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Original Article

Downregulation of growth differentiation factor-15 in trichostatin A-induced apoptosis could play a role in progression of gastric cancer

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Abstract: Aim: To investigate the effect of trichostatin A (TSA) on gastric cancer cell line BGC-823, and identify the differentially expressed genes induced by TSA, which might participate in the progression of gastric cancer. Methods: MTT, fluorescence microscopy, and flow cytometry were used to detect the effect of TSA on growth inhibition and apoptosis of BGC-823 cells. Using gene microarray, we analyzed the changes in gene expression. Change in growth differentiation factor-15 (GDF-15) was verified by qRT-PCR and Western blotting. The expression of GDF-15 in gastric cancer and adjacent normal tissues was detected by immunohistochemistry. Results: Apoptosis of BGC-823 cells induced by TSA (75 ng/mL for 48 h) was demonstrated by flow cytometry. There were significant variations between TSA treated groups and control groups ($P = 0.02$). Nuclear chromatin condensation and fluorescence intensity were observed by fluorescence microscopy. GDF-15 gene expression and protein level were significantly reduced in the TSA treated group (75 ng/mL for 48 h). Immunohistochemistry demonstrated that the expression of GDF-15 in gastric adenocarcinoma was significantly higher than in the surrounding normal tissues ($P < 0.05$). Conclusion: Lower GDF-15 gene expression due to TSA-induced apoptosis was found in gastric cancer cell line BGC-823. Higher GDF-15 gene expression was seen in gastric adenocarcinoma tissues.

Keywords: Gastric cancer, trichostatin A, BGC-823, apoptosis, growth differentiation factor-15

Introduction

Gastric cancer is a major threat to human health [1] with a low 5-year survival rate due to local invasion and metastasis. Epigenetics is a research hotspot and genetic changes in regulatory regions have been reported to play an important role in the susceptibility to certain type of diseases [2, 3]. Trichostatin A (TSA), a potent inhibitor of histone acetylases and deacetylases (HDAC), could inhibit proliferation and induce apoptosis of tumor cells [4-6].

Given that TSA can induce apoptosis in different cell types, we examined its apoptotic potential in the human gastric cancer cell line BGC-823. We also investigated the changes in gene expression in BGC-823 cells using gene microarray and real-time PCR analyses. Gene expression in human gastric adenocarcinoma tissue and normal adjacent tissue was examined by immunohistochemical assay to understand the mechanism by which TSA induces apoptosis in gastric cancer.

Material and methods

Reagents and antibodies

Jing xin ® cDNA amplification tag kit and 22K Human Genome Array were obtained from CapitalBio Corporation (Beijing, China). TSA, propidium iodide (PI) and Hoechst 33342 were purchased from Sigma-Aldrich (United Kingdom). High Capacity Reverse Transcriptase kit and ExScript™ RT-PCR kit were purchased from Applied Biosystems (USA). Anti-GDF-15 and anti-rabbit antibodies were purchased from Biosynthesis Biotechnology Co., Ltd (Beijing, China). Dimethylthiazol diphenyltetrazolium bromide (MTT) and 0.02% 3-amino-9-ethylcarbazole (AEC) were obtained from Zhongshan

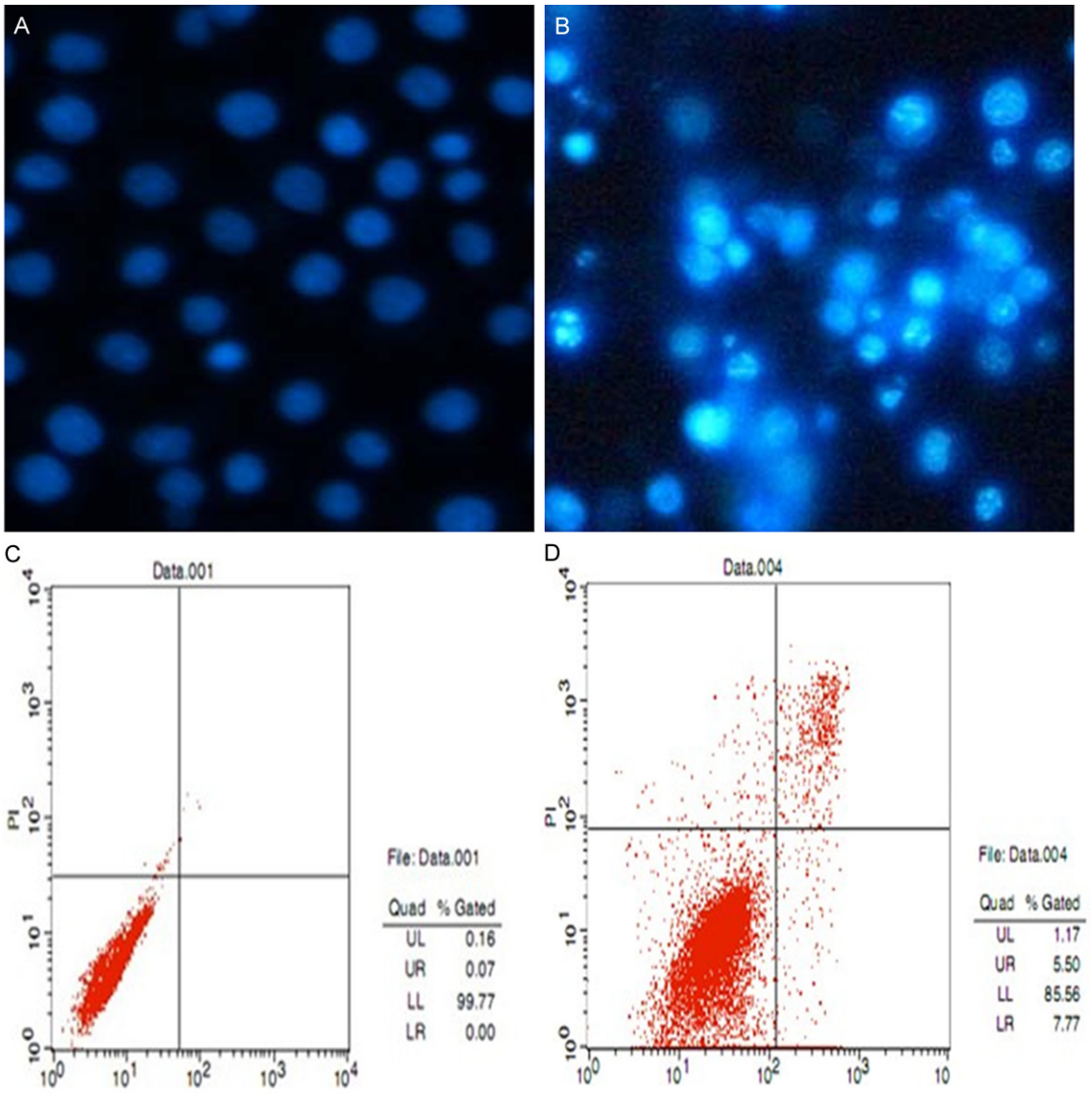

Figure 1. After 48 h, BGC-823 cells treated with (75 ng/mL) or without TSA were stained by Hoechst 33342 to test nuclear morphology. The result indicated that nuclei of most control group cells were stained into average slightly blue (A), while cells treated with TSA were stained into highly condensed, 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 cytometric. Apoptotic cells were increased from 0.07% (C) to 13.27% (D) in TSA treated group as compared to the control group.

Goldenbridge Biotechnology Co., Ltd (Beijing, China).

Cell culture and treatments

Human gastric epithelial carcinoma cell line BGC-823 was provided by the Institute of Tumor Research of Heilongjiang Province, and cultured in RPMI-1640 medium 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

The number of living cells with or without TSA treatment was detected by MTT assay. Cells were seeded in 96-well plates at an optimal density to ensure exponential growth for the duration of the assay. Six duplicate wells were set up for each sample. Treatment was conducted for 12 h, 24 h, 36 h, 48 h and 72 h with final TSA concentrations of 37.5 ng/mL and 75 ng/mL, respectively. MTT (6 g/L) 10 μL was added to each well for 4 h. After removal of the

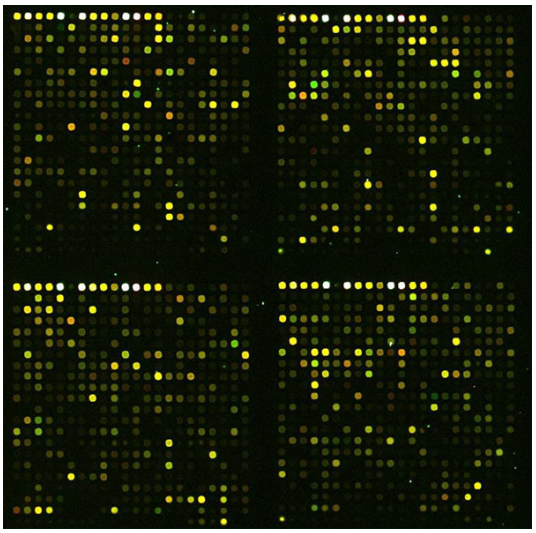

Figure 2. Microarray hybridization pseudo color chart. (Red: expression increased; green: expression decreased). Each array contained 25122 probes, of which 20449 correlated with unique genes. Expression of 82 genes was decreased in BGC-823 cells after treatment with 75 ng/mL TSA for 48 hours, of which GDF-15 expression was significantly lowered. The mean fold change in GDF-15 was 0.4583 (0.4448, 0.4534, 0.4632, respectively). *P*-value was 0.0218.

medium, MTT stabilization solution (dimethylsulphoxide: ethanol = 1:1) was added to each well, and shaken for 10 min until all crystals were dissolved. Optical density (OD) was detected at 550 nm wavelength using an ELISA microplate reader. Each assay was performed in triplicate. Data analyses were performed with AVONA. $P < 0.05$ was considered to be statistically significant.

Detection of chromatin condensation

Chromatin condensation was detected by nuclear staining with Hoechst 33342. BGC-823 cells were pelleted 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 with 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 performed.

Apoptosis assay

To detect apoptosis, cells were trypsinized, washed and fixed in ice-cold ethanol at 4°C for at least 12 h. The cells were permeabilized, stained with PI for 20 min, and subjected to FACS analysis using FACSCalibur Analytic flow cytometer.

Microarray

Total RNA from BGC-823 cells treated with or without TSA was isolated with Trizol according to the manufacturer's protocol, and then used for microarray analysis with LuxScan 10KA two-channel laser scanner and LuxScan image analysis software 3.0 (CapitalBio Corporation, Beijing, China). Microarray experiments were repeated three times. Each array contained 25122 probes, of which 20449 correlated with unique gene symbols. Data obtained from the microarray were analyzed by Significance Analysis of Microarrays (SAM), ArrayVision 7.0 software (Stanford University). False discovery rate (FDR) control was within 5%. $P < 0.05$ was considered to be statistically significant.

Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR)

Total RNA was transcribed to cDNA with Applied Biosystems High Capacity Reverse Transcriptase kit. Primers for qPCR were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized by Genechem chemical technology Co., LTD (Shanghai, China). The primers were as follows: GDF-15: 5'-GTTGCGGAAACGCTACGA-3' (F), 5'AACAGAGCCCGGTGAAGG-3' (R); GAPDH: 5'-TGACTTCAACAGCGACACCCA-3' (F), 5'-CACCCTGTTGCTGTAGCCAAA-3' (R). Amplicon sizes were 210 bp and 121 bp, respectively. The PCR reaction mix per reaction 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 RNAse-free H_2O, and 2 μl of 2 ng/μl cDNA. Reactions were seeded in a 384-well optical reaction plate (Applied Biosystems) and fluorescence was quantified in real-time with the 7300 Real-Time PCR System (Applied Biosystems, 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

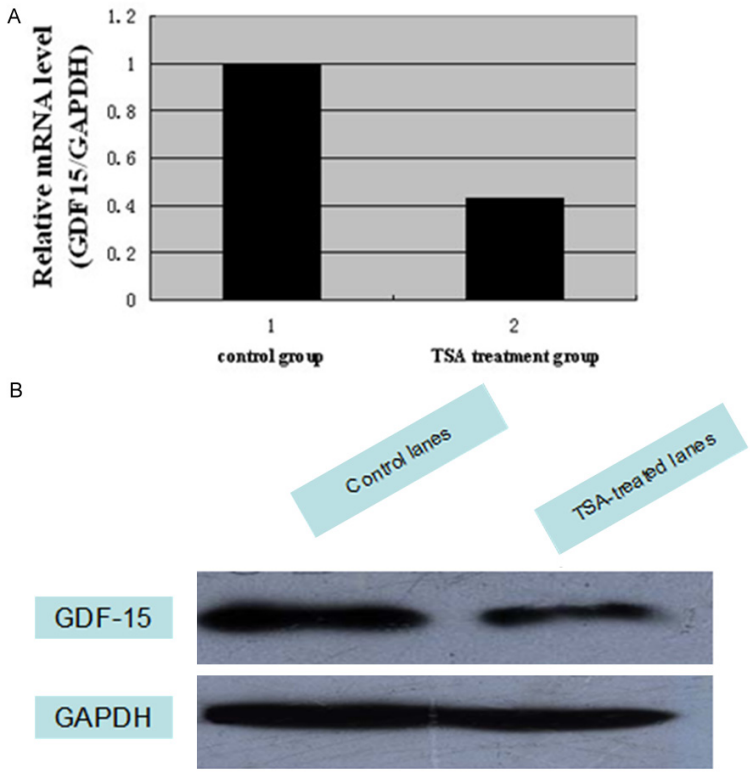

Figure 3. qRT-PCR and Western blot data shown expression of GDF-15 was decreased with TSA group (75 ng/mL for 48 h) in BGC-823 cell. qRT-PCR data analyses shown that $2^{-\Delta\Delta Ct}$ average value was 1.000 in control and 0.430 in TSA-treated group ($P < 0.05$) (A). Western blot data shown The expression of GDF-15 gene was decreased from 1.00 ± 0.20 to 0.59 ± 0.10 in control group, as compared to TSA treated group ($F = 6.214$, $P = 0.0018$) (B).

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.

Western blotting

Cells were harvested in 5 ml 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] and then incubated with anti-GDF-15 antibody at appropriate dilutions (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 membranes were then incubated at room temperature with horseradish peroxidase-conjugated secondary antibody. After extensive washing with TBS-T, the bands were visualized by enhanced chemiluminescence followed by exposure to autoradiography film.

Immunohistochemistry

One hundred cases each of advanced gastric adenocarcinoma and normal gastric tissue within 5 cm of carcinoma specimens were obtained from the pathology department of the Affiliated Hospital of Harbin Medical University from the archived surgical specimens from 2011 to 2013. All samples were fixed in 10% formaldehyde and embedded in paraffin. Each hematoxylin and eosin (H&E) stained sample was reviewed by two board-certified pathologists to confirm the specimen's histological consistency with advanced gastric adenocarcinoma or the absence of tumor cells in the adjacent normal specimens. The staining was performed according to the SP immunohistochemical staining procedure. GDF-15 staining was mainly seen in the cytoplasm. Positioning results was based on the assessment of positive cells by semi-quantitative method [7], as confirmed by positive cell percentage and staining intensity. According to the percentage of positive tumor cells, < 5% was 0 point, 5-25% was 1 point, 26-50% was 2 points, 51-75% was 3 points, > 75% was 4 points; According to staining inten-

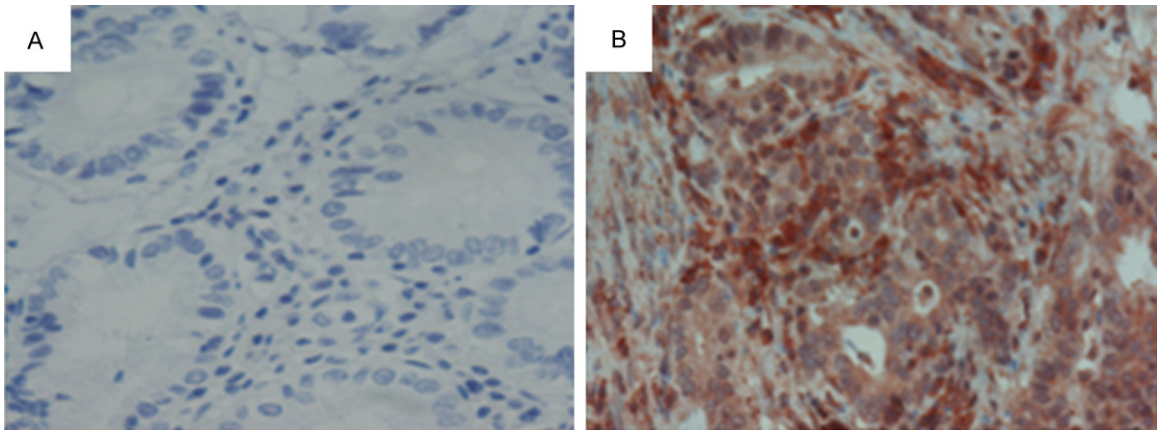

Figure 4. Expression of GDF-15 was higher in gastric adenocarcinoma tissues than that in normal tissues (magnification × 400). A GDF-15-negative case containing only rare positive cells (Scores ≤ 2) is shown in (A). (B) Shows a positive cases (Scores > 2), in which definitive cytoplasm staining was detectable.

Table 1. Inhibition effect of different concentrations of TSA conducted on BGC-823 gastric cancer cells. A. Cell proliferation of BGC-823 incubated with various concentrations of TSA for 72 h (mean ± SD). B. Cell proliferation of BGC-823 (37.5 ng/ml, 75 ng/ml) for12, 24, 48, and 72 h (mean ± SD)

A.						
TSA	1	2	3	4	5	Control
BGC-823	0.81±0.07[1]	0.75±0.08[1]	0.72±0.09[1]	0.14±0.03[2]	0.14±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; 5:600 ng/ml.						
B.						
TSA	12 h	24 h	48 h	72 h	Control	
37.5 ng/ml	1.13±0.08	1.13±0.06	1.12±0.06	0.82±0.061	1.14±0.04	
75 ng/m[l]	1.12±0.05	1.12±0.02	0.86±0.041	0.75±0.101		
[1]*P*=0.02 *vs.* control.						

sity rating, no color was 0 point, pale tan was 1 point, tan was 2 points, deep palm red was 3 points. For scores, ≤ 2 score was negative, > 2 score was positive. Qualitative data were analyzed using χ^2 test. $P < 0.05$ was considered to be statistically significant.

Results

TSA inhibited the proliferation of BGC-823 cells

BGC-823 cells were treated with 37.5 ng/mL or 75 ng/mL of TSA for 12 h, 24 h, 36 h, 48 h or 72 h, respectively. MTT assay showed a decline in live cell number with increasing concentration of TSA. The inhibition of cell growth was more obvious with 75 ng/mL TSA for 48 h, and the live cell number was 0.86 ± 0.04 as compared to the control group (1.14 ± 0.04, $P < 0.05$) (**Table 1**).

TSA induced apoptosis in BGC-823 cells

To investigate the effects of TSA on cytotoxicity, morphological changes of apoptosis were observed with fluorescence microscopy. At 48 h, BGC-823 cells treated with or without TSA were stained with Hoechst 33342 to test the nuclear morphology. The cells treated with TSA showed highly condensed, brightly stained nuclei; while the control group cells showed normal, slightly blue nuclei (**Figure 1A**, **1B**). TSA treatment (75 ng/mL for 48 h) induced apoptosis of BGC-823 cells, as demonstrated by the increase in apoptosis rates (from 0.07% to 13.27%) by flow cytometry analysis (**Figure 1C**, **1D**).

TSA treatment could inhibit GDF-15 expression

Decreased expression of 82 genes was seen in BGC-823 cells treated with 75 ng/mL TSA after

48 h by microarray (**Figure 2**). GDF-15 gene expression in BGC-823 cells was significantly decreased. Mean fold change in GDF-15 gene was 0.4583 (0.4448, 0.4534, 0.4632, respectively) and Q-value was 0.0218. $P < 0.05$ was considered to be statistically significant.

To verify the changes in GDF-15 expression with TSA treatment, we performed qRT-PCR with BGC-823 cells after 75 ng/mL TSA treatment for 48 h. Significant downregulation of GDF-15 gene expression was detected. 2-ΔΔCt average values were 1.000 vs 0.430 (control group vs TSA treated group) and $P = 0.0085$ by t-test (**Figure 3A**). Downregulation of GDF-15 protein expression was found in BGC-823 cells after 75 ng/mL TSA treatment for 48 h by Western blotting (1.00 ± 0.20 vs 0.59 ± 0.10, F = 6.214, $P = 0.0018$) (**Figure 3B**), which confirmed that TSA could inhibit GDF-15 expression.

Expression of GDF-15 was increased in gastric carcinoma

Positive expression of GDF-15 was found in 78 out of 100 (78%) gastric adenocarcinoma tissues. In the adjacent normal tissue, 25 out of 100 (25%) cases showed positive expression. GDF-15 was significantly higher in gastric adenocarcinoma than in normal tissue (χ^2 value 10.020, $P < 0.05$) (**Figure 4**).

Discussion

TSA can regulate gene expression by modulating the chromatin structure. It is known to induce growth arrest and apoptosis in cancer cells [8]. TSA is also an antifungal antibiotic and a specific inhibitor of HDAC activity. It has a tubular structure with a zinc atom at its base and it fits into this structure with the hydroxamic moiety of the inhibitor binding to the zinc. TSA can regulate gene transcription by changing the histone acetylation level [9].

Our study showed that TSA could induce apoptosis of BGC-823 cells. Microarray was used to study the changes in gene expression of BGC-823 cells. Among the differentially expressed genes, GDF-15 was chosen for qRT-PCR and western blotting, which showed that GDF-15 gene expression was significantly decreased in BGC-823 cells after treatment with TSA.

GDF-15, also known as macrophage inhibitory cytokine-1 (MIC-1) and anti-inflammatory drug-activated gene-1 (NAG-1), is a member of the transforming growth factor-β (TGF-β) superfamily [10-12]. The members of GDF are regulators of cell growth and differentiation in embryonic and adult tissues. GDF-15 plays a role in the regulation of cellular responses to stress signals and inflammation, and tissue repair after acute injuries. The dysregulation of GDF-15 expression and signaling pathways is associated with diverse human diseases and cancer progression. However, little is known about the interaction between GDF-15 and immune cells in the tumor microenvironment. GDF-15 is highly expressed in various malignant cancers, and is associated with the proliferation, metastasis and prognosis of colon cancer, ovarian cancer, oral squamous cell carcinoma, melanoma, and prostate cancer [13-17]. GDF-15 inhibits the ability of dendritic cells (DCs) to activate cytotoxic T lymphocyte (CTL) killing. CTLs can efficiently kill tumor cells. GDF-15 is known to inhibit the function of DCs serving as antigen presenting cells (APCs) that activate T cells. These DCs with functional defects may induce T cell tolerance and dysfunction, which suppresses the tumor-specific immune response suggesting a possible role of GDF-15 in tumor immune evasion. Our study showed that GDF-15 gene expression was significantly higher in advanced gastric carcinoma tissue than in the normal adjacent tissue suggesting that GDF-15 may play a role in gastric cancer. Apoptosis of BGC-823 cells and the inhibition of GDF-15 gene expression induced by TSA could be related.

In conclusion, GDF-15 may play a role in gastric cancer, and its overexpression may inhibit the apoptosis of BGC-823 cells. Future studies should aim to clarify the underlying mechanism.

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Disclosure of conflict of interest

None.

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References

[1] Hartgrink HH, Jansen EP, van Grieken NC, van de Velde CJ. Gastric cancer. Lancet 2009; 374: 477-90.

[2] Iuliano R, Vismara MF, Dattilo V, Trapasso F, Baudi F, Perrotti N. The role of microRNAs in cancer susceptibility. Biomed Res Int 2013; 2013: 591-93.

[3] Skeeles LE, Fleming JL, Mahler KL, Toland AE. The impact of 3'UTR variants on differential expression of candidate cancer susceptibility genes. PLoS One 2013; 8: e58609.

[4] Chatterjee N, Wang WL, Conklin T, Chittur S, Tenniswood M. Histone deacetylase inhibitors modulate miRNA and mRNA expression, block metaphase, and induce apoptosis in inflammatory breast cancer cells. Cancer Biol Ther 2013; 14: 658-71.

[5] Chen CQ, Chen CS, Chen JJ, Zhou LP, Xu HL, Jin WW, Wu JB, Gao SM. Histone deacetylases inhibitor trichostatin A increases the expression of Dleu2/miR-15a/16-1 via HDAC3 in non-small cell lung cancer. Mol Cell Biochem 2013; 383: 137-48.

[6] Liu Y, He G, Wang Y, Guan X, Pang X, Zhang B. MCM-2 is a therapeutic target of trichostatin A in colon cancer cells. Toxicol Lett 2013; 221: 23-30.

[7] Kyo S, Sakaguchi J, Ohno S, Mizumoto Y, Maida Y, Hashimoto M, Nakamura M, Takakura M, Nakajima M, Masutomi K, Inoue M Kyo S, Sakaguehi J. High twist expression is involved in infiltrative endometrial cancer and affects patient survival. Hum Pathol 2006; 7: 43l-38.

[8] Smith S, Fox J, Mejia M, Ruangpradit W, Saberi A, Kim S, Choi Y, Oh S, Wang Y, Choi K, Li L, Hendrickson EA, Takeda S, Muller M, Myung K. Histone deacetylase inhibitors selectively target homology dependent DNA repair defective cells and elevate non-homologous endjoining activity. PLoS One 2014; 9: e87203.

[9] Xiao Ming Z, Yun Long L, Hao W, Wu C, Xiao Lin L, Song Bin F, Hong Chi J. Gastric cancer cell lines induced by trichostatin A. World J Gastroenterol 2008; 14: 4810-15.

[10] Zhou Z, Li W, Song Y, Wang L, Zhang K, Yang J, Zhang W, Su H, Zhang Y. Growth differentiation factor-15 suppresses maturation and function of dendritic cells and inhibits tumor-specific immune response. PLoS One 2013; 8: e78618.

[11] Kaur S, Chakraborty S, Baine MJ, Mallya K, Smith LM, Sasson A, Brand R, Guha S, Jain M, Wittel U, Singh SK, Batra SK. Potentials of plasma NGAL and MIC-1 as biomarker(s) in the diagnosis of lethal pancreatic cancer. PLoS One 2013; 8: e55171.

[12] Baek SJ, Kim JS, Moore SM, Lee SH, Martinez J, Eling TE. Cyclooxygenase inhibitors induce the expression of the tumor suppressor gene EGR-1, which results in the up-regulation of NAG-1, an antitumorigenic protein. Mol Pharmacol 2005; 67: 356-64.

[13] Brown DA, Ward RL, Buckhaults P, Liu T, Romans KE, Hawkins NJ, Bauskin AR, Kinzler KW, Vogelstein B, Breit SN. MIC-1 serum level and genotype: associations with progress and prognosis of colorectal carcinoma. Clin Cancer Res 2003; 9: 2642-50.

[14] Joshi JP, Brown NE, Griner SE, Nahta R. Growth differentiation factor 15 (GDF15)-mediated HER2 phosphorylation reduces trastuzumab sensitivity of HER2-overexpressing breast cancer cells. Biochem Pharmacol 2011; 82: 1090-9.

[15] Zhang L, Yang X, Pan HY, Zhou XJ, Li J, Chen WT, Zhong LP, Zhang ZY. Expression of growth differentiation factor 15 is positively correlated with histopathological malignant grade and in vitro cell proliferation in oral squamous cell carcinoma. Oral Oncol 2009; 45: 627-32.

[16] Suesskind D, Schatz A, Schnichels S, Coupland SE, Lake SL, Wissinger B, Bartz-Schmidt KU, Henke-Fahle S. GDF-15: a novel serum marker for metastases in uveal melanoma patients. Graefes Arch Clin Exp Ophthalmol 2012; 250: 887-95.

[17] Rasiah KK, Kench JG, Gardiner-Garden M, Biankin AV, Golovsky D, Brenner PC, Kooner R, O'neill GF, Turner JJ, Delprado W, Lee CS, Brown DA, Breit SN, Grygiel JJ, Horvath LG, Stricker PD, Sutherland RL, Henshall SM. Aberrant neuropeptide Y and macrophage inhibitory cytokine-1 expression are early events in prostate cancer development and are associated with poor prognosis. Cancer Epidemiol Biomarkers Prev 2006; 15: 711-6.