Thalidomide inhibits proliferation and epithelial-mesenchymal transition by modulating CD133 expression in pancreatic cancer cells

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Abstract. Pancreatic cancer is a solid malignancy with a high mortality rate, on account of the high incidence of metastasis at the time of detection. The aggressiveness of pancreatic cancer may be partly driven by cancer stem cells (CSCs), which are characterized by the ability to self-renew and recapitulate tumors in the ectopic setting. However, although a number of drugs targeting CSCs are currently under clinical investigation, few effective drugs have been developed. The present study demonstrated that thalidomide inhibited cell proliferation and metastasis in pancreatic cancer cell lines through the inhibition of epithelial mesenchymal transition. The effect of thalidomide was more pronounced in cluster of differentiation 133 (CD133)+ SW1990 cells than in Capan-2 cells, in which CD133 expression was almost undetectable. The results revealed that CD133 is likely to serve a role in the antitumor effect of thalidomide and indicated that thalidomide could be developed as a CSC-specific adjuvant chemotherapy in pancreatic cancer.

Introduction

Pancreatic cancer is a disease with a high mortality rate that is characterized by the early metastasis to local and distant organs; the majority of patients present with unresectable disease at the time of initial diagnosis (1). Therefore, patients with pancreatic cancer have a poor prognosis, with an overall 5-year survival rate of <5% in the United States between 1975 and 2008 (2). No significant advances in the treatment of pancreatic cancer have been made in >10 years, largely due to of the resistance of the disease to conventional chemotherapy and radiation therapy (3-6). The involvement of cancer stem cells (CSC; also termed tumor-initiating cells) in the development of chemotherapy resistance has been reported in a number of types of malignancy (7-9). CSCs are a phenotypically distinct population of cells that are functionally defined by their ability to form tumors, self-renew and differentiate, as well as their resistance to chemotherapy (10,11). Therefore, the development of novel chemotherapeutic agents that are effective against CSCs is urgently required.

Thalidomide is a non-barbiturate sedative and hypnotic drug with anti-angiogenic and immunomodulatory properties (12). Thalidomide is currently used in the treatment of various types of malignant tumor, including prostate cancer (13), glioblastoma (14), glioma (15), renal cell carcinoma (16) and advanced breast cancer (17). The mechanism of action of thalidomide includes the inhibition of vascular endothelial growth factor, basic fibroblast growth factor and tumor necrosis factor-α, the inhibition of angiogenesis, and the stimulation of natural killer cells (12). However, to the best of our knowledge, the use of thalidomide in the treatment of pancreatic cancer has not been assessed and the potential mechanism of thalidomide-mediated inhibition of tumor cell viability remains to be elucidated. In the present study, the effect of thalidomide on pancreatic cancer cells was examined, alongside its mechanism of action.

Materials and methods

Cell lines and reagents. The human pancreatic cancer SW1990, Capan-2, Panc-1, Patu8988 and Aspc-1 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a

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humidified incubator with 5% CO_2. Thalidomide was obtained from Selleck Chemicals (Houston, TX, USA). The MTT reagent and anti-cluster of differentiation 133 (CD133) antibody for western blotting were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; cat. no. SAB2107606). The phycoerythrin-conjugated CD133 antibody for flow cytometry was purchased from MiltenyiBiotec, Inc., (Auburn, CA, USA; cat. no. 130-080-801). Transwell filter inserts were obtained from Corning (Corning In corporated, Corning, NY, USA). Rabbit anti-E-cadherin (cat. no. sc-7870), rabbit anti-N-cadherin (cat. no. sc-7939), and mouse anti-β-actin (cat. no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Thalidomide was dissolved in dimethyl sulfoxide (DMSO) to a storage concentration of 50 mmol/l. An equal amount (0.1% v/v) of DMSO was present in all the treatment groups, including the control.

Cell proliferation assay. Cell viability was measured using the MTT assay, according to the manufacturer's protocol. Cells were seeded at a density of $5x10^4$ cells/well in 96-well flat-bottom microtiter plates. Cells were treated with different concentrations (0, 6.25, 12.5, 25, 50 or 100 μmol/l) of thalidomide in 200 μl complete medium for 24, 48 or 72 h at 37°C in an incubator. Cells were then incubated with MTT (0.25 mg/ml) for 2-4 h at 37°C. The formazan crystals in the cells were solubilized with a solution containing 50% dimethylformamide and 20% SDS (pH 4.7). Finally, the level of MTT/formazan was determined by measuring absorbance at a wavelength of 570 nm using a microplate reader (SPECTRA; Tecan Group, Ltd., Mannedorf, Switzerland).

Cell migration assays. SW1990 and Capan-2 cells were plated in 6-well plates with various concentrations of thalidomide (0, 50, 100 μmol/l). At 48 h, the cells were harvested, washed once in PBS and resuspended in serum-free high-glucose DMEM. A 200-μl aliquot of cell suspension was added to Transwell filter inserts, resulting in a density of $2x10^4$ cells per insert. High-glucose DMEM containing 10% FBS was added to the lower wells. Migration was allowed to proceed for 48 h at 37°C. Cells that did not migrate through the filters were removed using cotton swabs, and cells that migrated through the inserts were fixed and stained with 1% crystal violetat room temperature. The number of migrating cells was observed with phase contrast microscopy (magnification, x200) and images were captured.

Flow cytometry. SW1990 and Capan-2 cells were plated in 6-well plates for 48 h with various concentrations of thalidomide (0, 50, 100 μmol/l), then were harvested, washed twice in cold PBS and incubated with a phycoerythrin-conjugated CD133 antibody (1:10) for 30 min at 4°C. Mouse IgG1-phycoerythrin (1:10; cat. no. IC002P; R&D Systems, Inc.) was used as an isotype control antibody. Non-viable cells were eliminated with 7-aminoactinomycin D (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China; cat. no. KGA219) staining. The labeled cells were analyzed by a BD FASCCanto II flow cytometer 338960 (BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's protocol and data was analyzed using FlowJo v10 software (Tree Star, Inc., Ashland, OR, USA). Gating was implemented on the basis of negative-control staining profiles.

Table I. Polymerase chain reaction primer sequences.

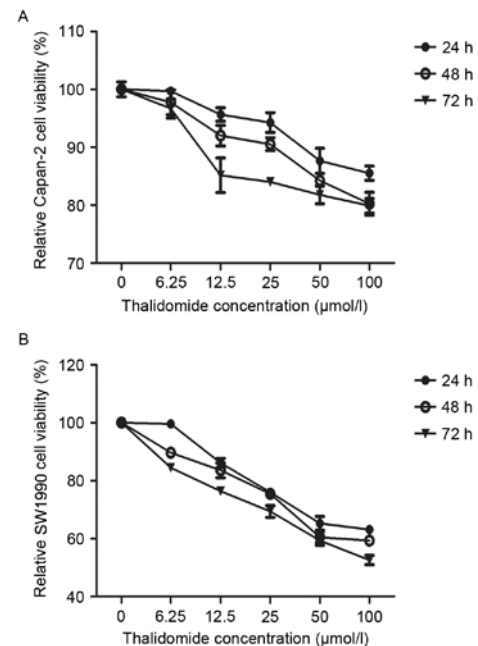| Gene	Primer sequence, 5'-3'
CD133	
Forward	TTCTTGACCGACTGAGACCCA
Reverse	TCATGTTCTCCAACGCCTCTT
E-cadherin	
Forward	TCGACACCCGATTCAAAGTGG
Reverse	GTGGGTTATGAAACCGTAGAGG
N-cadherin	
Forward	AGCCAACCTTAACTGAGGAGT
Reverse	GGCAAGTTGATTGGAGGGATG
β-actin	
Forward	CTGGAACGGTGAAGGTGACA
Reverse	AAGGGACTTCCTGTAACAATGCA

CD133, cluster of differentiation 133.

Figure 1. Thalidomide inhibits the cell viability of pancreatic cancer cell lines. The viability of (A) Capan-2 and (B) SW1990 cells treated with a range of thalidomide concentrations was measured with an MTT assay and compared with untreated cells. Thalidomide treatment induced a dose- and time-dependent inhibitionof cell viability. Results represent the mean ± standard deviation from six independent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. A density of $10x10^4$ SW1990 and Capan-2 cells were respectively treated with 100 μmol/l thalidomide for 24 h and harvested, and total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and subjected to reverse transcription using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). The mRNA expression was detected by qPCR with an ABI PRISM® 7900HT Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR-Green Dye (Takara Bio, Inc.).
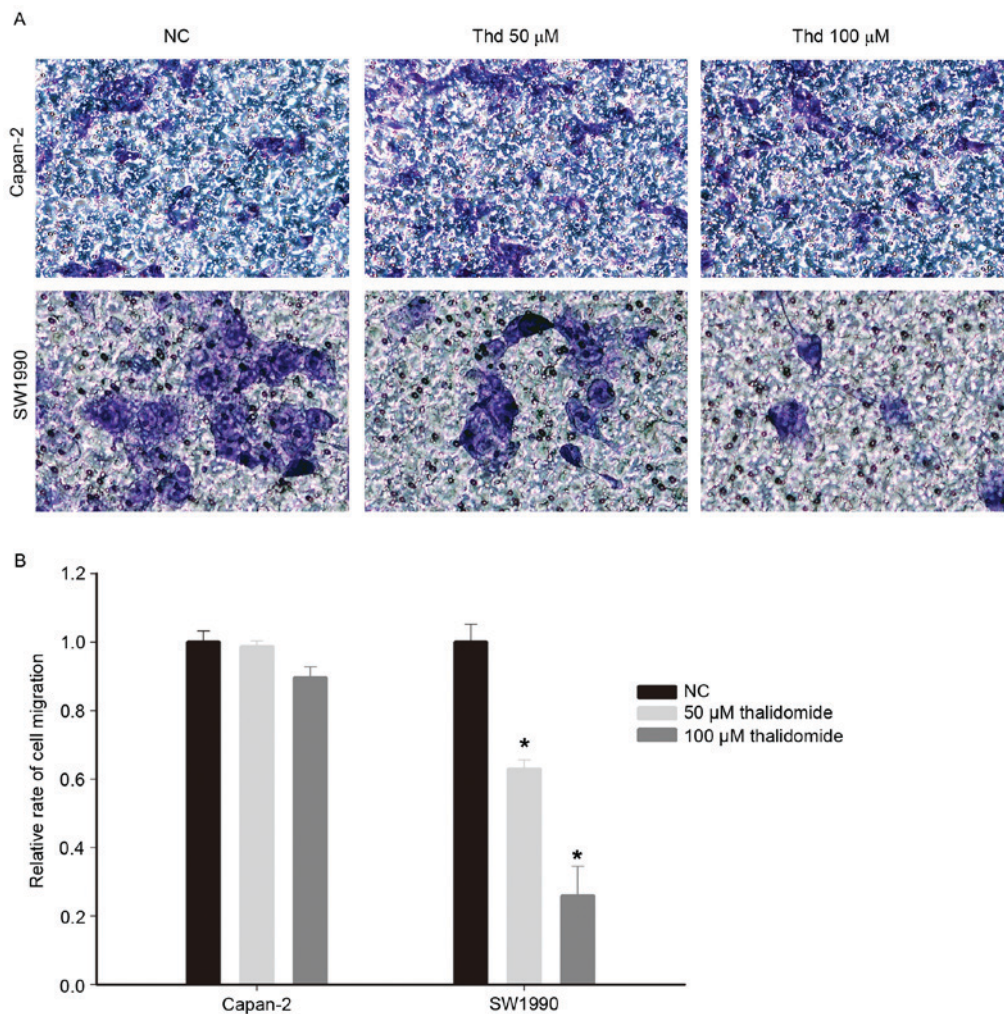
Figure 2. The effect of thalidomide on the migration of pancreatic cancer cell lines was assessed using Transwell filter inserts. (A) The relative proportion of migrated pancreatic cancer cells treated with thalidomide for 48 h was determined. Magnification, x200. (B) Analysis of cell migration. *P<0.05 compared with NC. NC, negative control.

Primers used for qPCR are listed in Table I. cDNA was amplified with 40 PCR cycles (0.5 sec at 95°C; 10 sec at 60°C; 10 sec at 72°C). The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (18). All experiments were repeated at ≥3 times.

Western blotting. SW1990 and Capan-2 cells were treated with 100 μmol/l thalidomide for 24 h and rinsed twice in PBS. The cells were harvestedand centrifuged at 2,000 x g for 5 min at 4°C, then lysed for 2 h in radioimmunoprecipitation analysis lysis buffer (Beyotime Institute of Biotechnology, Haimen, China; cat. no. P0013B) on ice, and centrifuged at 12,000 x g for 10 min at 4°C. The protein concentration was determined by using a bicinchoninic acid protein assay (BCA™ protein assay kit; Pierce; Thermo Fisher Scientific, Inc.). Aliquots of 40 μg protein were separated by 6% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Non-specific binding was blocked with 5% low-fat milk at room temperature for 1 h in a covered container. The membranes were incubated with the relevant primary antibodies overnight at 4°C (dilutions: E-cadherin, 1:200; N-cadherin, 1:400, CD133, 1:800; β-actin, 1:1,000). The membranes were washed in PBS with 0.1% Tween-20

and incubated with the appropriate HRP-conjugated secondary antibodies (goat anti-rabbit IgG-HRP; 1:2,000; cat. no. sc-2004; goat anti-mouse IgG-HRP; 1:2,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. The blots were developed using an enhanced chemiluminescence (ECL)-detection system (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), dried, and exposed to ECL film. All experiments were repeated ≥3 times, with similar results.

Statistical analysis. SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data were obtained from three independent experiments and expressed as the mean ± standard deviation. The statistical analyses performed included χ^2 test and one-way analysis of variance (ANOVA), which was followed by Student-Newman-Keuls (SNK) as a post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Thalidomide inhibits the proliferation of pancreatic cancer cells. The inhibitory effect of thalidomide on the growth of human pancreatic cancer cell lines was determined by an
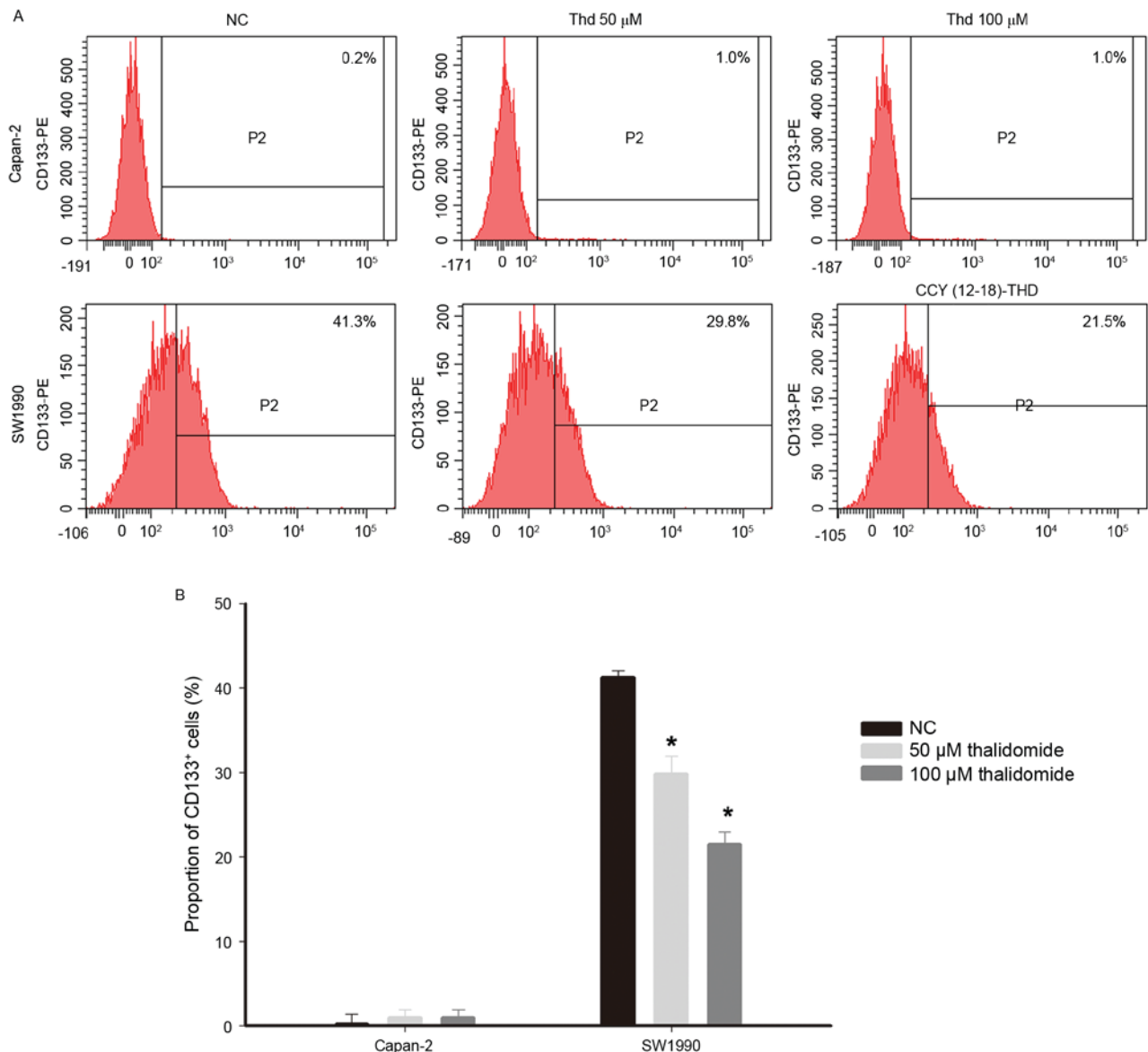
Figure 3. Flow cytometry analysis of the proportion of CD133+ cells in the Capan-2 and SW1990 cell lines following treatment with different concentrations of thalidomide. (A) Capan-2 cells were 0.2% CD133+, compared with 41.3% for SW1990 cells. (B) Thalidomide treatment reduced the CD133+ cell subpopulation in SW1990 cells; however, there was no effect on Capan-2 cells. *$P<0.05$ compared with NC. CD133, cluster of differentiation 133; NC, negative control.

MTT assay. In Capan-2 cells treated with thalidomide, a dose-dependent decrease in cell number was observed, with a 4-14, 7-19 and 14.8-20% inhibition in cell growth, respectively (Fig. 1A). Thalidomide caused acomparatively higher dose- and time-dependent inhibition of cell growth in SW1990 cells, with 13-36, 16-40, and 23-47% of growth at 24, 48 and 72 h at 6.25-100 μmol/l, respectively (Fig. 1B). These data indicated that the mechanism for growth inhibition by thalidomide may differ between the two cell lines.

The proliferation of three other pancreatic cancer cell lines (Panc-1, Patu8988 and Aspc-1) was assessed in preliminary experiments using the MTT assay (data not shown). Results obtained from this analysis demonstrated that the proliferation of these three pancreatic cancer cell lines did not differ significantly following administration of thalidomide, whereas the proliferation of the Capan-2 and SW1990 cell lines were inhibited following treatment. Therefore, Capan-2 and SW1990 cells were selected for the present study.

Effects of thalidomide on migration in pancreatic cancer cells. To examine the effect of thalidomide on the motility of pancreatic cancer cells, a cell migration assay was conducted with Transwell filter inserts. The motility of SW1990 cells treated with thalidomide for 48 h was reduced compared withthe control cells (Fig. 2A and B; $P<0.05$). However, the Capan-2 cells exposed to thalidomide treatment exhibited no significant decrease in motility (Fig. 2A and B), which was consistent with the pattern of growth inhibition in the two cell lines.

Thalidomide reduces the proportion of CD133+ cell subpopulations in vitro. CD133 is a cell surface marker of CSCs in pancreatic cancer (19,20). The effect of thalidomide on the proportion of pancreatic cancer cells with the CD133+ antigenic phenotype was analyzed by flow cytometry. The proportion of CD133+ cells in the Capan-2 and SW1990 lines was different, with <1% of cells exhibiting CD133-positivity

in the Capan-2 cell line compared with >40% in SW1990. This indicated that the SW1990 line has a higher proportion of CSCs than the Capan-2 line. The proportion of CD133+ cells in the Capan-2 cell line were not significantly altered in response to thalidomide treatment (negative control, 0.2% vs. thalidomide, 1.0%), whereas thalidomide treatment significantly decreased the proportion of CD133+ cells in the SW1990 cell line (negative control, 41.3% vs. thalidomide 50 μmol/l, 29.8% and 100 μmol/l, 21.5%; Fig. 3; P<0.05).

Thalidomide inhibits EMT in pancreatic cancer cells. The dysregulation of the EMT program contributes to tumor initiation, invasion and metastatic spread, and is associated with an increase in tumor cell stemness (21). The teratogenic effect of thalidomide is well-documented; EMT governs morphogenesis and is activated during embryogenesis (22). Therefore, the EMT status was assessed in cells exposed to thalidomide. The primary molecular feature of cancer cells undergoing EMT is the upregulation of characteristic mesenchymal genes, including N-cadherin and E-cadherin (23). On the basis of the previous results that thalidomide could reduce the proportion of CD133+ cells, the expression of N-cadherin, E-cadherin and CD133 were detected by RT-qPCR and western blotting.

The results of western blotting indicated that the protein expression of CD133 in Capan-2 cells was markedly lower than in SW1990 cells. Thalidomide treatment downregulated CD133 and N-cadherin, and upregulated E-cadherin mRNA and protein expression in SW1990 cells (Fig. 4). However, thalidomide did not significantly affect the expression of E-cadherin, N-cadherin and CD133 mRNA in Capan-2 cells. Therefore, it was concluded that thalidomide inhibited the EMT program in CD133+ cells, whereas it exhibited no significant effect in cells with low CD133 expression.

Discussion

Pancreatic cancer is the fourth-leading cause of cancer-associated mortality in the United States (24). Unlike other types of cancer, the survival rate of patients with this disease has not improved substantially in ~40 years, largely due to its aggressiveness (25). Emerging evidence indicates that the aggressiveness of pancreatic cancer may be partly driven by CSCs, phenotypically distinct cell populations (10,26,27). CD133 is the most commonly expressed CSC marker in several cancer types, including pancreatic cancer (18,19,28-30).

In the present study, it was demonstrated that thalidomide inhibits the growth of human pancreatic cancer cells *in vitro*. However, the sensitivity to thalidomide differed between SW1990 and Capan-2 cells, which exhibit different levels of CD133 expression. The SW1990 cell line, in which the proportion of CD133+ cells was >40%, was more sensitive to thalidomide than the Capan-2 cell line, of which <1% were CD133+ cells, as demonstrated by the increased inhibition of cell growth and migration. These results indicated that the sensitivity of pancreatic cancer cells to thalidomide may dependent on the expression of CD133, and therefore, on the proportion of CSCs. Thalidomide reduced the CD133+ cell subpopulation in the SW1990 cell line and downregulated the mRNA and protein expression of CD133. Taken together, the results indicated that thalidomide may inhibit the proliferation and metastasis of
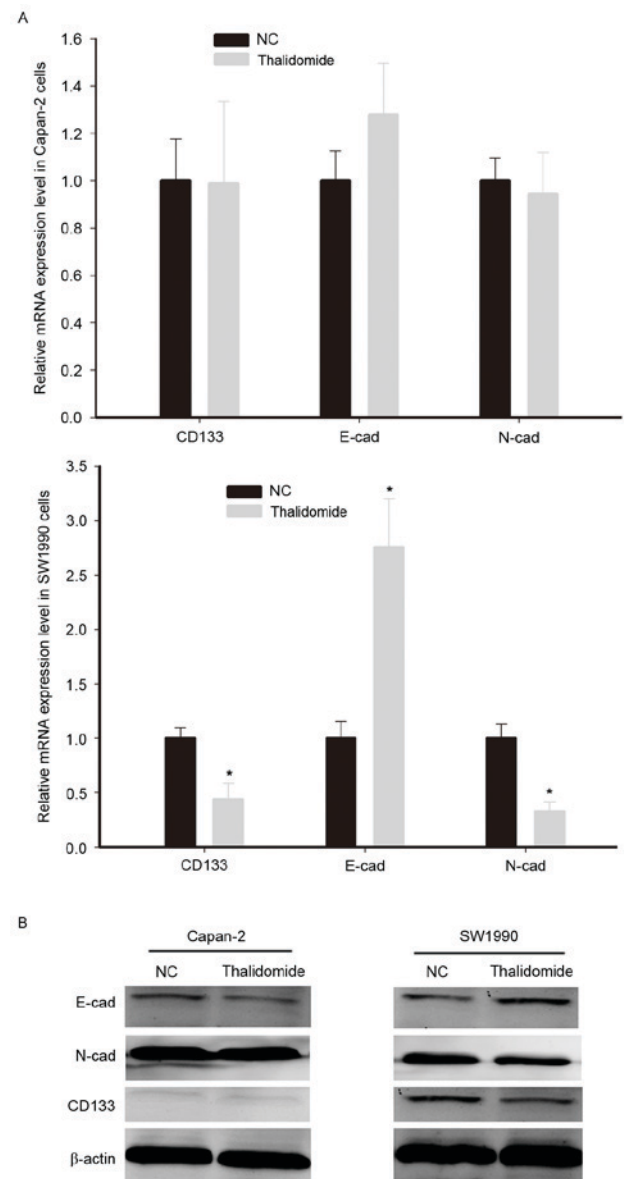

Figure 4. Thalidomide inhibited EMT and downregulated CD133 expression in SW1990 cells. The expression levels of E-cad, N-cad and CD133 were detected by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting. Thalidomide downregulated CD133 and N-cad mRNA and protein expression and upregulated E-cad mRNA and protein expression in SW1990 cells; however, it had no effect on the levels of these markers in Capan-2 cells. *P<0.05 compared with NC. EMT, epithelial-mesenchymal transition; CD133, cluster of differentiation 133; E-cad, E-cadherin; N-cad, N-cadherin; NC, negative control.

pancreatic cancer cells partly by modulating the expression of CD133, which suggests that the antitumor effects of thalidomide are partially mediated by inhibiting CSCs.

The epithelial-mesenchymal transition (EMT) is a process implicated in drug resistance, metastasis and the generation of CSCs (21,31,32). The present study analyzed the mRNA and protein expression of the EMT markers E-cadherin and N-cadherin in pancreatic cancer cells treated with thalidomide. The results of this analysis revealed that thalidomide upregulated E-cadherin expression and downregulated N-cadherin expression in SW1990 cells, whereas there was no significant effect on these markers in Capan-2 cells. These results

suggested that thalidomide inhibited EMT in SW1990 cells and not in Capan-2 cells. Previous studies have demonstrated that CD133 is part of the regulatory network that facilitates EMT; CD133 is a transmembrane protein associated with the signaling pathways regulating the EMT program, particularly the cadherin switch (28,33). In liver cancer cells, CD133^{+} cells exhibited upregulated N-cadherin expression and down-regulated E-cadherin expression compared with CD133^{-} cells, which indicates that EMT occurs more frequently in CD133^{+} cells than in CD133^{-} cells (34). Ding *et al* (29) reported that CD133 serves an important role in the regulation of N-cadherin and Slug expression. However, other molecules are involved in the regulation of EMT, in addition to CD133. Further study is required to characterize this regulatory loop, which implies that thalidomide may inhibit EMT at least partly through the modulation of CD133.

In summary, the data of the present study indicate that the *in vitro* efficacy of thalidomide at inhibiting pancreatic cancer cell growth and migration is mediated by the down-regulation of CD133 and the inhibition of EMT. The efficacy of thalidomide was dependent on the expression of CD133, as demonstrated by the different sensitivity to thalidomide of the SW1990 and Capan-2 pancreatic cancer cell lines, which express different levels of CD133. The results indicated that thalidomide may partly target CSCs. However, the differential response of SW1990 and Capan-2 cells to thalidomide warrants further investigation at the molecular level in order to further characterize the mechanisms underlying their sensitivity to thalidomide. Furthermore, assessing the effect of a combination of CSC-targeting and non-CSC-targeting therapies could be of value in optimizing the tumor response, enhancing long-term disease control and ultimately improving patient survival rates.

Acknowledgements

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