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ORIGINAL CONTRIBUTIONS

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EFFECT OF TRICHOSTATIN A ON VIABILITY AND microRNA EXPRESSION IN HUMAN PANCREATIC CANCER CELL LINE BxPC-3

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Aim: **To investigate the influence of trichostatin A (TSA) on inhibition of cell proliferation and induction of apoptosis in human pancreatic cancer cells.** ***Methods:*** **MTT-based cytotoxicity assay was used to evaluate the cell viability after treatment with TSA. Cell cycle distribution and apoptosis were examined by means of flow cytometry. Expression of microRNA was determined with microRNA array. Expression of miR-200c and miR-21 was detected by Northern blotting.** ***Results:*** **TSA significantly inhibited the proliferation of BxPC-3 human pancreatic cancer cells in a time- and dose-dependent manner. BxPC-3 cells treated with TSA were arrested in G_0/G_1 phase and were characterized by increased apoptotic rate, accompanied by differential expression of microRNAs.** ***Conclusions:*** **The results suggest that TSA may activate expression of microRNAs that may act as tumor suppressor in human pancreatic cancer cell line BxPC-3.**

Key Words: **pancreatic cancer, trichostatin A, microRNA.**

Pancreatic cancer is one of the most aggressive human cancers. Advanced pancreatic cancer is associated with a poor prognosis although surgical resection or radiotherapy is potentially curative for localized disease. 5-year survival rates for patients with pancreatic cancer are less than 5% and the median survival time is less than 6 months [1]. Multimodality treatments, including surgery, chemotherapy, and post-operative radiation therapy, have resulted in only an incremental increase in survival. Novel therapeutic approaches that can change the course of the disease are urgently needed.

Histone deacetylase (HDAC) inhibitors seem to be a new class of anticancer agents. In numerous cancers, alterations in histone acetyl transferase (HAT) or HDAC activity occur and overactivation of the HDAC enzymes results in histone hypoacetylation. By altering the acetylation status of an array of substrates, including histones, transcription factors, and chaperone proteins, HDAC inhibitors have been shown to induce growth arrest, differentiation, and/or apoptosis of proliferating cancer cells [2–4]. Trichostatin A (TSA), the most common HDAC inhibitor, has been shown to have antitumor effects on pancreatic cancer, either alone or in combination with gemcitabine. These effects may result from alteration of the transcriptional profile where genes such as p21, which promotes cell cycle arrest, are up-regulated, whereas genes, such as 5'-nucleotidase UMPH (uridine monophosphate phosphohydrolase) type α gene, which prevents the formation of the active forms of gemcitabine, are down-regulated [5–9]. However, until now, therapy with HDAC inhibitors has been based on classic protein-coding tumor-suppressor genes, and only a few genes were found to be affected. Thus, to explore additional mechanisms of HDAC inhibitors influence, changes of the microRNAs (miRNAs) expression profile in pancreatic cancer cell line BxPC-3 following treatment with TSA were examined.

miRNAs are ~22 nucleotide (nt) noncoding RNAs that regulate gene expression by translational repression when partially complementary sequences are present in the 3'untranslated regions (3'UTR) of the target mRNAs or by directing mRNA degradation. miRNAs are expressed in a tissue-specific manner and are considered to play important roles in cell proliferation, apoptosis, and differentiation [10–13]. Moreover, altered expression of miRNAs has been shown to be associated with many human diseases including cancer. Aberrant miRNAs expression in pancreatic cancer contributes to tumor cell proliferation and survival [14–17]. In this study, we examined whether the HDAC inhibitor TSA can affect cell growth, apoptosis and alter expression of miRNAs in pancreatic cancer cell line BxPC-3.

MATERIALS AND METHODS

Materials. The human BxPC-3 cell line was obtained from the American Type Culture Collection (Manassas, VA). TSA was purchased from Sigma, USA, dissolved in absolute ethanol and stored at –20 °C.

Cell culture. BxPC-3 cells were cultured in RPMI 1640 supplemented with 20 mM glutamine, 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and were incubated at 37 °C in a humidified incubator with 5% CO_2.

Cell viability assay. Cell viability was assessed by the MTT-based cytotoxicity assay. BxPC-3 cells were trypsinized and seeded in 96-well plates at a density of 2×10^3 cells/well and cultured overnight. Cells were then treated with different concentrations of TSA (0.1, 0.5, 1.0, 2.0 μmol/L) or control (0.1% ethanol) for 24–72 h. At the completion of incubation, media was replaced with fresh complete media (100 μl). 4 h before the end of the incubation period, 20 μl of PBS containing MTT (5 mg/mL) were

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Abbreviations used: HAT – histone acetytransferase; HDAC – histone deacetylase; HDACIs – histone deacetylase inhibitors; PI – propidium iodide; TSA – trichostatin A; miRNAs – microRNAs; UMPH – uridine monophosphate phosphohydrolase.

added to each well. Following this, the plates were centrifuged at 200 × g for 5 min and media was removed. The precipitate was then resuspended in 150 µl of DMSO. The absorbance was measured on a plate reader at 570 nm. Each experiment was performed in triplicates.

Cell cycle analysis. For cell cycle assay, BxPC-3 cells (at least 1×10^6 cells) treated with TSA at concentration of 1.0 µmol/L or 0.1% ethanol for 24 h were harvested and washed with PBS, and fixed in 90% ethanol for 1 h at – 20 °C. Prior to analysis, the cells were washed and resuspended in PBS, and incubated with 1 g/L of RNase I and 20 g/L of propidium iodide (PI) at 37 °C for 30 min. Fluorescence was quantified on a flow cytometry, and the percentage of cells in each phase was calculated using ModFit software (BD Biosciences).

Apoptosis assessment by Annexin V staining. Annexin V-FITC kit (Jingmei Biotech) was used to measure the percentage of apoptotic cells induced by 1.0 µmol/L TSA. After 24 h incubation, cells were harvested and washed with PBS at 4 °C and then resuspended in 100 µl of the staining solution containing 5 µl Annexin V-FITC and 10 µl PI. After incubation at room temperature for 15 min, stained cells were analyzed by flow cytometry.

miRNA microarray analysis. miRNA expression profiling was carried out according to the manufacturer's introduction. In brief, miRNA was isolated from untreated cells and cells treated with 1.0 µmol/L TSA for 6 h using mirVana miRNA isolation kit according to manufacturer's instructions (Ambion). Purified miRNA was labeled with Cy3 and then hybridized to the miRNA microarray chip containing 576 human miRNA probes. Each probe on the microarray slide is printed in duplicate with positive and negative controls. Following hybridization, the slides were washed, dried and scaned on a LuxScan 10K/A Scanner (CapitalBio Corp., China). Database calculations were done and expression maps were generated with Significance Analysis of Microarrays (SAM) for Excel.

Northern blotting. To verify the reliability of the expression changes detected by the profiling analysis using the microRNA array, Northern blotting with the same RNA samples that had been used for the microarray was performed for the elective number of microRNAs. RNA samples (20 µg each) were separated on 15% denaturing polyacrylamide gel and then electroblotted onto a Zeta-Probe G1 Blotting Membrane (Bio-Rad). Following transfer, the membrane was dried and UV cross-linked. The probes were prepared using the StatFne Oligonucleotide Labeling System (Integrated DNA Technologies) according to the manufacturer's protocol. The blots were hybridized at 50 °C in a buffer containing 5 × SSC, 20 mmol/L Na_2PO_4 (pH 7.2) 7% SDS, 1 × SSC/1% SDS buffer for 16 h. The probe sequences are as follows: miR-200c 5'-ACATCGTTACCAGACAGTGTTA-3', miR-21 5'-TCAACATCAGTCTGATAAGCTA-3'. U6 RNA (5'-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3') was used to normalize.

Statistical analysis. SPSS statistical software (version 12.0) was used for analysis. Statistical significance was determined using the analysis of variance (ANOVA). Data are expressed as mean ± standard deviation (SD). A P-value < 0.05 was considered statistically significant.

RESULTS

TSA inhibited pancreatic cancer cell viability. To examine the antiproliferative effect of TSA on BxPC-3 pancreatic cancer cells, we treated the cells with TSA at concentrations from 0.1 µmol/L to 2.0 µmol/L for 24–72 h. As shown in Fig. 1, TSA significantly inhibited the viability of BxPC-3 cells significantly in a time- and dose-dependent manner. Inhibition of cell viability was observed even at the lowest concentration. Exposure to TSA for 72 h at the concentration of 2.0 µmol/L caused a 72.6% (± 0.2) decrease of cell viability.

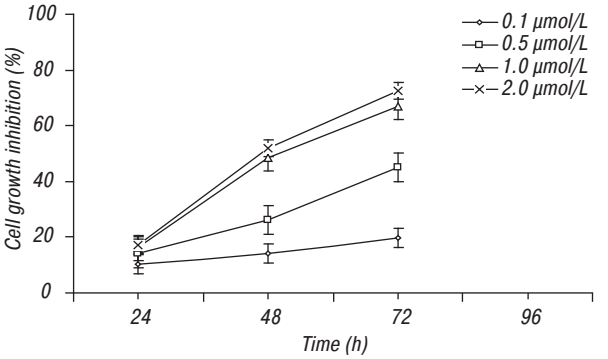

Fig. 1. The cell viability curve of pancreatic cancer cells BxPC-3 treated with TSA. BxPC-3 cells were treated with TSA at various concentrations for 24–72 h and cell viability was measured with MTT. TSA inhibited cell viability in a dose- or time-dependent manner, n = 3

Induction of cell cycle arrest and apoptosis by TSA. Cell cycle analysis showed that 24 h after the treatment of BxPC-3 cells with 1.0 µmol/L TSA, 19% increase of cells in the G_0/G_1 phase ($P < 0.05$, Table 1) was observed, indicating arrest of the cells at the G_0/G_1 transition. The flow cytometry analysis with Annexin V-FITC and PI staining showed that TSA induced the apoptosis of BxPC-3 cells. The apoptosis rate was increased significantly up to 25% in BxPC-3 cells treated with 1.0 µmol/L TSA *vs* 5% in the control cells (Fig. 2).

Table 1. Effect of TSA (1.0 µmol/L) on the cell cycle distribution of BxPC-3 cells (%, mean ± SD)

Groups	Cell cycle distribution		
	G_0/G_1	S	G_2/M
Blank	42.5 ± 2.2	33.2 ± 1.9	24.3 ± 3.1
Ethanol	47.3 ± 3.4	27.4 ± 2.3	25.3 ± 1.3
TSA	61.8 ± 2.5*	24.9 ± 4.2	13.3 ± 1.8

Notes: Experiment was carried out in triplicates (10^6 cells per each sample). Mean ± SD, $^*P < 0.05$ *vs* Blank or Ethanol.

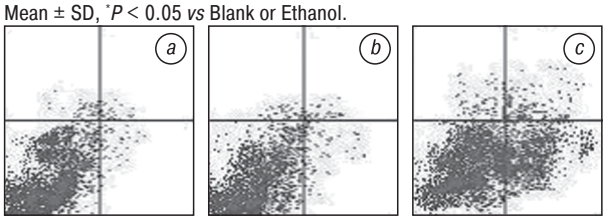

Fig. 2. TSA induced cell apoptosis in pancreatic cancer cells BxPC-3. 24 h after treatment, cell apoptosis was detected using FCM. *a*, Mock cells; *b*, Cells treated with ethanol control; *c*, Cells treated with 1.0 µmol/L TSA. AnnexinV-FITC and PI staining showed that TSA caused an apoptosis effect. The percentage rate of apoptosis was significantly increased to 25% in BxPC-3 cells treated with 1.0 µmol/L TSA, compared with 5% in control ($P < 0.001$)

Alteration of miRNAs levels following treatment with TSA. To assess the response of miRNAs to TSA, the expression profile of miRNAs from the pancreatic cancer cell line BxPC-3 treated for 6 h with 1.0 µmol/L TSA was determined by miRNA microarray analysis. Hi-

erarchical clusterings of miRNAs expression in the TSA-treated and untreated cells are shown in Fig. 3. Upon TSA treatment, the expression levels of BxPC-3 miRNAs were altered: 24 miRNAs were down-regulated and 5 miRNAs were up-regulated. To validate these miRNA microarray results, Northern analysis for several of the most abundantly expressed miRNAs was performed. As could be seen from Fig. 4, Northern blots showed the up-regulation of miR-200c and the down-regulation of miR-21 after TSA treatment of the cells confirming the miRNA microarray result for these miRNAs.

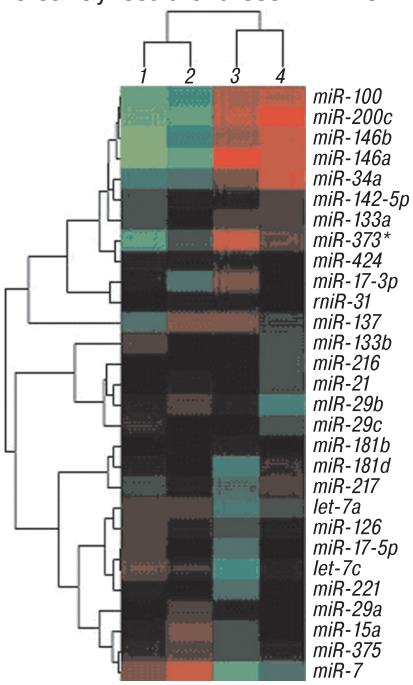

Fig. 3. Hierarchically clustered (average linkage) heat map of TSA-induced changes in miRNA expression in BxPC-3 cells. Lane 1–2, untreated; Lane 3–4, TSA-treated. Red, significantly higher in treated cells; green, significantly lower in treated cells in comparison with untreated cells

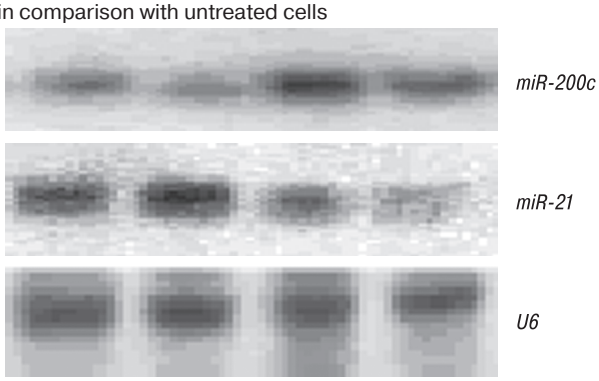

Fig. 4. Northern blots validating array results for miR-200c, which is up-regulated, and miR-21, which is down-regulated. Blots were normalized with a probe for U6. Lane 1–2, untreated; Lane 3–4, TSA-treated

DISCUSSION

Accumulating evidence is showing that the HADCs play an important role in the carcinogenesis, and HADCIs emerge as a new class of potential anticancer drugs, because they can induce the growth inhibition, cell cycle arrest and apoptosis in cancer cell as well as increase the sensitivity of cancer cell to chemotherapy and ionizing radiation. The mechanism of HDACIs action, however, can vary from cell line to cell line [18–21]. Mechanistic understanding of the antitumor programs initiated by HDACIs through the transcriptional regulation of key cellular genes, at both transcript and protein levels, remains a challenging problem.

TSA has been reported to have growth inhibition effect on pancreatic cancer cells, but the exact mechanisms have not been well studied. The present studies have shown that TSA has a dramatic effect on the viability and apoptosis of pancreatic cancer cells.

Several investigators have reported that epigenetic mechanisms such as DNA methylation and histone modifications, can affect the expression of miRNAs [22–25]. LAQ824, one of HDACIs, can lead to a rapid change in miRNA expression profile in breast cancer cell line SKBr3 [26]. In particular, miR-127, which can down-regulate BCL6, was found to be remarkably up-regulated in cancer cell lines after the treatment with 5-Aza-CdR, a potent DNA methylation inhibitor, and 4-phenylbutyric acid, a histone deacetylase inhibitor [27]. It was demonstrates that epigenetic drugs may exert their antitumor effects on two fronts: they not only turn on the tumor-suppressor genes that were aberrantly silenced epigenetically, but they also turn on tumor-suppressor miRNAs that down-regulate target oncogenic mRNAs. In contrast to these investigations, Diederichs *et al*. [28] did not find significant alterations in miRNA expression patterns following either DNA demethylation or HDAC inhibitor treatment in A549 lung cancer cells.

In our experiment, using miRNA microarray analysis, we found that 6 h treatment of cells with TSA induced altered expression of miRNAs: 29 miRNAs including miR-21, -181b, -181d, -221, -126, -375, let-7a, and let-7c were significantly down-regulated and miR-200c, -100, -34a, -146a, and -146b were up-regulated. By Northern blot analysis we showed that miR-200c was up-regulated and miR-21 was down-regulated following TSA treatment. miR-21 is found to be antiapoptotic, and it is up-regulated in hepatocellular cancer [29], breast cancer [30], and glioblastomas [31]. Considering the expression changes of miR-21 associated with TSA, we postulate that the post-transcriptional effects of HDACIs may play an important role in mediating their anticancer activity. Park *et al* [32] have demonstrated that increasing miR-200 levels may induce mesenchymal-to-epithelial transition and reduce the aggressiveness in human cancer cell lines.

In conclusion, the results of this study show that TSA is a potent HDACI that is effective in inhibiting the growth of pancreatic cancer cells *in vitro*. miRNA expression profiling of pancreatic cancer cell line treated with TSA should provide valuable information for further research on the therapeutic potential of histone modifications and miRNAs.

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ВЛИЯНИЕ ТРИХОСТАТИНА А НА ВЫЖИВАЕМОСТЬ КЛЕТОК РАКА ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ BxPC-3 И ЭКСПРЕССИЮ микроРНК

Цель: **изучить влияние трихостатина А (TSA) на ингибирование пролиферации клеток и индукцию апоптоза в клеточной линии рака поджелудочной железы человека.** ***Методы:*** **для оценки жизнеспособности клеток после их обработки TSA применяли основанный на MTT цитотоксический тест. Распределение клеток по фазам клеточного цикла и процент апоптических клеток определяли с помощью проточной цитофлуориметрии. Экспрессию микроРНК изучали с использованием микроРНК-чипа. Экспрессия miR-200c и miR-21 исследована с помощью Нозерн-блот анализа.** ***Результаты:*** **TSA значительно ингибировал пролиферацию клеток линии рака поджелудочной железы человека BxPC-3, и этот процесс зависел от времени инкубации и концентрации препарата. Клетки BxPC-3, обработанные TSA, были остановлены в G_0/G_1-фазе клеточного цикла, увеличивалось количество апоптотических клеток, что сопровождалось изменением экспрессии микроРНК.** ***Выводы:*** **полученные результаты позволяют предположить, что TSA может активировать экспрессию микроРНК, которые в свою очередь выступают онкосупрессорами опухоли в клетках линии рака поджелудочной железы BxPC-3.**

Ключевые слова: **рак поджелудочной железы, трихостатин А, микроРНК.**