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Original Article

Differential expression of glycoprotein non-metastatic melanoma protein B (GPNMB) involved in trichostatin A-induced apoptosis in gastric cancer

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Abstract: In this study, we investigated the effect of trichostatin A (TSA) on the gastric cancer cell line BGC-823.The effect of TSA on growth inhibition and apoptosis of BGC-823 cells was examined. The gene expression profile was determined by microarray. Western blotting was used to study the levels of acetylated histone H4 and Glycoprotein non-metastatic melanoma protein B (GPNMB) proteins. GPNMB gene expression was measured by real-time PCR. GPNMB protein levels in gastric adenocarcinoma tissues and adjoining normal tissues were detected by immunohistochemistry. The results showed that a significant decrease in cell population following treatment with 75 ng/mL TSA for 48 h (0.87 ± 0.04) as compared to control (1.14 ± 0.06) (P = 0.02). Apoptotic cells were increased in TSA (75 ng/mL for 48 h) treated group as compared to the control group (from 2.02% to 19.74%) by flow cytometry. The expression of acetylated histone H4 was increased in TSA treated (75 ng/mL for 48 h) group (from 1.00 ± 0.26 to 1.87 ± 0.33, F = 5.862, P = 0.0038) as compared to the control group by Western blotting. After 48 h TSA treatment (75 ng/mL), BGC-823 cells showed decrease in GPNMB gene expression (from 1.00 ± 0.21 to 0.59 ± 0.11, F = 6.214, P = 0.0018). Immunohistochemistry showed that GPNMB expression in gastric adenocarcinoma was significantly higher than the adjoining normal tissues (P = 0.000). To conclusion, our results support that TSA can induce apoptosis, and increase acetylated histone H4 in BGC-823 cells. GPNMB expression is decreased in BGC-823 cells after TSA treatment. GPNMB is overexpressed in gastric adenocarcinoma tissue. GPNMB involved in TSA-induced apoptosis might participate in gastric cancer.

Keywords: Trichostatin A, BGC-823, apoptosis, GPNMB, histone H4

Introduction

Gastric cancer is one of the most common types of cancer [1], and is frequently undetected in the early stage. Owing to local invasion and metastasis, patients usually need further treatment after surgery. However, radiation therapy or chemotherapy does not significantly increase the duration or quality of life in patients with advanced gastric cancer, and the 5-year survival rate is low (10-20%). Therefore, it is important to identify a new effective adjuvant treatment.

Epigenetics is a research hotspot in recent years. The reversible acetylation and deacetylation of the N-terminal histone tails by specific histone acetylases and deacetylases (HDAC) is involved in the regulation of gene expression. Trichostatin A (TSA) is a specific inhibitor of histone acetylation transferase. It can inhibit the growth of several types of malignant tumors at low concentration, and promote tumor cell apoptosis [2, 3]. But the mechanism of action of TSA on tumors is unclear. Given that TSA can induce apoptosis in different cell types, we detected apoptosis in gastric cancer cell line BGC-823 after TSA treatment. We also measured the level of acetylated histone H4. We examined changes in gene expression levels in BGC-823 cells treated with TSA in comparison with untreated control using microarray. Furthermore, real time-PCR and western blotting were performed to measure the outcome of the selected genes. Additionally, protein expression of the selected genes was detected by immunohistochemistry in gastric carcinoma tissue and adjoining normal tissue.

Materials and methods

Reagents and antibodies

Jing Xin ® cDNA amplification tag kit and 22K Human Genome Array were used obtained from CapitalBio Corporation (Beijing, China). TSA, propidium iodide (PI) and Hoechst33342 were purchased from Sigma-Aldrich (United Kingdom). High Capacity Reverse Transcriptase kit and ExScript™ RT-PCR kit were purchased from Applied Biosystems (CA, USA). Anti-Growth differentiation factor-15 (GDF-15) antibody and anti-rabbit antibody were purchased from Biosynthesis Biotechnology Co., Ltd (Beijing, China). Dimethylthiazol diphenyl tetrazolium bromide (MTT) and AEC (0.02% 3-amino-9-ethylcarbazole) were obtained from Zhongshan Goldnbridge Biotechnology Co., Ltd (Beijing, China).

TSA, propidium iodide (PI) and Hoechst33342 were purchased from Sigma-Aldrich (United Kingdom). Jingxin ® cDNA amplification tag kit and 22K Human Genome Array were used obtained from CapitalBio Corporation (Beijing, China). Anti-GAPDH and anti-acetyl-histone H4 antibodies were purchased from Upstate Biotechnology (USA). Trizol, Applied Biosystems High Capacity Reverse Transcriptase kit and ExScript™ RT-PCR kit were obtained from Invitrogen (USA) and Applied Biosystems (USA), respectively. Anti-human GPNMB polyclonal antibody and secondary antibody, and 0.02% 3-amino-9-ethylcarbazole (AEC) were purchased from Biosynthesis Biotechnology Co. Ltd (China) and Zhongshan Goldenbridge Biotechnology Co. Ltd (China), respectively.

Cell culture and treatments

Human gastric epithelial cell line BGC-823 was provided by the Institute of Tumor Research of Hei long-king. The cell line was cultured in RPMI-1640 supplemented with 10% (v/v) fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in humidified 5% CO_2.

MTT assay

Cells were seeded in 96-well plates at a predetermined optimal cell density to ensure exponential growth for the duration of the assay. After 24 h incubation, growth medium was replaced with experimental medium containing TSA or medium alone as a control. Six duplicate wells were set up for TSA or control. Treatment was conducted for 12, 24, 48 and 72 h with final TSA concentrations of 37.5, 75, 150, 300 and 600 ng/mL, respectively. After incubation, 10 μL MTT (from 6 g/L stock solution) was added to each well for 4 h at 37°C. After removal of the medium, MTT stabilization solution (dimethyl sulphoxide:ethanol = 1:1) was added to each well, and the plates were shaken for 10 min until all crystals were dissolved. Then, optical density (OD) was detected using a microplate reader at 550 nm. The negative control well had no cells, and was used as zero absorbance. Each assay was performed in triplicate. Data analyses were performed using the SPSS software (version 13.0). $P < 0.05$ was considered to be statistically significant.

Detection of chromatin condensation

Chromatin condensation was detected by nuclear staining with Hoechst 33342. BGC-823 cells were collected by centrifugation (500 g for 5 min at 4°C), and washed twice with PBS. Cells were fixed in 10% formaldehyde, and stored at 4°C. For analysis, cells were washed in PBS, and Hoechst 33342 (5 mg/L) was directly added to the medium by gentle shaking at 4°C for 5 min. Stained nuclei were visualized with a Zeiss Axiophot fluorescence microscope at 400× magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm. Four independent replicates were used. Apoptotic cells appeared bright blue due to their chromatin condensation, while normal cells appeared light blue.

Apoptosis assays

BGC-823 cells were treated as indicated. Floating and adherent cells were collected by centrifugation (500× g for 5 min at 4°C), and washed twice with PBS. Cells were fixed in 90% ethanol, and stored at -20°C. For analysis, cells were washed in PBS, and stained by resuspending in PI (50 μg/mL) containing RNase A (2 μg/mL) for 30 min at 4°C. Stained cells were analyzed on a FACScan (Becton-Dickinson, Germany).

Western blotting

Cells were treated as indicated, harvested in 5 mL of medium, pelleted by centrifugation

(1000× g for 5 min at 4°C), washed twice with ice-cold PBS, and lysed in ice-cold HEPES buffer [50 mM HEPES (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, a cocktail of protease inhibitors and 1 μg/mL TSA] on ice for 30 min. Lysates were clarified by centrifugation (15000× g for 10 min at 4°C), and the supernatants were either analyzed immediately or stored at -80°C. Equivalent amounts of protein (50 μg) from total cell lysates were resolved by SDS-PAGE using precast 12% Bis-Tris gradient gels, and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked overnight at 4°C in blocking buffer [5% (v/v) nonfat dried milk, 150 mM NaCl, 10 mM Tris (pH 8.0) and 0.05% (v/v) Tween 20]. Proteins were detected by incubation with appropriate dilutions of primary antibodies (anti-acetylated histone H4 and anti-GPNMB; 1:1000) in blocking buffer overnight at 4°C. Unbound antibody was removed by washing with Tris-buffered saline (pH 7.2) containing 0.5% Tween 20 (TBS-T). The membrane was then incubated at room temperature with horseradish peroxidase-conjugated secondary antibody. After extensive washing with TBS-T, bands were visualized by enhanced chemiluminescence followed by exposure to autoradiography film. The activity was expressed as integral optical density (IOD) ratio, and hybridization signals were analyzed with Lab works software (UVP, INC). Data analyses were performed with the SPSS software (version 13.0). $P < 0.05$ was considered to be statistically significant.

Microarray

Trizol one-step extraction of total RNA from the cells was performed according to the manufacturer's protocol. RNA concentrations and integrity were quantified by spectrophotometer and formaldehyde degeneration electrophoresis. Microarray was performed by CapitalBio corporation (China) with LuxScan 10Ka two-channel laser scanner (CapitalBio corporation). The microarray experiment was repeated three times, and the data was viewed using LuxScan image analysis software 3.0 (CapitalBio corporation). Data was analyzed by SAM (Significance Analysis of Microarrays, ArrayVision 7.0) software (Stanford University). False discovery rate (FDR) control within 5% and $P < 0.05$ were considered to be statistically significant.

RT-qPCR

Reverse transcription of RNA to cDNA was performed with Applied Biosystems High Capacity Reverse Transcriptase kit (USA). Subsequently, quantitative PCR (qPCR) was performed on the resulting cDNA. Primers for qPCR were designed with Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast).

All primers were synthesized by Genechem chemical technology co. Ltd. (China). Primer sequences are as follows: (1) GPNMB Forward: 5'-ACAAGGAATACAACCCAATA-3', GPNMB Reverse: 5'-ATAGCCACTCCAGCACA-3'. (2) GAPDH Forward: 5'-TGACTTCAACAGCGACACCCA-3', GAPDH Reverse: 5'-CACCCTGTTGCTGTAGCCAAA-3'. Amplicon sizes were 221 bp and 121 bp, respectively. Briefly, each PCR reaction mix contained 5 μL of Applied Biosystems SYBR ® green PCR master mix, 2.5 μL of 3 μM forward and reverse primer mix, 0.5 μL of RNAse-free H_2O, and 2 μL of 2 ng/μL cDNA. Reactions were performed in a 384-well optical reaction plate (Applied Biosystems), and fluorescence was quantified in real-time with the Applied Biosystems 7300 Real Time PCR System (USA) under the following conditions: 50°C for 2 min, 95°C for 10 min, (95°C for 15 sec, 60°C for 30 sec) ×45 cycles. Melt curves were calculated for every reaction. Relative mRNA expression was calculated by the comparative ΔΔCt method, using GAPDH as a reference transcript. Statistical differences in mRNA levels were determined with an unpaired, two-tailed Student's t-test using Graphpad Prism 4.0. $P < 0.05$ was considered to be statistically significant.

Immunohistochemistry

100 cases of advanced gastric adenocarcinoma and 5 cm normal gastric tissue adjacent to each carcinoma specimen were derived from surgically resected specimens collected from 2011 to 2013, and archived by our pathology department. All samples were fixed in 10% formaldehyde and embedded in paraffin. Each Hematoxylin and Eosin (H&E) stained sample was reviewed by a board-certified pathologist to confirm its histological consistency with advanced gastric adenocarcinoma, and also confirm that the adjoining normal specimens contained no tumor cells. All staining was performed according to the SP immunohistochemi-

Table 1. Proliferation of BGC-823 cells incubated with various concentrations of TSA for 72 h (mean ± SD)

TSA	1	2	3	4	5	control
BGC-823	0.82 ± 0.08[1]	0.76 ± 0.09[1]	0.72 ± 0.10[1]	0.15 ± 0.03[2]	0.15 ± 0.02[2]	1.13 ± 0.07

[1]*P* = 0.02 *vs* control; [2]*P* = 0.005 *vs* control. 1: 37.5 ng/mL; 2: 75 ng/mL; 3: 150 ng/mL; 4: 300 ng/mL; and 5: 600 ng/mL.

Table 2. Proliferation of BGC-823 cells incubated with 37.5 ng/mL and 75 ng/mL TSA for 12, 24, 48, and 72 h (mean ± SD)

TSA	12 h	24 h	48 h	72 h	control
37.5 ng/mL	1.13 ± 0.10	1.13 ± 0.09	1.12 ± 0.06	0.84 ± 0.06[1]	1.14 ± 0.06
75 ng/mL	1.12 ± 0.04	1.11 ± 0.02	0.87 ± 0.04[1]	0.75 ± 0.13[1]	

[1]*P* = 0.02 *vs.* control.

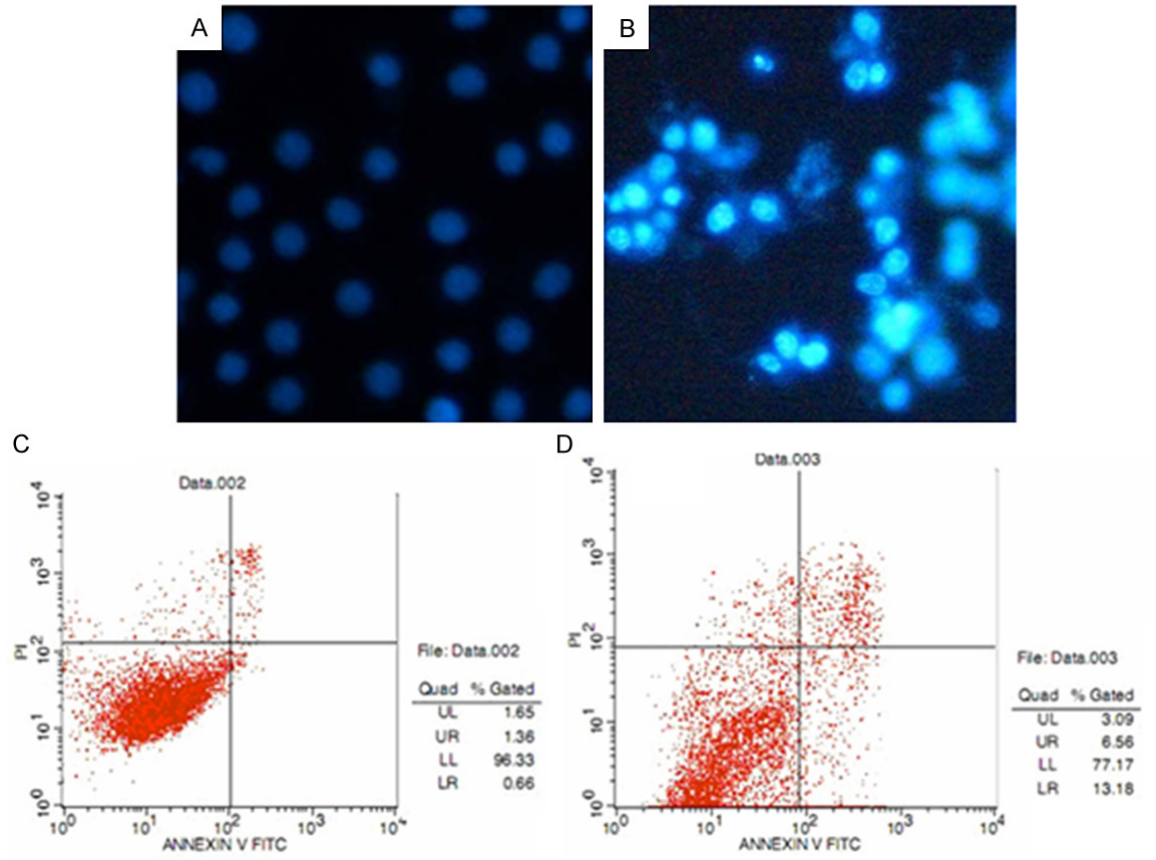

Figure 1. After 48 h, BGC-823 cells treated with (75 ng/mL) or without TSA were stained by Hoechst 33342 to test nuclei morphology. The result indicated that nuclei of most control group cells were stained into average slightly blue (A), while cells treated with TSA were stained into highly condensed, brightly staining nucleus (B). TSA treatment (75 ng/mL/48 h) sensitively induced apoptosis of BGC-823 cells, which was demonstrated by the increment of apoptosis rates in flow cytometry. Apoptotic cells were increased from 2.02% (C) to 19.74% (D) in TSA treated group as compared to the control group.

cal staining procedure. GPNMB staining was primarily in the cytoplasm. Positioning results were based on judging the positive cells by a semi-quantitative method [4], with percentage and staining intensity assessment for positive expression. Based on the percentage of positive tumor cells, < 5% was graded as 0 point, 5-25% as 1 point, 26-50% as 2 points, 51-75% as 3 points, and > 75% as 4 points. The intensity of staining was rated as no color = 0 point,

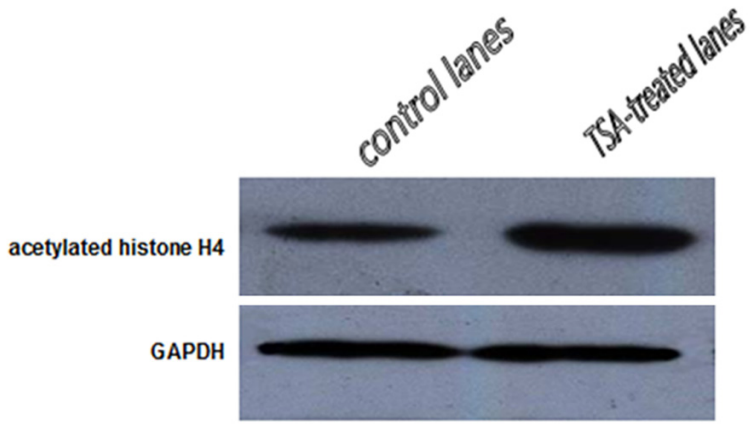

Figure 2. TSA (75 ng/mL for 48 h) upregulated the level of acetylated histone H4 protein in BGC-823 cells. The expression of acetylated histone H4 was increased from 1.00 ± 0.26 to 1.87 ± 0.33 in TSA treated group, as compared to the control group by western blotting (F = 5.862, P = 0.0038).

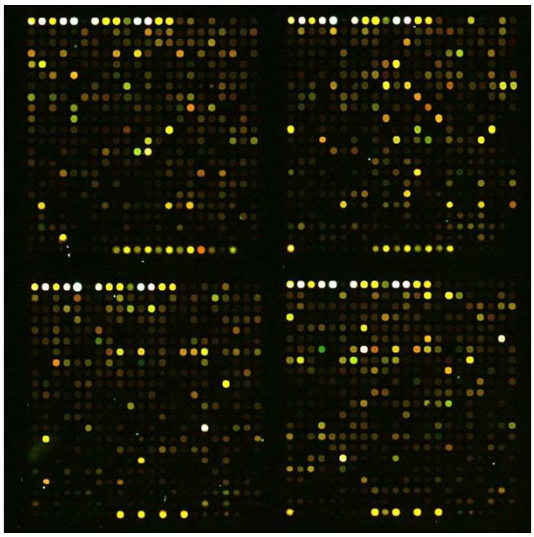

Figure 3. Microarray hybridization pseudo color chart. (Red: expression increased; green: expression decreased). Each array contained 25122 probes, of which 20449 correlated with unique genes. Expression of 82 genes was decreased in BGC-823 cells after treatment with 75 ng/mL TSA for 48 hours, of which GPNMB expression was significantly lowered. The mean fold change in GPNMB was 0.4431 (0.4232, 0.4503, 0.4558, respectively). *P*-value was 0.0372.

pale tan = 1 point, tan = 2 points, deep palm red = 3 points. Scores were classified as ≤ 2 = negative, and > 2 = positive. Qualitative data were analyzed using χ^2 test. Analyses were performed with the SPSS software (version 13.0). $P < 0.05$ was considered to be statistically significant.

Results

TSA inhibited the proliferation of BGC-823 cells

TSA inhibited cellular proliferation and survival of BGC-823 cells, and a significant decrease in cell population following treatment with 75 ng/mL TSA for 48 h has compared to control was observed (**Tables 1**, **2**).

TSA induced apoptosis of BGC-823 cells

To investigate the effects of TSA-induced cytotoxicity, morphological changes related to apoptosis were observed with a fluorescence microscope. At 48 h, BGC-823 cells treated with or without TSA were stained by Hoechst 33342, a classical method for identifying apoptotic cells, to test nuclei morphology. The results indicated that most cells treated with TSA showed highly condensed, brightly stained nuclei, while the cells in the control group showed normal slightly blue nuclei (**Figure 1A**, **1B**). TSA treatment (75 ng/mL for 48 h) induced apoptosis of BGC-823 cells, which was demonstrated by the increase in apoptosis rates (from 2.02% to 19.74%) by flow cytometry (**Figure 1C**, **1D**).

TSA Up-regulated the level of acetylated histone H4 in BGC-823 cells

Western blotting was used to detect the level of acetylated histone H4 protein in BGC-823 cells treated with 75 ng/mL TSA for 48 h. Results showed that TSA induced an increase of acetylated histone H4 protein in BGC-823 cells (from 1.00 ± 0.26 to 1.87 ± 0.33, F = 5.862, P = 0.0038) (**Figure 2**).

TSA treatment could inhibit GPNMB expression

Each array contained 25122 probes, of which 20449 correlated with unique genes. Expression of 82 genes was decreased in BGC-823 cells after treatment with 75 ng/mL TSA for 48 hours (**Figure 3**), of which GPNMB expression was significantly lowered. The mean fold change in GPNMB was 0.4431 (0.4232,

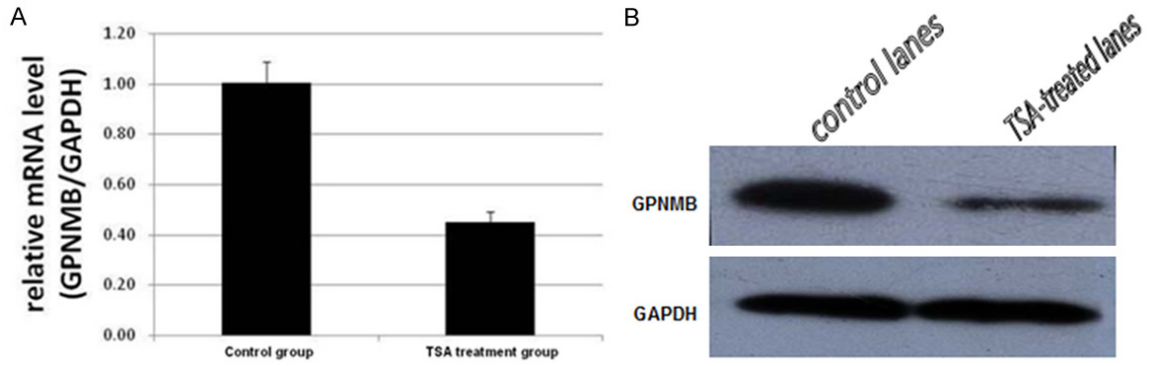

Figure 4. qRT-PCR and Western blot data shown expression of GPNMB was decreased with TSA group (75 ng/mL for 48 h) in BGC-823 cell. qRT-PCR data analyses shown that $2^{-\Delta\Delta Ct}$ average value was 1.002 in control and 0.450 in TSA-treated group, and standard deviation was 0.0834 for control and 0.0396 for TSA group ($P < 0.05$) (A). Western blot data shown The expression of GPNMB gene was decreased from 1.00 ± 0.21 to 0.59 ± 0.11 in control group, as compared to TSA treated group ($F = 6.214$, $P = 0.0018$) (B).

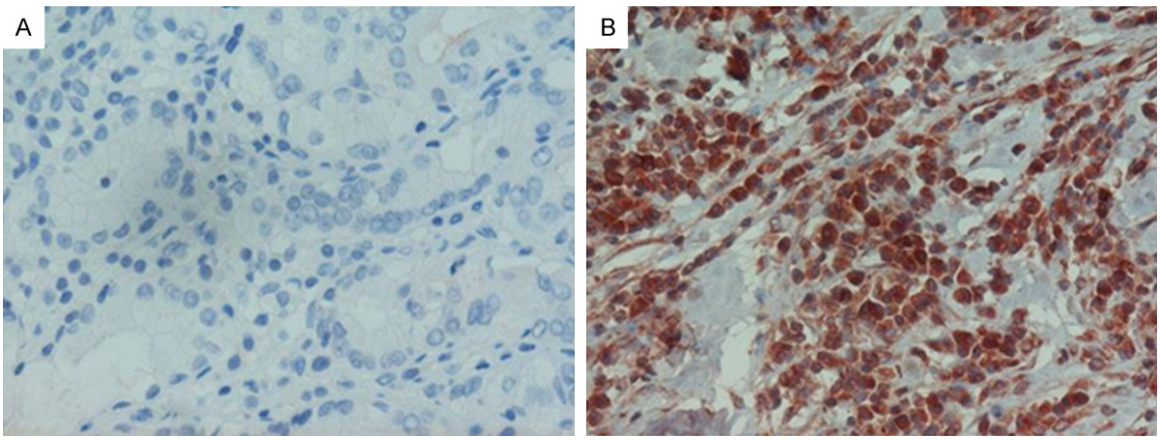

Figure 5. Expression of GPNMB was higher in gastric adenocarcinoma tissues than that in normal tissues (magnification × 400). A GPNMB-negative case containing only rare positive cells (Scores ≤ 2) is shown in (A). (B) show a positive cases (Scores > 2), in which definitive cytoplasm staining was detectable.

0.4503, 0.4558, respectively). q-value was 0.0372. $P < 0.05$ was considered to be statistically significant.

Downregulation of GPNMB gene expression was detected in BGC-823 cells after treatment with 75 ng/mL TSA for 48 hours, and the difference was statistically significant. qRT-PCR data analyses showed that $2^{-\Delta\Delta Ct}$ average value was 1.002 in control and 0.450 in TSA-treated group, and standard deviation was 0.0834 for control and 0.0396 for TSA group ($P < 0.05$) (**Figure 4A**). Western blot data shown The expression of GPNMB gene was decreased from 1.00 ± 0.21 to 0.59 ± 0.11 in control group, as compared to TSA treated group ($F = 6.214$, $P = 0.0018$) (**Figure 4B**).

Expression of GPNMB was increased in gastric adenocarcinomas

Positive expression of GPNMB was found in 70 out of 100 (70%) gastric adenocarcinoma specimens. In contrast, only 5 out of 100 (5%) normal tissue specimens showed positive expression. Therefore, GPNMB expression was significantly higher in gastric adenocarcinoma as compared to adjoining normal tissue (χ^2 value 41.835, $P = 0.000$ and $P < 0.05$) (**Figure 5**).

Discussion

As an antifungal drug, the role of TSA in epigenetics has been previously investigated. Our study showed that TSA induced apoptosis, and promoted the expression of acetylated histone

H4 in gastric cancer cell line BGC-823. After treatment with 75 ng/mL TSA for 48 hours, BGC-823 cells showed downregulation of GPNMB gene expression. The protein level of GPNMB was also decreased in BGC-823 cells treated with TSA. Immunohistochemistry showed that the expression of GPNMB in gastric adenocarcinoma was significantly higher as compared to adjoining normal tissues.

Acetylation, methylation, and phosphorylation are suggested to be involved in the regulation of gene expression, cell division, nucleosome assembly and DNA repair processes via alterations in the nucleosome architecture [5, 6]. Acetylation of histones is predominantly implicated in the regulation of gene transcription due to nucleosome remodeling [7]. Transcription is controlled by the acetylation of histones, which is controlled by the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities [8]. TSA is a potent and specific inhibitor of HDAC activity. Numerous studies have reported that TSA can induce apoptosis in cancer cells [9-11]. We found that TSA can significantly reduce proliferation and induce apoptosis in BGC-823 cells.

Accumulating evidence suggests that inhibition of HDAC activity leads to relaxation of chromatin structure associated with a specific set of programmed genes. The relaxed chromatin structure allows these genes to be expressed, which, in turn, arrests tumor cell growth [12-14]. These findings suggest that induction of histone hyperacetylation by HDAC inhibitors is responsible for their antiproliferative activity through selective induction of genes that play important roles in the cell cycle and morphology [15].

Reversible acetylation of lysine on histone H4 was shown to play an important role in regulating gene transcription [16]. Our findings show that acetylated histone H4 expression levels increased in BGC-823 cells following TSA treatment. Increased acetylated histone H4 expression levels could be important in mediating the apoptosis induced by TSA. These findings led us to investigate the mechanism of action of TSA in BGC-823 cells. We used microarray technology to detect GPNMB gene expression in BGC-823 cells after treatment with TSA, and found that GPNMB expression was significantly decreased. In order to investigate the expression of GPNBM gene *in vivo*, clinical specimens of advanced gastric cancer were examined and GPNMB expression in gastric carcinoma tissue was found to be significantly higher as compared to adjoining normal tissue.

GPNMB, also known as osteoactiven, is a type I transmembrane protein, which was identified in 1995 in a human melanoma cell line [17, 18]. Its structure comprises of extracellular, transmembrane and cytoplasmic regions [19]. It is widely expressed in various normal tissues [20, 21], and participates in physiological functions of cells. GPNMB is also involved in several diseases. Higher expression of GPNMB gene was found in tumor cells, including glioma in melanoma [22, 23], neuroglioma [19], liver tumors [24], breast tumors [25, 26] and colorectal tumors [27]. GPNMB is considered to be a potential target for the treatment of malignancies [28]. Solinas G et al. [29] showed that treatment of macrophages with tumor-cell conditioned media induced a 83-fold increase in GPNMB expression. These tumor-conditioned macrophages adopted a phenotype similar to M2-type macrophages [29, 30], which are known to promote tumor progression [31, 32]. Additionally, GPNMB can inhibit the activation of tumor associated T cells, which enable cancer cells to evade immune recognition [33]. Furochi H et al. [34] and Ogawa T et al. [35] have reported that GPNMB can activate fibroblasts by inducing upregulation of pro-invasive matrix metalloproteases, such as MMP-3 and MMP-9, via Erk-dependent signaling. These studies suggest that GPNMB helps in sustaining the tumor microenvironment. It remains to be seen if stromal GPNMB can directly influence tumor progression [36]. GPNMB-positive tumors are characterized by a high endothelial cell density as compared to tumors that lack GPNMB. *In vitro* studies have revealed that soluble GPNMB ECD is biologically active since it is capable of inducing endothelial cell migration [37]. These data suggest that GPNMB could regulate the ability of tumor cells to recruit vasculature to facilitate tumor growth and metastasis. The role of GPNMB in malignant tumors has been studied. A GPNMB-targeted therapeutic agent glembatumumab vedotin is an antibody-drug conjugate, which is also known as CR001-vcMMAE (CR001) [38]. Upon GPNMB binding and internalization, the drug is released and induces cell cycle arrest and apoptosis of the target cells.

Our findings showed that TSA induces apoptosis, increases histone H4 acetylation level, and inhibits expression of GPNMB in BGC-823 cells. It would be interesting to see if these phenomena are related to each other. We hypothesize that increase in histone H4 acetylation levels induces chromatin structural changes, which influences the expression of genes. This could be one of the reasons for apoptosis of BGC-823 cells after TSA treatment. GPNMB gene expression in advanced gastric carcinoma tissue was found to be significantly higher as compared to adjoining normal tissue. These findings suggest that GPNMB could play a role in gastric cancer.

In summary, TSA can induce apoptosis, and increase acetylated histone H4 level in BGC-823 cells. GPNMB expression is decreased in BGC-823 cells after TSA treatment. Additionally, GPNMB is overexpressed in gastric adenocarcinoma tissue. These support that GPNMB involved in Trichostatin A-induced apoptosis might participate in gastric cancer.

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Disclosure of conflict of interest

None.

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