Methylation-mediated Silencing of *TMS1* in Pancreatic Cancer and its Potential Contribution to Chemosensitivity

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Abstract. *Background: Resistance to chemotherapeutic agents, resulting in part from epigenetic silencing of pro-apoptotic genes, is one of the causes of treatment failure of pancreatic cancer. We examined whether epigenetic silencing of target of methylation induced silencing 1 (TMS1) contributes to resistance to chemotherapy in pancreatic cancer. Materials and Methods: Methylation analysis was performed by methylation-specific PCR (MS-PCR) and gene expression was analyzed by quantitative reverse transcriptase PCR (qRT-PCR). MIA PaCa-2 cells were transfected with pCMV6-XL5/TMS1 plasmid and the effect of TMS1 expression on sensitivity to gemcitabine and docetaxel was determined. Cell viability was measured using Cell Titer Blue assay. Results: TMS1 expression was repressed in MIA PaCa-2 cells by DNA methylation. Up-regulation of TMS1 by recombinant gene expression in MIA PaCa-2 cells or by pre-treatment of these cells with 5-azacytidine resulted in enhanced sensitivity to gemcitabine and docetaxel. Conclusion: Our results suggest that TMS1 is a potential therapeutic target in pancreatic cancer.*

Pancreatic cancer remains the fourth leading cause of death in the United States with an estimated 43,140 new cases and 36,800 pancreatic cancer-related deaths in 2010 (1), indicating that little has changed over the last 30 years. This is a stark contrast to the therapeutic advances and improvements in survival rates of other types of gastrointestinal cancer over the years. Lack of early diagnostic markers and high failure rate of most conventional treatments are among the factors contributing to the poor prognosis of pancreatic cancer. Hence there is a strong need for a better understanding of the genes that contribute to sensitivity to chemotherapeutic drugs which can identify novel targets for therapeutic intervention.

Gemcitabine (2'-deoxy-2'difluorodeoxycytidine, Gemzar) is a deoxycytidine analog used in the treatment of pancreatic cancer. The median survival time of patients treated with gemcitabine is 6.3 months (2). A primary mechanism of gemcitabine cytotoxicity is the blocking of DNA synthesis by inhibiting DNA polymerase activity. Inhibition of DNA synthesis leads to cell growth inhibition or cell death (3). Treatment of tumor cells with gemcitabine has been shown to induce apoptosis (4). Different studies have demonstrated that gemcitabine induced apoptosis is mediated through caspase activation, blocking of nuclear factor kappa B (NFκB), extracellular signal-regulated kinase (ERK), AKT, BCL-2 and p38 mitogen-activated protein kinase (MAPK) pathways (3). Docetaxel as a single agent has been shown to have modest efficacy in treatment of pancreatic cancer with a response rate of 6-15% and a median survival of 7 months (5, 6). Docetaxel binds to microtubules reversibly with high affinity and this binding stabilizes microtubules. Because microtubules do not disassemble in the presence of docetaxel, they accumulate inside the cell and initiate apoptosis (7, 8). Apoptosis is also induced by the inhibition of apoptosis-blocking BCL-2 oncoprotein (9). The molecular mechanisms of gemcitabine- and docetaxel-induced apoptosis have not been fully elucidated. Changes that contribute to carcinogenesis include alterations in DNA damage, cell cycle regulation and apoptotic pathways. Defects in apoptotic machinery are one of the causes of resistance to therapeutic agents in many types of cancer, including pancreatic cancer. Strategies to activate the apoptotic machinery could result in novel approaches for rational molecular-based therapy in pancreatic cancer.

Target of methylation-induced silencing 1 (*TMS1*), also known as ASC (apoptosis speck-like protein containing a caspase recruitment domain (CARD)), is a pro-apoptotic gene that has been shown to play an important role in the progression of many types of cancer. *TMS1* encodes a

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protein-containing pyrin domain (PYD) in the *N*-terminus and a CARD in the *C*-terminus, both of which are members of the death domain-fold superfamily. It is believed that *TMS1* induces apoptosis *via* the caspase-9 pathway (10, 11). Previous studies have shown that *TMS1* is epigenetically silenced by methylation in many tumor types and thus may have an important role in chemoresistance, tumorogenesis, and progression of the disease (12). Aberrant methylation of *TMS1* also correlated with gene silencing in 70% of small cell lung cancer lines (13). Methylation-mediated silencing of *TMS1* was also observed in ovarian cancer cell lines and correlated with a survival advantage of tumor cells, indicating *TMS1* may contribute to ovarian tumorigeneisis (14). In this study, we examined the role of DNA methylation mediated silencing of *TMS1* in chemoresistance of pancreatic cancer cell lines.

Materials and Methods

Cell culture. Pancreatic cancer cell lines were purchased from the American Type Culture Collection (ATCC, VA, USA). MIA PaCa-2, ASPC1 and PANC1 pancreatic cancer cells were routinely cultured in RPMI-1640 medium (Mediatech, VA, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine (Invitrogen, CA, USA) and 100 μg/ml penicillin-streptomycin (Invitrogen) in a humidified incubator at 37˚C with 5% CO_2.

Drug treatment. MIA PaCa-2 cells were treated with different concentrations of gemcitabine and docetaxel (LKT, MN, USA) and cell viability measured after 24, 48, and 72 h following treatment. MIA PaCa-2 cells were treated with different concentrations of the DNA methyltransferase (DNMT) inhibitor 5-azacytidine (Sigma Aldrich, St. Louis, MO, USA) for 72 h, after which RNA was extracted. Cell viability was measured after 24, 48, and 72 h of 5-azacytidine treatment. For combination treatment, MIA PaCa-2 cell lines were seeded in 96-well plates and treated with 5-azacytidine for 72 h followed by treatment with gemicitabine or docetaxel for 72 h. Cell viability was measured on the final day of treatment and cell survival was compared in the cells that were pre-treated with 5-azacytidine to the cells that received no pre-treatment.

Cell viability assay. Cells were incubated with RPMI medium containing Cell Titer Blue (Promega, WI, USA) for 5 h and fluorescence ($560_{Ex}/590_{Em}$) was measured in a Biotek Synergy HT multi-task plate reader.

Transfection. MIA PaCa-2 cells were transfected with either pCMV6-XL5/TMS1 plasmid or pCMV6-XL5/Mock plasmid (Origene, MD, USA). Transfection was carried out using Amaxa Nucleofection system (Amaxa Inc., MD, USA). Cells were then seeded in 96-well plates and treated with either gemcitabine or docetaxel for 72 h. Cell viability was measured after 24, 48 and 72 h.

DNA extraction, bisulfite treatment, and methylation-specific PCR (MS-PCR). Methylation patterns of the pancreatic cell lines were analyzed by MS-PCR. DNA was extracted using the Masterpure DNA purification kit (Epicentre Biotechnologies, WI, USA) as per manufacturer's instructions. Bisulfite conversion of genomic DNA was carried out as previously described (15, 16). The bisulfite conversion reaction was carried out by incubating 5 μg DNA with a 5 M bisulfite solution and 100 mM hydroquinone, pH 5.0 at 55˚C for 4 h. This was followed by desulfonation by the addition of 3 M NaOH, and desalting using a QIAquick PCR purification kit (Qiagen, CA, USA). After bisulfite conversion, the DNA template was amplified with primers sets specific for the *TMS1* promoter sequence. The primer set specific for the methylated product was as follows: forward, 5' F CGA TTT TGG CGT TTT TCG ACG GTT 3', and reverse, 5' CCG CTC ACC CCG CTA CAA CCG C 3'. The primer set specific for the unmethylated product was as follows: forward, 5' TTG TTG GAG GGT AAT GGA TT 3', and reverse, 5' CCC ACA AAA ATA CAC CCA TA 3'. The PCR product was then analyzed by agarose gel electrophoresis and visualized under UV light.

Bisulfite genomic sequencing. Bisulfite-converted DNA template was amplified with primers sets: forward, 5' GGT TTG GGT GGG AGG GGA TTA A 3', and reverse, 5' CCC ATA ACT CCA AAA TCC C 3' for the proximal promoter region between positions −126 and +76 with respect to the transcription start site. The PCR product was run on a 3% agarose gel and purified using Wizard SV gel and PCR clean-up system (Promega). The purified PCR product was sequenced using Thermosequenase Radiolabeled Terminator cycle sequencing kit (USB Corporation, OH, USA) and run on a 7 M urea/5% polyacrylamide gel. The gel was dried and analyzed using Phosphoimager (GE Healthcare, NJ, USA).

RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR). RNA was extracted from cells using the Epicentre Masterpure RNA purification kit (Epicentre Biotechnologies) as per the manufacturer's instructions and reverse transcribed using MMLV Reverse Transcriptase (USB Corporation). Real-time PCR amplification was performed in triplicates with cDNA using primers for *TMS1*. The average C_t was then used to quantitate relative mRNA levels by the comparative C_t method. The levels of *TMS1* were normalized to the housekeeping gene, glyceraldehye phosphate dehydrogenase (*GAPDH*). The primer sequences used to amplify *TMS1* in MIA PaCa-2 were: forward, 5' GCA GCC AAG CCA GGC C 3', and reverse, 5' CCA GCA GCC ACT CAA CGT T 3' and the sequences used to amplify *GAPDH* were: forward, 5' GGT ATC GTG GAA GGA CTC ATG AC 3', and reverse, 5' CAC GCC ACA GTT TCC CGG A 3'. The PCR reaction was carried out in a volume of 25 μl using iQ™ SYBR® Green Supermix in a MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad, CA, USA).

Results

Methylation of the promoter region of TMS1 correlates inversely with expression in MIA PaCa-2 cells. Epigenetic silencing of pro-apoptotic genes is one of the mechanisms of development of resistance of cancer cells to chemotherapy. We examined the methylation and expression pattern of *TMS1*, a gene involved in cell death pathway in pancreatic cancer cell lines. The promoter region of *TMS1* was found to be completely methylated in MIA PaCa-2 cells and unmethylated in PANC1 and ASPC1 cells (Figure 1 A, B). To determine whether *TMS1* expression is regulated by

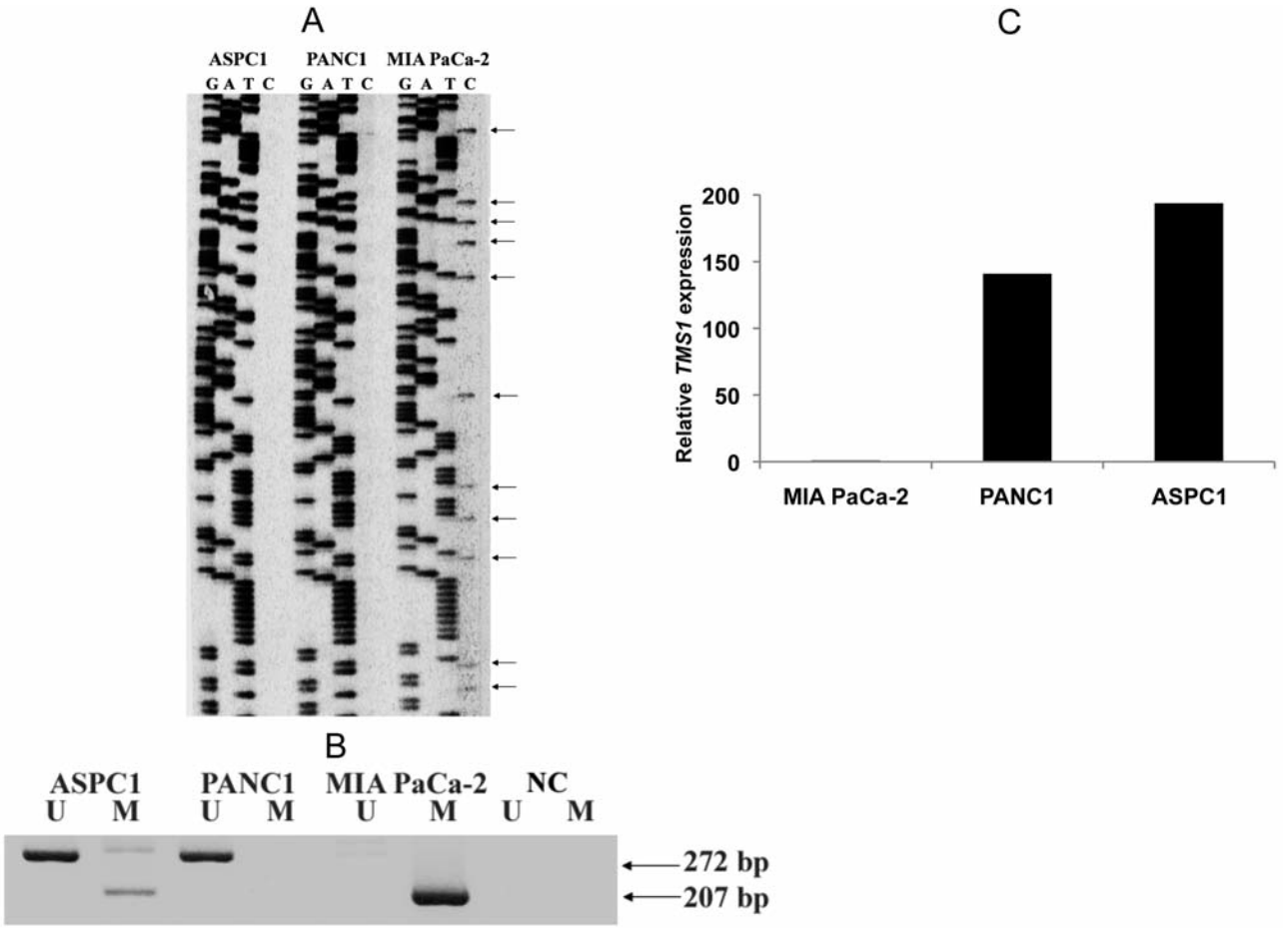

Figure 1. *A: Bisulfite genomic sequencing. DNA extracted from cells was bisulfite treated and amplified using primers flanking the TMS1 proximal promoter region between positions –126 and +76 with respect to the transcription start site and the PCR product was purified and sequenced. CpGs are indicated by arrows. B: MS-PCR analysis. Genomic DNA was extracted from pancreatic cancer cells, bisulfite treated and analyzed by MS-PCR using primers specific for unmethylated (U) and methylated (M) sequences. Water was used as negative control (6). C: Gene epression analysis. RNA extracted from breast cancer cells was reverse transcribed and amplified by qRT-PCR. TMS1 expression levels were normalized to that of the housekeeping gene, GAPDH.*

methylation of promoter region in these cells, we examined gene expression levels by real-time RTPCR. *TMS1* expression was down-regulated in MIA PaCa-2 cells compared to PANC1 and ASPC1 cells (Figure 1C). Levels of expression correlated inversely with methylation of the promoter region in these cells.

Treatment with DNMT inhibitor resulted in demethylation and re-expression of TMS1. To verfy the role of DNA methylation in regulation of *TMS1* expression, we determined the effect of treatment with the DNMT inhibitor, 5-azacytidine. MIA PaCa-2 cells were treated with 2.5 μM 5-azacytidine for 72 h, following which promoter methylation and expression levels of *TMS1* were determined. Treatment with 5-azacytidine results in a degree of demethylation of the promoter region of *TMS1* in MIA PaCa-2 cells (Figure 2A), concomitant with an increase in expression of *TMS1* (Figure 2B).

Overexpression of TMS1 results in enhanced sensitivity of MIA PaCa-2 cells to gemcitabine and docetaxel treatment. Although the role of *TMS1* in apoptosis has been well documented, its role in sensitivity to gemcitabine and docetaxel is not well understood. We determined the effect of recombinant expression of *TMS1* in MIA PaCa-2 cells on sensitivity to gemcitabine and docetaxel. MIA PaCa-2 cells were transfected with *TMS1* expression vector or empty vector (mock) and treated with either gemcitabine or docetaxel for 72 h, after which cell viability was measured. Cells transfected with *TMS1* expression vector showed

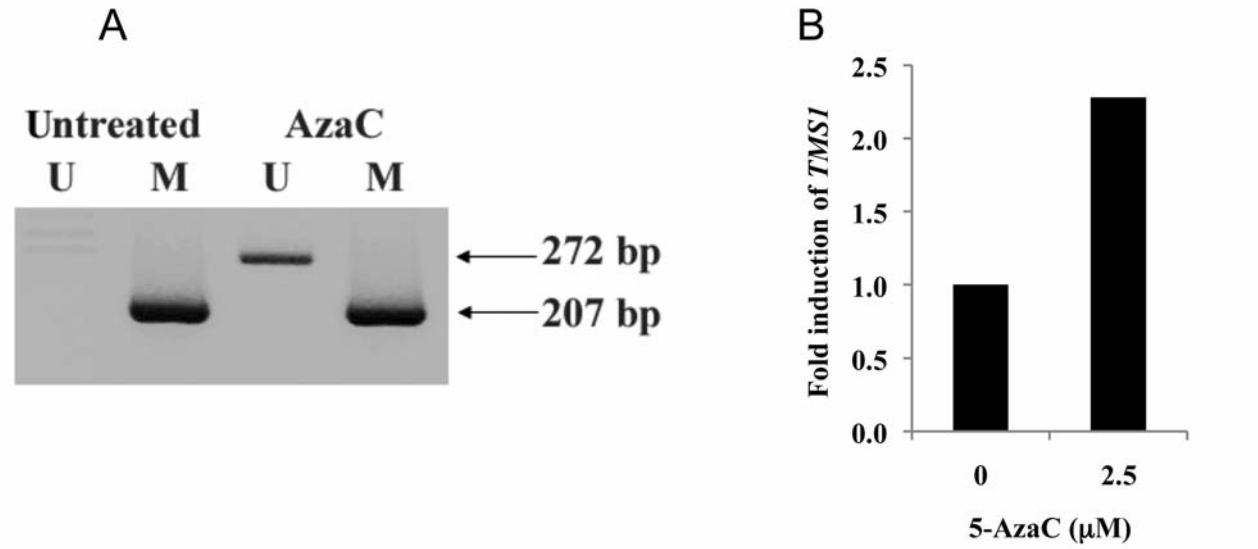

Figure 2. *Effect of 5-azacytidine treatment. MIA PaCa-2 cells were treated with 2.5 μM 5-azacytidine for 72 h, following which DNA and RNA were extracted. A: MS-PCR analysis. Genomic DNA was bisulfite treated and analyzed by MS-PCR using primers specific for unmethylated (U) and methylated (M) sequences. Water was used as negative control (6). B: Gene expression analysis. RNA extracted from cells was reverse transcribed and amplified by PCR.*

increased sensitivity to both gemcitabine and docetaxel treatment when compared to mock-transfected cells. Increased cell death was observed in MIA PaCa-2 cells expressing *TMS1* as compared to mock-transfected cells when treated with 40 nM gemcitabine (34% *vs*. 7%) and with 80 nM gemcitabine (60% *vs*. 36%). (Figure 3A). Similarly increased cytotoxicity was observed in MIA PaCa-2 cells expressing *TMS1* as compared to mock-transfected cells when treated with 1.25 nM docetaxel (49% *vs*. 20%) and with 5 nM docetaxel (63% *vs*. 38%) (Figure 3B).

Pre-treatment with 5-azacytidine results in enhanced cytotoxicity to gemcitabine and docetaxel treatment in MIA PaCa-2 cell lines. Our previous studies in prostate and breast cancer cells have shown that combined treatment with 5-azacytidine and chemotherapeutic drugs improve the antitumor activity of chemotherapeutic drugs (17, 18). We hypothesized that induction of *TMS1* expression by 5-azacytidine pretreatment would increase sensitivity of MIA PaCa-2 cells to docetaxel and gemcitabine chemotherapy. MIA PaCa-2 cells were treated with concentrations of 5-azacytidine ranging from 1.25 μM to 20 μM and cell survival measured after 24, 48, and 72 h (data not shown). Quantitative RT-PCR was used to measure the corresponding gene expression after 72 h. Treatment with 2.5 μM 5-azacytidine resulted in 76% viable cells after 72 h and ~2.5-fold increase in *TMS1* mRNA levels. Hence, we chose the sub-cytotoxic dose of 2.5 μM 5-azacytidine for pretreatment of the cells.

MIA PaCa-2 cells were pre-treated with 5-azacytidine for 72 h followed by treatment with either gemcitabine or docetaxel for 72 h, following which cell viability was measured. Pre-treatment with 5-azacytidine resulted in increased cytotoxicity of both gemcitabine and docetaxel. MIA PaCa-2 cells pre-treated with 5-azacytidine showed a 27% and 36% increase in cell death when treated with 40 nM and 80 nM gemcitabine, respectively compared to cells with no pretreatment (Figure 4A). Similarly, cells pre-treated with 5-azacytidine showed an increase in cytoxicity at 1.25 nM and 2.5 nM docetaxel by 18% and 33% compared to cells that were treated with docetaxel alone (Figure 4B).

Discussion

We examined the role of *TMS1* in sensitivity to chemotherapeutic drugs in MIA PaCa-2 pancreatic cancer cells. Loss of expression of *TMS1* was associated with complete methylation of the promoter region in MIA PaCa-2 cells. Up-regulation of *TMS1* by recombinant expression or by pre-treatment with 5-azacytidine resulted in enhanced sensitivity to docetaxel and gemcitabine in these cells.

Apoptosis is a process of programmed cell death that occurs in various physiological and pathological conditions. Cancer cells harbor DNA modifications or mutations in pro-apoptotic genes, leading to disruption of the apoptotic pathway, which in turn contributes to resistance to chemotherapeutic drugs. DNA methylation is a predominant epigenetic phenomenon which has an

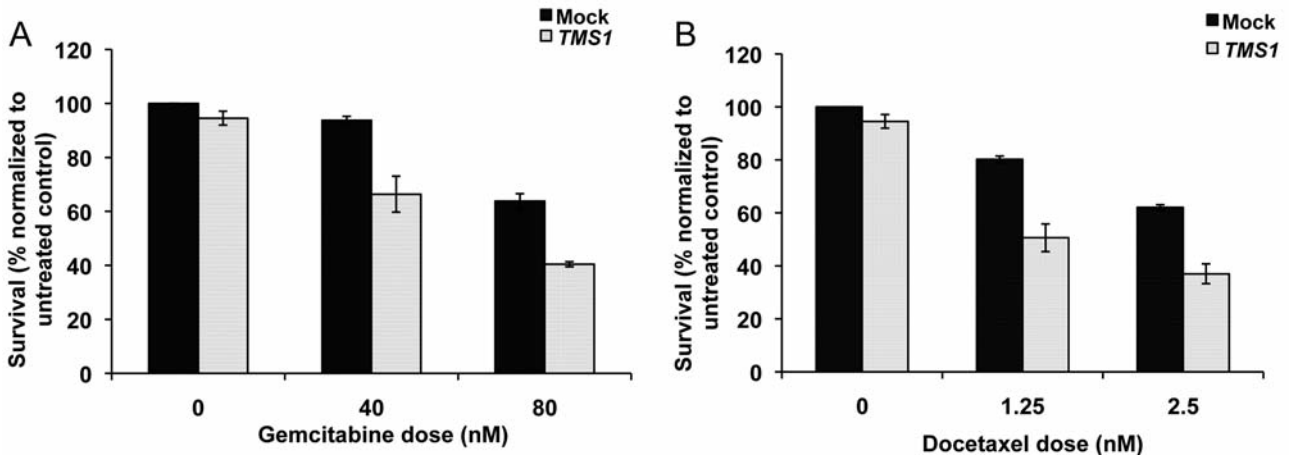

Figure 3. *Effect of TMS1 expression on sensitivity to chemotherapeutic agents. MIA PaCa-2 cells were transfected with TMS1 expression vector or vector alone (mock) using Amaxa nucleofection kit and seeded in 96-well plates for 24 h, following which they were treated with gemcitabine (A) or docetaxel (B) for 72 h. Cell viability was assayed by Cell Titer Blue assay.*

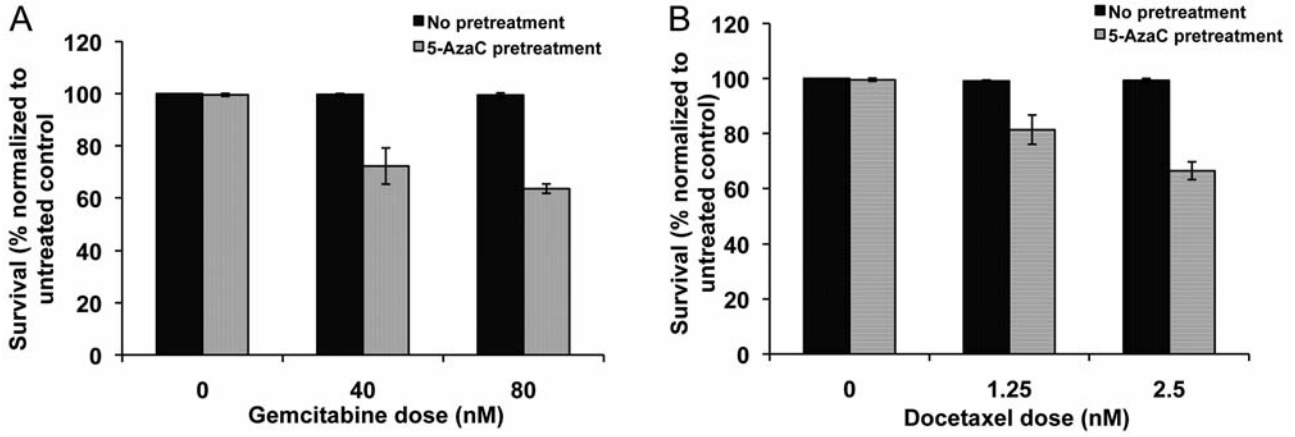

Figure 4. *Increased sensitivity of MIA PaCa-2 cells to gemcitabine (A) and docetaxel (B) by pretreatment with 5-azacytidine. Cells seeded in 96-well plates were treated with 2.5 μM 5-azacytidine for 72 h, following which they were treated with gemcitabine or docetaxel for 72 h. In parallel, control cells were left untreated for 72 h following which they were treated with the docetaxel or gemcitabine for 72 h. Cell viability was assayed by Cell Titer Blue assay.*

important role in the etiology of cancer (19). Changes in DNA methylation patterns, particularly in the promoter region of genes, contributes to regulation of gene expression (19). Hypermethylation in the promoter region of genes is involved in down-regulation of gene expression. Several genes have been shown to be hypermethylated in pancreatic cancer, and a large number of these are tumor suppressor genes, genes involved in cell cycle regulation and apoptosis. Examples of such genes that are silenced by DNA methylation in pancreatic cancer include *p16*, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (*BNIP3*), human MutL homolog 1 (*hMLH1*), E-cadherin (*ECAD*) and *Reprimo* (20, 21). *TMS1* has been shown to be down-regulated by DNA methylation in several types of carcinoma including the prostate, ovary and breast (14, 22-24). We have previously shown that methylation of *TMS1* is a frequent event in prostate cancer and that loss of *TMS1* gene expression was associated with complete methylation of the promoter region in LNCaP prostate cancer cells (22). More recently, we described DNA methylation-mediated silencing of *TMS1* in SKBR3 and Hs578t breast cancer cells (17). In this study, we show that *TMS1* is down-regulated by DNA methylation in MIA PaCa-2 cells. Treatment with DNMT inhibitor leads to demethylation of the *TMS1* promoter and up-regulation of gene expression.

Although *TMS1* has been shown to participate in apoptosis, its precise role in the apoptotic pathway is still unclear. It has been previously shown that recombinant expression of *TMS1* results in caspase-8 dependent apoptosis (25-27). Furthermore, it has been demonstrated that TMS1-induced apoptosis is dependent on the instrinsic mitochondrial pathway (11). More recently, Parsons *et al*. (28) showed that *TMS1* plays a role in anoikis and that siRNA-mediated silencing of TMS1 leads to resistance to anoikis, possibly mediated by failure to up-regulate the BH3-only protein BIM, ERK activation and inhibition of cleavage of pro-caspase-8. We observed that recombinant expression of *TMS1* in MIA PaCa-2 cells results in increased sensitivity to gemcitabine and docetaxel, indicating that *TMS1* may play a role in apoptosis induced in response to treatment with these chemotherapeutic agents.

Due to their reversible nature, epigenetic modifications in cancer have been identified as therapeutic targets. DNMT inhibitors and histone deacetylase inhibitors have been used in the treatment of various types of cancer because of their ability to restore expression of epigenetically repressed tumor suppressor and pro-apoptotic genes (29, 30). We have previously shown that methylation of *TMS1* was found to correlate with down-regulation of gene expression in the breast cancer cell line, SKBR3. Pre-treatment with a DNMT inhibitor, 5-azacytidine, increased the sensitivity of these cells to docetaxel, most likely due to the up-regulation of *TMS1* (17). In a prior study, we described methylation-mediated silencing of growth arrest and DNA damage inducible alpha (*GADD45A*), a gene involved in the apoptotic pathway and cell cycle control, in DU145 prostate cancer cells. Up-regulation of *GADD45A* either by recombinant gene expression or by treatment with 5-azacytidine resulted in enhanced sensitivity to docetaxel treatment (18). Based on these findings, we are currently conducting a phase I/II clinical trial using a combination of 5-azacytidine, docetaxel and prednisone in patients with docetaxel-refractory metastatic castration-resistant prostate cancer. Our present results show that pretreatment of MIA PaCa-2 cells with 5-azacytidine resulted in increased cytotoxicity in response to docetaxel and gemcitabine treatment, indicating 5-azacytidine may improve the efficacy of conventional chemotherapeutic drugs in pancreatic cancer.

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