilla C, Villa R, Gandellini P, Vignati S, Paduano F, et al. Antisense oligonucleotide-mediated inhibition of htert, but not hterc, induces rapid cell growth decline and apoptosis in the absence of telomere shortening in human prostate cancer cells. Eur J Cancer 2005;41: 624-34.
[24] Ludwig A, Saretzki G, Holm PS, Tiemann F, Lorenz M, Emrich T,et al. Ribozyme cleavage of telomerase mrna sensitizes breast epithelial cells to inhibitors of topoisomerase. Cancer Res 2001;61: 3053-61.
[25] Yuan Z, Mei HD. Inhibition of telomerase activity with htert antisense increases the effect of cddp-induced apoptosis in myeloid leukemia. Hematol J 2002;3: 201-5.
[26] Kraemer K, Fuessel S, Kotzsch M, Ning S, Schmidt U, Wirth MP, Meye A. Chemosensitization of bladder cancer cell lines by human telomerase reverse transcriptase antisense treatment. J Urol 2004;172: 2023-28.
[27] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. Methods 2001;25: 402-8.
[28] Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: Causes and therapies. Nat Rev Cancer 2001;1: 194-2.
[29] Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. Hest2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 1997;90: 785-95.
[30] Avilion AA, Piatyszek MA, Gupta J, Shay JW, Bacchetti S, Greider CW. Human telomerase rna and telomerase activity in immortal cell lines and tumor tissues. Cancer Res 1996;56: 645-50.
[31] Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, et al. Telomerase catalytic subunit homologs from fission yeast and human. Science 1997;277: 955-59.
[32] Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, et al. Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279: 349-52.
[33] Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura H, et al. Telomerase activation by htrt in human normal fibroblasts and hepatocellular carcinomas. Nat Genet 1998;18: 65-68.
[34] Vaziri H, Benchimol S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol 1998;8: 279-82.
[35] Counter CM, Meyerson M, Eaton EN, Ellisen LW, Caddle SD, Haber DA, et al. Telomerase activity is restored in human cells by ectopic expression of htert (hest2), the catalytic subunit of telomerase. Oncogene 1998;16: 1217-22.
[36] Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, et al. Inhibition of telomerase limits the growth of human cancer cells. Nat Med 1999;5:1164-70.
[37] Natarajan S, Chen Z, Wancewicz EV, Monia BP, Corey DR. Telomerase reverse transcriptase (htert) mrna and telomerase rna (htr) as targets for downregulation of telomerase activity. Oligonucleotides 2004;14: 263-73.

[38] Pallini R, Sorrentino A, Pierconti F, Maggiano N, Faggi R, Montano N, et al. Telomerase inhibition by stable rna interference impairs tumor growth and angiogenesis in glioblastoma xenografts. Int J Cancer 2006;118: 2158-67.
[39] Keith WN, Jeffry Evans TR, Glasspool RM. Telomerase and cancer: Time to move from a promising target to a clinical reality. J Pathol 2001;195: 404-14.
[40] Sharma GG, Gupta A, Wang H, Scherthan H, Dhar S, Gandhi V, et al. Htert associates with human telomeres and enhances genomic stability and DNA repair. Oncogene 2003;22: 131-46.
[41] Lawrence TS, Eisbruch A, Shewach DS. Gemcitabine-mediated radiosensitization. Semin Oncol 1997;24: S7-24-S27-28.
[42] Xi L, Chen G, Zhou J, Xu G, Wang S, Wu P, et al. Inhibition of telomerase enhances apoptosis induced by sodium butyrate via mitochondrial pathway. Apoptosis 2006;11: 789-98.



Pancreatic Cancer - Molecular Mechanism and Targets

Edited by Prof. Sanjay Srivastava

ISBN 978-953-51-0410-0
Hard cover, 432 pages
Publisher InTech
Published online 23, March, 2012
Published in print edition March, 2012

This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen