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Original Research

Effect of GDF11 on proliferation and apoptosis of esophageal cancer cells

Biao Gu[1], Qi Wang[1], Weijie Dai[2], Gang Ma[2], Yaqi Song[3], Yaxin Song[3], Qinquan Wu[1*]

[1] Department of Thoracic Surgery, The Affiliated Huai'an No.1 People's Hospital of Nanjing Medical University, 6 Beijing West Road, Huai'an, Jiangsu 223300, China
[2] Department of Gastroenterology, The Affiliated Huai'an No.1 People's Hospital of Nanjing Medical University, 6 Beijing West Road, Huai'an, Jiangsu 223300, China
[3] Department of radiation oncology, The Affiliated Huai'an No.1 People's Hospital of Nanjing Medical University, 6 Beijing West Road, Huai'an, Jiangsu 223300, China

Correspondence to: endcase@163.com

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Abstract: This study was aimed at investigating the effect of growth differentiation factor 11 (GDF11) on the proliferation and apoptosis of esophageal cancer cells. Serum levels of GDF11 in esophageal cancer patients were determined with ELISA kits, and the correlation between serum GDF11 and pathological features of esophageal cancer were determined. The effect of recombinant GDF11 on the growth of esophageal cancer cells was measured by CCK6 method. In order to investigate the effect of recombinant GDF11 on the proliferation and apoptosis of esophageal cancer cells, the expression of apoptosis-promoting protein Bax and proliferative-associated protein Bcl-2 in esophageal cancer cells were determined using western blot. Moreover, GDF11 was used to treat esophageal cancer cells, and its effect on proliferation and apoptosis was determined with MTT assay and flow cytometry, respectively. The serum content of GDF11 was much less in esophageal cancer patients than in the control group. Esophageal GDF II in cancer patients was correlated with cancer differentiation: the higher the degree of differentiation, the higher the content of GDF11. GDF11 inhibits proliferation and apoptosis of esophageal cancer cells.

Key words: Growth differentiation factor 11; Esophageal cancer; Clinico-pathological features; Apoptosis.

Introduction

Esophageal cancer is one of the most fatal malignancies of the gastrointestinal tract. In the past two decades, there has been little change in poor prognosis of patients with esophageal cancer which 5-year survival is still far below 20 % (1). However, within the same period, the relative frequencies of two major histological types of esophageal cancer have undergone profound changes. Thus, although the incidence of esophageal squamous cell carcinoma has decreased significantly, that of esophageal adenocarcinoma has increased more than twice since 1975 (2, 3). GERD3 has been confirmed as a strong risk factor for esophageal cancer (4). Over 40 % of adult Americans have common symptoms of GERD (5). In some individuals, chronic esophageal inflammation induced by GERD leads to intestinal metaplasia, a condition known as Barrett's esophagus. The metaplastic epithelium is prone to malignancies, and majority of esophageal adenocarcinomas are considered to be derived from Barrett's esophagus (6).

Growth differentiation factor 11 (GDF11), a protein of transforming growth factor β (TGFβ), superfamily has a significant protective effect on cardiovascular and nervous systems (7, 8). The protective effects of GDF11 on heart and muscle are related to its regulation of several signaling pathways, including the MAPK-p38-mioglianin pathway (9). *In vitro* studies have enhanced understanding of mechanisms behind the changes observed. Following treatment of a culture of older mouse myocytes with rGDF11, the number of mitochondria increased, and the multinucleated muscle fibers representing the syncytial of myoblasts began to form (10, 11). Thus, it was presumed that rGDF11 not only induces mitochondrial reaction but also promotes mitochondrial defects to remove muscle fibers in old mice.

This study determined the content of GDF11 in blood of esophageal cancer patients, and determined cellular activity, expression of proliferation and apoptosis-related proteins, and expression of cytokines after treating esophageal cancer cells with recombinant GDF11. Through determination of cell activity, proliferation and apoptosis, the effect of GDF11 on the proliferation and apoptosis of esophageal cancer cells was assessed.

Materials and Methods

Experimental materials

The chemicals and assay kits used in this study were fetal bovine serum (FBS, Gemini Biologicals, Calabasas, Canada); glutamine (Life Technologies, Inc., Gaithersburg, USA); Amphotericin (Life Technologies, Inc., USA); GDF11 ELISA kit (R&D Systems, USA); SDS-PAGE pre-cast gel, and nitrocellulose membrane (Bio-Rad, Hercules, Canada). Others were goat anti-human mAb Bax and Bcl-2 antibodies, horseradish oxidase-labeled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, Canada), Chemiluminescence

assay kit (Pierce, Rockford, IL, USA), and Cell death ELISA assay system (Boehringer Mannheim, Indianapolis, IN); Spectramax M5 microplate reader (Molecular Devices, Canada).

Methods

Case sources and cell culture

A total of 86 serum samples from 43 esophageal cancer patients and 43 healthy persons from XXX hospital were collected and used in the study. It is known that large differences exist in the content of GDF11 in people as a function of age. In order to reduce interference from age on the results, esophageal cancer patients or healthy individuals aged from 35 to 45 years were selected for the study (mean age = 41 years). The esophageal adenocarcinoma cell line ECa9706 was obtained from the Clinical Cancer School of Wuhan University. The cells were cultured in DMEM containing 10 % FBS, double antibody, 1-glutamine and amphotericin, and placed in an incubator containing 5 % CO_2 at 37 °C.

Determination of GDF11 content in serum

The level of GDF11 protein in serum of patients with clinical esophageal cancer was determined using a commercial GDF11 ELISA kit. Whole blood samples from clinical cases stored in disposable tubes containing pyrogen and endotoxin were centrifuged at 30 °C for 30 min, and the supernatants were collected. The principle of GDF11 detection in this kit is based on double antibody sandwich ELISA. First, clinical serum samples and standards were added to the microtiter plate for reaction to take place. The unbound fraction was then rinsed off with a wash solution, and anti-human GDF11 antibody was reacted with the horseradish peroxidase-labeled biotinylated secondary antibody. Washed free fraction was added to the TMB chromogenic substrate solution for color development. Finally, the stop solution was added and absorbance was measured at a wavelength of 450 nm in a microplate reader. The concentration of GDF11 in the test sample was calculated from standard calibration curve.

Determination of cell activity

Cells in logarithmic phase of growth were counted and their density was adjusted to 6×10^4 cells/ml. Then, 100 ul of the cell suspension was added into each well in a 96-well plate and cultured for 24 h. Subsequently, the medium was changed and replaced with 200 uL of serum-free medium. After 6 h, the medium was discarded. About 100 uL of serum-free medium was added to the experimental group and the control group. After culturing for 24 h, 48 h and 72 h, the cells were incubated with CCK8 kit reagent for 2 h, and the absorbance was read in a fully automatic microplate reader.

Western bolt

Cell lysate was prepared with lysis buffer for 20 min, centrifuged at 4 °C, and the supernatants were collected. After measuring the protein concentration of the supernatant, the protein (100 μg) from the esophageal cancer cell line was separated on a 10 % SDS-PAGE (precast gel) overnight. The separated proteins were transferred to a nitrocellulose membrane, and incubated with 1:2000 dilution of goat anti-human monoclonal antibody Bax or Bcl-2 antibodies. A horseradish peroxidase-labeled secondary antibody was used at a 1:5000 dilution, after which the bands were determined with chemiluminescence using the SuperSignal West Fento assay kit according to the manufacturer's instructions. All experiments were repeated.

Determination of cell proliferation

Live cell uptake and the ability to convert soluble MTT to formazan crystals were assessed with MTT assay. The cells were seeded in 96-well plates at an initial density of 5 x 10^3 cells/well, treated with rGDF11 and a negative control, and cultured and maintained for a scheduled time. Then, MTT (0.2 mg/ml) was added to the culture, and the culture was further incubated for 2 h. The medium was replaced with acidified isopropanol (0.04 N HCl in isopropanol) and the plates were incubated for 1 h at room temperature, after which the absorbance of the samples were read in a Spectramax M5 plate reader. All experiments were performed in triplicate and repeated at least three times.

Determination of cell apoptosis

The same amount of cells was seeded in a 6-well DMEM plates containing 10 % FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 12.5 μg/ml amphotericin. Tumor cell lines were incubated with rGDF11 (10 ng) for 36 h. Tumor cells incubated in the vehicle only were used as negative controls. Apoptosis ratios were determined using a cell death ELISA assay kit according to the manufacturer's instructions.

Statistical analysis

All data were processed by software GraphPad 5 and expressed as mean ± SD. Statistical analysis was performed using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Plasma GDF11 content in esophageal cancer patients and the control group

The GDF11 content of clinical samples was determined using ELISA kit. The results showed that the content of GDF11 was 0.76 ± 0.05 mg/L in plasma of patients with esophageal cancer, while the GDF11 in the control group plasma was up to 1.02 ± 0.18 mg/L. Thus, the content of GDF11 was much lower in the esophageal cancer patients than in the control group, suggesting that GDF11 is lowly expressed in patients with

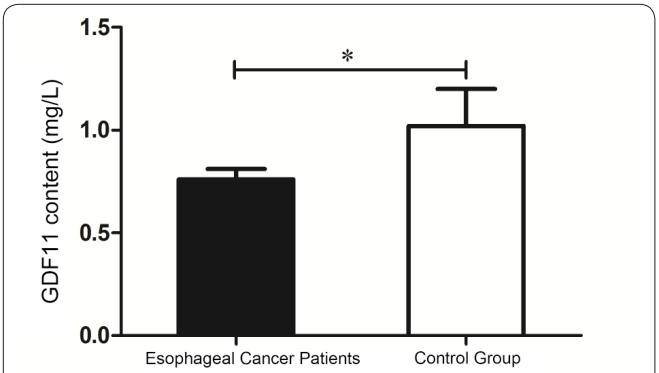

Figure 1. Plasma content of GDF11 in esophageal cancer patients and control. *$p < 0.05$.

esophageal cancer (Figure 1).

Correlation between GDF11 content in esophageal cancer patients and their pathological features

Correlation analysis showed that GDF11 was correlated only with the degree of differentiation. The contents of GDF11 in low, moderate and high differentiation stages were 0.65 ± 0.08, 0.84 ± 0.19 and 1.07 ± 0.21 mg/L, respectively. The higher the degree of differentiation, the higher the content of GDF11 (Figure 2).

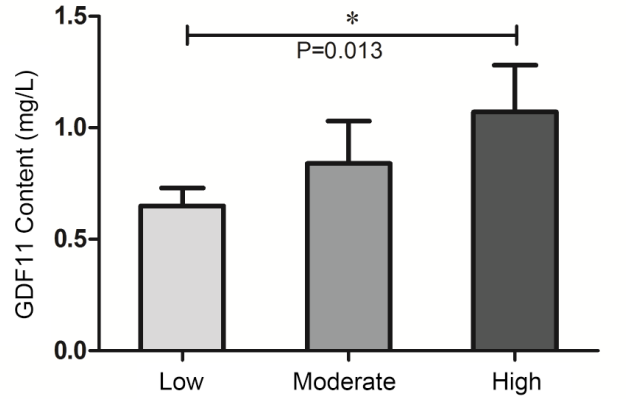

Figure 2. Correlation between plasma GDF11 content of esophageal cancer patients and degree of differentiation. *$p < 0.05$.

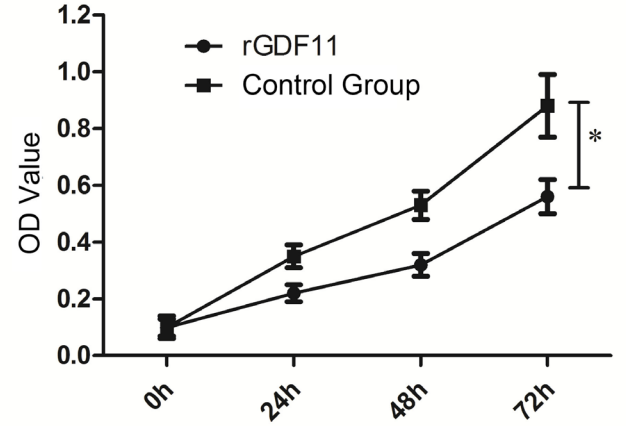

Figure 3. Activity of recombinant GDF11-treated esophageal cancer cells *$p < 0.05$.

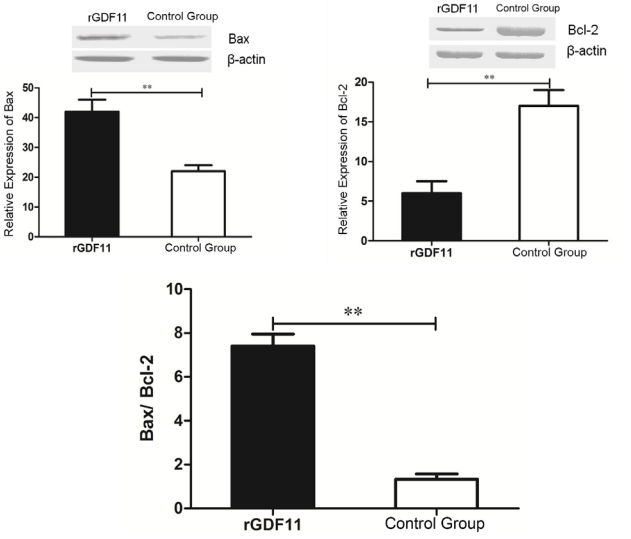

Figure 4. Effect of GDF11 on the expressions of proliferation-related protein Bcl-2 and apoptosis-related protein Bax in esophageal cancer patients. **$p < 0.01$.

ECa9706 activity of recombinant GDF11-treated esophageal cancer cells

The activity of esophageal cancer cells treated with recombinant GDF11 was determined by CCK8. The results revealed that cell activity was significantly reduced in esophageal cancer cells treated with recombinant GDF11, when compared to the control group ($p < 0.05$) (Figure 3).

Effect of recombinant GDF11 treatment on proliferation-related and apoptosis-related proteins

In order to investigate the effect of recombinant GDF11 on the proliferation and apoptosis of esophageal cancer cells, cell proliferation-related protein Bcl-2 and apoptosis-related protein Bax were determined with western blot. The expression of Bax was increased after treatment with recombinant GDF11. The ratio of Bax/Bcl-2 represented the effect of recombinant GDF11 on cell proliferation and apoptosis: the higher the ratio, the stronger the apoptosis-inducing effect, and the lower the ratio, the stronger the proliferation-promoting effect. In this study, the ratio of Bax/Bcl-2 was significantly higher in patients with esophageal cancer than in the control group. Thus, recombinant GDF11 could promote apoptosis of esophageal cancer cells (Figure 4).

Proliferation of recombinant GDF11-treated esophageal cancer cells

The proliferation of recombinant GDF11-treated esophageal cancer cells was determined with MTT assay. The formazan products of the cells at 0, 24, 48, 72 and 96 h after recombinant GDF11 treatment were quantified colorimetrically. The results revealed that the cell proliferation of the recombinant GDF11-treated group was not obvious: the proliferation was significantly inhibited, when compared with the control group (Figure 5).

Apoptosis of recombinant GDF11-treated esophageal cancer cells

The effect of GDF11 on apoptosis on esophageal cancer cell group and the control group were measured by flow cytometry. The results revealed that the percentage of apoptosis was as high as 13.4 ± 1.7 % in the esophageal cancer group, while the mean apoptosis was 4.5 ± 0.5 % in the control group. Thus, percentage of apoptosis was significantly higher in the esophageal

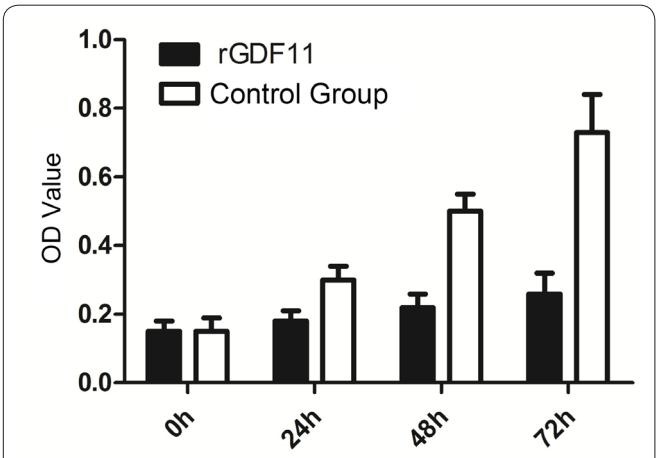

Figure 5. Effect of GDF11 on proliferation of esophageal cancer cells.

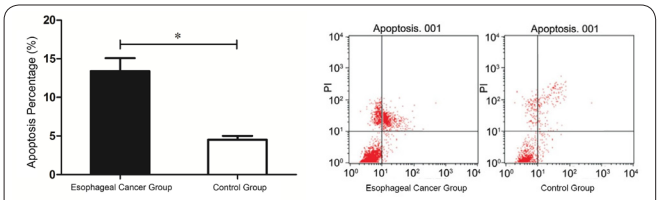

Figure 6. Effect of GDF11 on apoptosis of esophageal cancer cells. *$p < 0.05$.

cancer group than in the control group ($p < 0.05$). This shows that recombinant GDF11 promotes apoptosis in esophageal cancer cells (Figure 6).

Discussion

More than 400,000 people died of esophageal malignant cancers worldwide in 2005 (12). Indeed, esophageal cancer ranks eighth amongst the most common cancers in the world, and accounts for more than 80 % of all deaths in developing countries (13). There were over 490,000 new cases of esophageal cancer worldwide in 2005. The incidence of many other types of cancer is expected to decline over the next 10 years, while that of esophageal cancer is expected to increase by 140 % (12). According to the National Cancer Institute, there were approximately 17,990 new cases of esophageal cancer, and 15,210 esophageal cancer deaths in 2013 (14). Notwithstanding the numerous advances in diagnosis and treatment, the 5-year survival of patients diagnosed with esophageal cancer is between 15 and 20 % (15).

Growth differentiation factor 11 (GDF11) was discovered 20 years ago. Its recombinant analog rGDF11 is a disulfide-bonded dipolymer with a molecular weight of 25 kDa. Each polypeptide chain of this dipolymer contains 109 amino acid residues. Studies have shown that rGDF11 protected nerves, and also enhanced the functional activity of skeletal muscle in mice (16 -18).

It has been reported that GDF11 cytokines blocked erythrocyte maturation in the erythrocyte phase and gave rise to early apoptosis in patients with βthalassemia (19). This is due to an autocrine amplification loop that produces oxidative stress and leads to the precipitation of alpha globulin. The expression of GDF11 is increased in the spleen erythrocytes of thalassemia mice and in serum of patients with beta thalassemia. Inactivation of GDF11 reduces oxidative stress and the deposition of membrane protein α globin, thereby enhancing erythropoiesis (20). Currently, scientists are trying to unravel the mechanisms involved in the protective effect of GDF11 in mice. However, extant literature suggests that GDF11 has neuro-protective property and can activate the function of heart and skeletal muscle (12, 22). Consequently, the significant improvement in cognitive function resulting from GDF11 treatment may be associated more with recovery of synaptic plasticity than the enhancement and development of nerve cells. A few hours after its administration, GDF11 is able to alter the expression of 4,700 genes, including the expression of cell cycle genes. Besides, GDF11 inhibits genes involved in the regulation of the cytoskeleton, such as fascin, LIM, and SH3 region protein 1 (LASP1) (23). Moreover, GDF11 is considered as a regulator of tissue senescence which was established in the course of evolution (24). The protective effects of GDF11 may be related to the cascade reaction of p38 and MAPK nuclear protein, and the latter can activate mammalian myostatin (18). Hence, GDF11, apart from preventing cardiomegaly by activating p38-MAPS cascade reaction and regulating nucleolus function, can also protect other tissues from aging. However, in mice, myostatin occurs mainly in the skeletal muscle, while GDF11 is widely expressed in various tissues. Although the highest expression level of GDF11 is observed in the spleen, skeletal muscle is the most abundant tissue in the organism, accounting for 40 to 50 % of the body mass (25).

To date, studies on the relationship between GDF11 and the proliferation and apoptosis of esophageal cancer cells are limited. The results obtained in the present study have established the inhibitory effect of GDF11 on the proliferation of esophageal cancer cells, and its enhancing effect on their apoptosis.

Interest conflict

There is no conflict of interest to be declared by the author.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yaxin Song, Qingquan Wu; Qi Wang, Weijie Dai, Gang Ma, Yaqi Song collected and analysed the data; Biao Gu wrote the text and all authors have read and approved the text prior to publication.

References

1. Luo J, Wang W, Tang Y, Zhou D, Gao Y, Zhang Q, et al. mRNA and methylation profiling of radioresistant esophageal cancer cells: the involvement of Sall2 in acquired aggressive phenotypes. J Cancer 2017; 8: 646.
2. Shridhar R, Almhanna K, Meredith KL, Biagioli MC, Chuong MD, Cruz A, et al. Cancer Control 2013; 20: 97-110.
3. Tewari A. A cranberry proanthocyanidin inhibits cancer-related processes in human esophageal adenocarcinoma cells. Ohio State Univ 2006; 13: 51-55.
4. Kaur BS, Khamnehei N, Iravani M, Namburu SS, Lin O, Triadafilopoulos G. Rofecoxib inhibits cyclooxygenase 2 expression and activity and reduces cell proliferation in Barrett's esophagus. Gastroenterol 2002; 123: 60-67.
5. Cohen E, Bolus R, Khanna D, Hays RD, Chang L, Melmed GY, et al. GERD symptoms in the general population: prevalence and severity versus care-seeking patients. Dig Dis Sci 2014; 59: 2488-2496.
6. Zhang HY, Spechler SJ, Souza RF. Esophageal Adenocarcinoma Arising in Barrett Esophagus. Cancer Lett 2009; 275: 170-177.
7. Khavinson VK, Kuznik BI, Tarnovskaya SI, Linkova NS. GDF11 protein as a geroprotector. Biol Bull Rev 2016; 6: 141-148.
8. Andersson O, Reissmann E, Ibáñez CF. Growth differentiation factor 11 signals through the transforming growth factor-β receptor ALK5 to regionalize the anterior–posterior axis. Embo Rep 2006; 7: 831-837.
9. Bulterijs S. Metformin as a geroprotector. Rejuvenation Res 2011; 14: 469-482.
10. Bitto A, Kaeberlein M. Rejuvenation: it's in our blood. Cell Me-

tabol 2014; 20: 2-4.
11. Sinha M, Jang YC, Oh J, Khong D, Wu EY, Manohar R, et al. Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. Sci 2014; 344: 649.
12. Lambert R, Hainaut P. The multidisciplinary management of gastrointestinal cancer. Epidemiology of oesophagogastric cancer. Best Pract Res Clin Gastroenterol 2007; 21: 921-945.
13. Herszényi L, Tulassay Z. Epidemiology of gastrointestinal and liver tumors. Eur Rev Med Pharmacol Sci 2010; 14: 249-258.
14. Chin H, Miller M, Kong CY, Emily CD, Kevin JN, Michelle D, et al. Trends in esophageal adenocarcinoma incidence and mortality. Cancer 2013; 119: 1149-1158.
15. Allen DC. Oesophageal Carcinoma. S Afr Med J 1973; 47: 348.
16. Harmon EB, Apelqvist AA, Smart NG, Gu X, Osborne DH, Kim SK. GDF11 modulates NGN3+ islet progenitor cell number and promotes beta-cell differentiation in pancreas development. Dev 2004; 131: 6163.
17. Ge G, Hopkins DR, Ho WB, Greenspan DS. GDF11 Forms a Bone Morphogenetic Protein 1-Activated Latent Complex That Can Modulate Nerve Growth Factor-Induced Differentiation of PC12 Cells. Mol Cell Biol 2005; 25: 5846-5858.
18. Liu JP, Laufer E, Jessell TM. Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. Neuron 2001; 32: 997-1012.
19. Shamsian BS, Arzanian MT, Shamshiri AR, Alavi S. Frequency of Red Cell Alloimmunization in Patients with β-Major Thalassemia in an Iranian Referral Hospital. Iran J Pediatr 2008; 18: 149-153.
20. Dussiot M, Maciel TT, Fricot A, Chartier C, Negre O, Veiga J, et al. An activin receptor IIA ligand trap corrects ineffective erythropoiesis in β-thalassemia. Nat Med 2014; 20: 398-407.
21. Rochette L, Zeller M, Cottin Y, et al. Growth and differentiation factor 11 (GDF11): Functions in the regulation of erythropoiesis and cardiac regeneration. Pharmacol Ther 2015; 156: 26-33.
22. Anisimov VN, Popovich IG, Zabezhinski MA, Anisimov SV, Vesnushkin GM, Vinogradova IA. Melatonin as antioxidant, geroprotector and anticarcinogen. Biochim Biophys Acta 2006; 1757: 573-589.
23. Williams G, Zentar MP, Gajendra S, Sonego M, Doherty P, Lalli G. Transcriptional Basis for the Inhibition of Neural Stem Cell Proliferation and Migration by the TGFβ-Family Member GDF11. Plos One 2013; 8: 78478.
24. Estrada E, Colón M, Báez P, Ruiz A, Seto E, Flores I, et al. Expression of the Cell Cycle Regulator GDF11 is Downregulated in Endometriosis. Soc Gynecol Investig 2011; 18: 190-191.
25. Zhang YH, Feng C, Du XT, Gao JL, Xiao XL, Li N, et al. GDF11/BMP11 activates both smad1/5/8 and smad2/3 signals but shows no significant effect on proliferation and migration of human umbilical vein endothelial cells. Oncotarget 2016; 7: 12063-12074.