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Original research paper

Astragaloside IV inhibits pathological functions of gastric cancer-associated fibroblasts through regulation of the HOXA6/ZBTB12 axis

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ABSTRACT

Cancer-associated fibroblasts (CAFs) play critical roles in the tumor microenvironment and exert tumor-promoting or tumor-retarding effects on cancer development. Astragaloside IV has been suggested to rescue the pathological impact of CAFs in gastric cancer. This study aimed to investigate the potential mechanism of astragaloside IV in the regulation of CAF pathological functions in gastric cancer development. Homeobox A6 (HOXA6), and Zinc Finger and BTB Domain Containing 12 (ZBTB12) are highly expressed in gastric CAFs compared with normal fibroblasts (NFs) based on the GSE62740 dataset. We found that astragaloside IV-stimulated CAFs suppressed cell growth, migration, and invasiveness of gastric cancer cells. HOXA6 and ZBTB12 were downregulated after astragaloside IV treatment in CAFs. Further analysis revealed that HOXA6 or ZBTB12 knockdown in CAFs also exerted inhibitory effects on the malignant phenotypes of gastric cells. Additionally, HOXA6 or ZBTB12 overexpression in CAFs enhanced gastric cancer cell malignancy, which was reversed after astragaloside IV treatment. Moreover, based on the hTFtarget database, ZBTB12 is a target gene that may be transcriptionally regulated by HOXA6. The binding between HOXA6 and ZBTB12 promoter in 293T cells and CAFs was further confirmed. HOXA6 silencing also induced the downregulation of ZBTB12 mRNA and protein in CAFs. Astragaloside IV was demonstrated to regulate the expression of ZBTB12 by mediating the transcriptional activity of HOXA6. Our findings shed light on the therapeutic value of astragaloside IV for gastric cancer.

Keywords: astragaloside IV, cancer-associated fibroblasts, HOXA6, ZBTB12

Gastric cancer ranks fifth in cancer incidence globally, with nearly 1.1 million new cases in 2020 (1). Gastric cancer is a heterogeneous disease characterized by distinct pathogeneses and active tumorigenic pathways (2). *Helicobacter pylori* infection, age, high salt

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intake, and low fruit and vegetable intake are common risk factors for gastric cancer (3). Currently, treatment options including endoscopic resection, surgery, chemotherapy, immunotherapy, and targeted therapy are mainly recommended for gastric cancer patients (4). However, the clinical outcome of gastric cancer patients remains dismal, and median survival for those at an advanced stage is no more than one year (3). Therapy of advanced gastric cancer patients is challenged by the limited understanding of the underlying pathogenesis. Thus, the development of novel therapeutic strategies may contribute to the improvement of gastric cancer treatment.

Tumor microenvironment (TME) attracted increasing attention for its role in cancer progression. Cancer is a dynamic process with reciprocal interactions between the cancer cells and the TME, which consists of endothelial cells, fibroblasts, macrophages, lymphocytes, and extracellular matrix components (ECM) (5). Cancer-associated fibroblasts (CAFs) are the main TME component and promote cancer development by secreting growth factors, modifying ECM, facilitating angiogenesis, and reducing sensitivity to antitumor therapy (6). CAF accumulation is related to cancer cell growth, invasion as well as migration ability in gastric cancer (7). For example, CAF secretes miR-522 in TME to suppress ferroptosis and enhance acquired chemoresistance in gastric cancer (8). CAFs facilitate the development of malignant phenotypes in gastric cancer *via* the HSF1-mediated extracellular vesicles (8). Increasing evidence indicates that CAFs are promising targets for gastric cancer therapy (9–11), while the underlying mechanism of CAFs in gastric cancer is far from clear.

Traditional Chinese medicine (TCM) has long been used in cancer treatment with the advantages of low toxicity and side effects as well as good tolerance (12, 13). Accumulating studies have revealed that TCM may regulate the TME to enhance the antitumor effects (14–16). As a natural saponin derived from Astragali radix, astragaloside IV possesses anti-inflammatory, antitumor, antioxidative, and immune-regulatory properties (17, 18). Astragaloside IV is a glycoside component and an oligosaccharide of xylose and glucose. The chemical structure of astragaloside IV is shown in Fig. 1. Astragaloside IV inhibits cancer progression mainly by inducing cell cycle arrest at the G_0/G_1 phase and cell apoptosis and suppressing cell proliferation, invasion, and metastasis through various pathways (17, 19). The impact of astragaloside IV is restrained to tumor cells without cytotoxic effects on normal cells (20). Astragaloside IV protects the gastric mucosa of rats with gastric precancerous lesions by regulating p53 levels for the activation of the Ambra1/Beclin1 complex (21). Astragaloside IV is also indicated to inhibit proliferation and metastasis by inhibiting the circDLST/miR-489-3p/EIF4A1 axis in gastric cancer (22). It is revealed that astragaloside IV suppresses the promoting effects of CAFs on gastric cancer cell malignancy by downregulating miRNA-301a and upregulating miRNA-214 (23).

HOXA6 belongs to the HOX (Homeobox) family of transcription factors (TFs). Based on the analysis of the GSE62740 dataset in the GEO database, its upregulation in gastric cancer tissue samples and cell lines is closely associated with the differentiation, lymph node metastasis, AJCC as well as TNM stage, and adverse survival outcomes in gastric cancer (24). HOXA6 is predicted to be a transcription factor for ZBTB12. Moreover, the analysis of the GSE62740 dataset also identified the upregulation of ZBTB12 in CAFs, and ZBTB12 is predicted with higher expression in gastric cancer tissues, suggesting ZBTB12 as a potential oncogene transcriptionally regulated by HOXA6 in gastric cancer.

The aim of the present study was to understand the impact and possible mechanisms of astragaloside IV on gastric cancer. We assumed that astragaloside IV suppressed gastric cancer development by regulating HOXA6 and ZBTB12 in CAFs. The results may deepen our understanding of the astragaloside IV in anticancer therapy of gastric cancer.

EXPERIMENTAL

Chemicals

Astragaloside IV (chemistry formula: $C_{41}H_{68}O_{14}$, Fig. 1, HPLC ≥ 98 %) was purchased from Absin (China). Triton X-100, collagenase IV, DMEM, Roswell Park Memorial Institute 1640 (RPMI-1640) medium, Lipofectamine 3000 reagent, anti-FAP antibody, and RIPA lysis buffer were obtained from Thermo Fisher (USA). Paraformaldehyde, Cell Counting Kit-8 reagent, FBS , and antibiotics were obtained from Beyotime Biotechnology (China). Antibodies, except the anti-FAP antibody, were all purchased from Abcam (China). DAPI was purchased from Sigma-Aldrich (China). HyClone DMEM/F12 was obtained from Logan (USA). A chemiluminescent reagent was obtained from Pierce (USA).

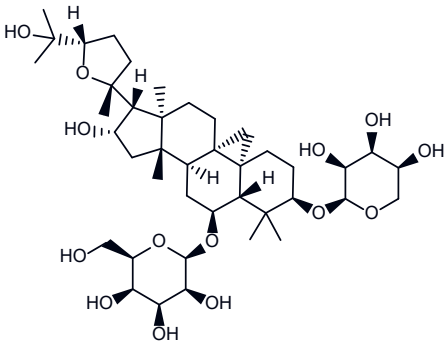

Fig. 1. Chemical structure of astragaloside IV.

Isolation and incubation of NFs and CAFs

Gastric cancer tissue samples and normal gastric tissue samples were collected from gastric cancer patients during surgery at the Affiliated Lianyungang Oriental Hospital of Xuzhou Medical University. The Ethics Committee of the Hongze District People's Hospital of Huai'an City Hospital has approved this study. For the isolation of NFs and CAFs, tissues were sectioned, and trypsinized by collagenase IV for 3 h at 37 °C. Next, a 70-μm mesh was applied to filtrate the samples, and then the samples were transferred to an Eppendorf tube. Then the filtrate was centrifuged at 450 g for 10 minutes and washed with sterile PBS thrice to eliminate red blood cells. The obtained cells were maintained in a humidified incubator with DMEM and 10 % FBS at 37 °C with 5 % CO_2. The adherent cells were harvested after 3 h and incubated in other dishes for NFs and CAFs (23).

Immunofluorescence

The identification of NFs and CAFs was conducted using immunofluorescence. NFs and CAFs were plated into 24-well culture plates, fixated with 4 % paraformaldehyde, followed by PBS washing. Next, cells were stimulated by 0.1 % Triton X-100 for 10 minutes, and then blocked with 5 % goat serum for 60 minutes at room temperature. Cells were then incubated with anti-S100A4 (#ab197896, 1/250) at 4 °C overnight. Subsequently, cells were cultured with the FITC-conjugated goat anti-rabbit IgG for 60 minutes at room temperature. DAPI was applied for nucleus staining. An Olympus fluorescent microscope was applied to capture the images of NFs and CAFs.

Cell culture and treatment

Gastric cancer cell lines, MGC803 and BGC-823, and human embryonic kidney 293T cells (cat. No. C6008) were provided by Beyotime. BGC-823 and 293T cells were kept in Roswell Park Memorial Institute 1640 (RPMI-1640) medium and MGC803 cells were incubated in HyClone DMEM/F12. Both the culture medium contained 10 % FBS and 1 % P/S and were kept at 37 °C with 5 % CO_2. Astragaloside IV was dissolved in DMSO to different concentrations (10, 20, 40 μmol L^{-1}) and CAFs were stimulated by astragaloside IV or DMSO (0.2 %, control) for 3 days.

Cell transfection

Short hairpin RNAs (shRNAs) for HOXA6 (sh-HOXA6-1/-2) and ZBTB12 (sh-ZBTB12-1/-2) were obtained from GenePharma (China). For HOXA6 or ZBTB12 overexpression, pcDNA3.1 plasmids were introduced with empty pcDNA3.1 vectors as the negative control. BGC-823 and MGC803 cells were grown in 12-well plates and cell transfection was conducted using Lipofectamine 3000 reagent. After 48 h, cells were harvested for the following experiments.

Coculture of CAFs and gastric cancer cells

CAFs were cocultured with gastric cancer cells in Transwell chambers in 24-well plates. The treated CAFs were seeded into the top chambers and gastric cancer cells were plated into the bottom chambers (5×10^5 cells per well). Cells were incubated with the RPMI-1640 medium with 10 % FBS and 1/100 P/S for 24 h. Subsequently, BGC-823 and MGC803 cells were harvested for further analysis.

RT-qPCR

TRIzol reagent was applied for total RNA collection from gastric cancer cells and CAFs. Then cDNAs were synthesized with a cDNA synthesis kit (Toyobo, Japan). qPCR was conducted with the Light Cycler Fast Start DNA MasterPlus SYBR Green I Kit (Roche Diagnostics, UK) on the StepOne Real-Time PCR System (Thermo Fisher). The mRNA levels of HOXA6 and ZBTB12 were measured using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. Sequences of primers are presented below:

HOXA6

Forward: 5′-GAAACTGAAAGTCCAACTCGG-3′,
Reverse: 5′-CCTTATCAGAATAGAAACACGAGG-3′;

ZBTB12

Forward: 5′-CCACTGGATGAAGACTTGG-3′,
Reverse: 5′-CGTCCTCATCCTCATCCTC-3′;

GAPDH

Forward: 5′-TCATTTCCTGGTATGACAACGA-3′,
Reverse: 5′-GTCTTACTCCTTGGAGGCC-3′.

Western blot

RIPA buffer was applied for the isolation of total proteins. Protein concentration was quantified using a BCA protein concentration kit (Beyotime, China) following the manufacturer's protocol. The same amount of protein sample (40 μg) was loaded on 10 % SDS-PAGE and then electro-transferred onto nitrocellulose membranes (Invitrogen). Next, 5 % non-fat milk was applied to block the membranes which were subsequently incubated with anti-α-SMA (#ab124964, 1/10000), anti-Vimentin (#ab92547, 1/1000), anti-FAP (#PA5-51057, 1/1000) and anti-S100A4 (#ab197896, 1/1000) for 12 h at 4 °C with GAPDH as the internal reference. Subsequently, the membranes were cultured with the HRP-conjugated secondary antibodies for 60 minutes at ambient temperature. The chemiluminescent reagent was applied for the visualization of protein bands. Relative protein expression was determined and normalized to GADPH.

Cell viability

Gastric cancer cell growth assessment was subject to Cell Counting Kit-8 (Beyotime) in accordance with the manufacturer's protocol. Briefly, treated MGC803 and BGC-823 cells were plated into 96-well plated at 4×10^3 cells/well. Then the cells were cultured for 24, 48, and 72 h, and 10 μL of CCK-8 solution (Beyotime) was supplemented into each well and for another 2-h incubation at 37 °C. Finally, a microplate analyzer (NanoDrop, USA) was applied to determine the OD value at 450 nm.

Cell proliferation

The proliferation potential of gastric cancer cells occulted with the CAFs was evaluated using colony-formation assays. The treated BGC-823 and MGC803 cells were inoculated in six-well plates (5×10^3 cells/well) and cultured for two weeks at 37 °C with 5 % CO_2. Next, cells were fixed with 4 % paraformaldehyde for 10 minutes and dyed using 0.5 % crystal violet for 15 minutes. The images were taken using a light microscope.

Transwell assays

Transwell chamber (Corning, USA) pre-coated with or without Matrigel mix (BD Biosciences, USA) was applied for the evaluation of gastric cancer cell invasion and migration,

respectively. Briefly, the top Transwell chamber was added with 3×10^5 treated BGC-823 and MGC803 cells and serum-free culture media. The bottom chamber was supplemented with complete culture media. After 48 h, paraformaldehyde was used to fasten the migrated or invaded cells. The fixed cells were subsequently dyed with 0.3 % crystal violet. The images of cell migration or invasion were captured under a light microscope.

Dual-luciferase reporter assays

The binding between HOXA6 and ZBTB12 promoter was examined by luciferase reporter assays. The pcDNA3.1 empty vector (control), pcDNA3.1/HOXA6 were co-transfected in CAFs and 293T cells with pGL3-based construct containing ZBTB12 promoter sequences or pGL3 empty vectors using the Lipofectamine 3000 reagent. After 24 h, the relative luciferase reporter activity was determined using Dual-Luciferase Reporter Assay System (Promega, USA) in each sample.

Chromatin immunoprecipitation (ChIP) assays

A ChIP assay kit (Beyotime) was applied to examine the binding between HOXA6 and ZBTB12 promoter following the manufacturer's protocol. Briefly, 5×10^6 CAFs cells and 293T cells were allowed to cross-link using 1 % formaldehyde (Sigma-Aldrich) for ten minutes, followed by ultrasonic treatment and centrifugation at 4 °C, 30,237×g. Then the supernatant was collected and incubated with anti-IgG (negative control) or anti-HOXA6 at 4 °C for 12 h. Then the complexes were digested. Finally, the enrichment of the ZBTB12 promoter fragment was subject to RT-qPCR analysis.

Statistical analysis

Graph Pad Prism 8.0 was applied for data analysis. The results were shown as the mean ± SD from at least three replicates ($n = 3$). Student's t-test was used to analyze the statistical difference between two groups and one-way ANOVA followed by Tukey's *post hoc* test was applied for comparison of two or multiple groups. $p < 0.05$ indicated statistical significance.

RESULTS AND DISCUSSION

Astragaloside IV inhibits the promoting impact of CAFs on gastric cancer cell malignancy

CAFs are the predominant component of stroma in TME with different functions and gene expression patterns from NFs (25, 26). α-SMA, S100A4, Vimentin, and FAP are representative markers of CAFs. Studies have revealed that CAFs concurrently evolve with the tumor cells and a cancer-promoting phenotype was acquired, which provides support for their survival and facilitates cancer progression (26). They can affect cancer cell proliferation, tumor immunity, angiogenesis, ECM remodeling, and metastasis. Accumulating evidence suggested that CAFs also exert tumor-retarding effects in some systems (27, 28). The exploration of CAF biology promisingly contributes to the diagnosis and targeted therapy for gastrointestinal cancer (29). Lu *et al.* have reported that CNN1 upregulated in

CAFs is associated with adverse survival outcomes. CNN1 silencing in CAFs is revealed to enhance the sensitivity to 5-Fu in a gastric cancer mouse model (30). In our study, we isolated NFs and CAFs from gastric normal or cancer tissue samples, respectively. The NFs and CAFs were identified by immunofluorescence with S100A4 (Fig. 2a). Then we examined the levels of fibroblast markers. Western blot analysis showed that the expression of α-SMA, Vimentin, FSP, and S100A4 proteins in CAFs was significantly higher compared with the NFs ($p < 0.01$) (Fig. 2b,c).

Astragaloside IV, a main active component of astragalus, is reported with antitumor activities against various tumors including colorectal cancer, hepatocellular carcinoma, and breast carcinoma (31–33). Additionally, previous studies have also revealed that astragaloside

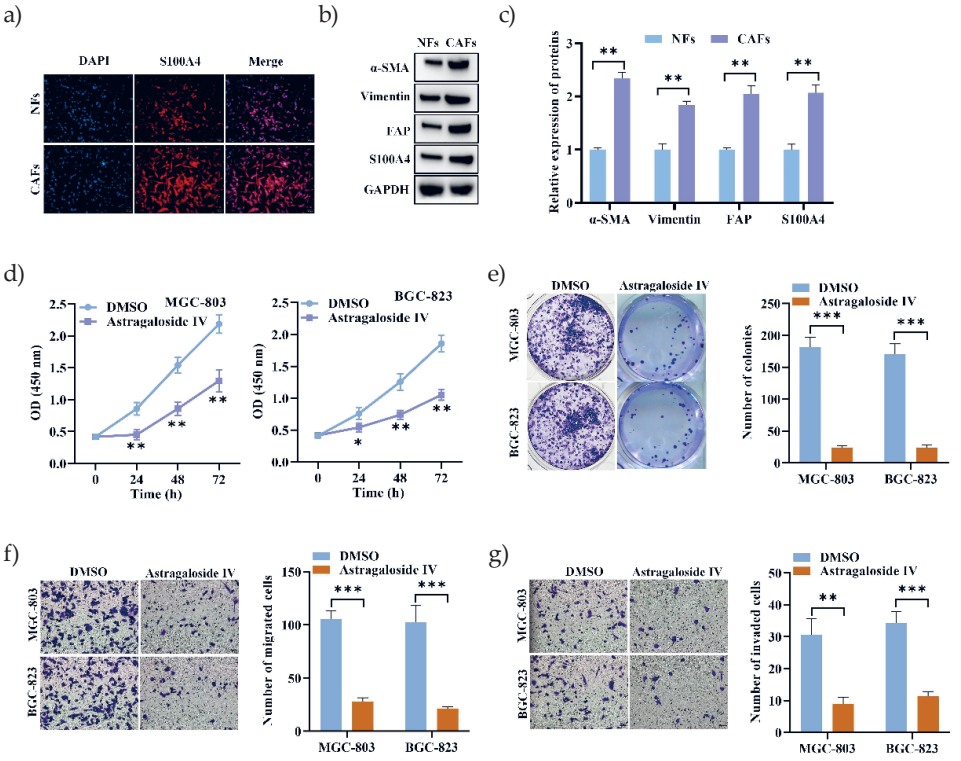

Fig. 2. Astragaloside IV treatment of CAFs inhibits gastric cancer cell malignancy *in vitro*. a) Immunofluorescence was conducted to identify NFs and CAFs marked by S100A4; b-c) Western blot was used to detect the protein expression of fibroblast markers (α-SMA, Vimentin, FSP, S100A4) in NFs and CAFs; d) CCK-8 assays were performed to examine the viability of BGC-823 and MGC803 cells cultured with CAFs treated with 40 μmol L^{-1} astragaloside IV or 0.2 % DMSO for 72 h; e) colony formation assays were used to evaluate the proliferation of gastric cancer cells in each group; f) Transwell assays were conducted to examine the migration ability of gastric cancer cells under indicated treatment; g) the invasion ability of gastric cancer cells was detected using Transwell assays; $^{**}p < 0.01$, $^{***}p < 0.001$.

IV directly inhibited gastric cancer progression. For example, astragaloside IV suppressed cell growth, migration, invasiveness as well as tumorigenesis by modulating the circDLST/miR-489-3p/EIF4A1 axis in gastric cancer (22). Astragaloside IV is also demonstrated to inhibit the EMT process of gastric cancer cells induced by TGF-β1 *via* the inactivation of the PI3K/Akt/NF-κB pathway (34). Astragaloside IV suppresses the pathological functions of CAFs, which are revealed to induce cell growth, migration, and invasiveness in gastric cancer (23). The impact and mechanism of astragaloside IV on gastric cancer cells were further investigated in our study. CAFs were stimulated by 40 μmol L^{-1} astragaloside IV or 0.2 % DMSO and cultured with MGC803 and BGC-823 cell lines for 72 h. CCK-8 assays showed that the viability of BGC-823 and MGC803 cells was reduced by half following the astragaloside IV treatment compared with the DMSO treatment ($p < 0.01$) (Fig. 2d). Consistently, colony formation assays also revealed that the number of colonies in the astragaloside IV group was over 7 folds the size of the DMSO group, which suggested that astragaloside IV treatment significantly inhibited the proliferation potential of BGC-823 and MGC803 cells ($p < 0.001$) (Fig. 2e). Moreover, Transwell assays demonstrated that astragaloside IV stimulation on CAFs significantly reduced the migrated cell number by over 70 % and decreased the invaded cell number by over 60 %, which indicated that astragaloside IV suppressed the migration ability and invasiveness of BGC-823 and MGC803 cells *in vitro* ($p < 0.001$) (Fig. 2f,g). The results of our study were consistent with the previous findings.

Astragaloside IV downregulates HOXA6 and ZBTB12 in CAFs in gastric cancer

Based on the analysis of the GSE62740 dataset from the GEO database, we identified that HOXA6 (adj.P.Val=0.00281, logFC=2.82) and ZBTB12 (adj.P.Val=0.00281, logFC=1.51) were both highly expressed in the gastric cancer CAFs (Fig. 3a,b). The UALCAN database also predicted that ZBTB12 was expressed at high levels in gastric cancer tissues (Fig. 3c). Furthermore, we detected the expression of HOXA6 and ZBTB12 in NFs and CAFs. RT-qPCR analysis showed that the mRNA expression of HOXA6 in CAFs was over one-fold higher than NFs ($p < 0.001$) (Fig. 3d). Consistently, Western blot analysis revealed that the protein level of HOXA6 in CAFs was nearly three times higher than that in NFs ($p < 0.001$) (Fig. 3e). RT-qPCR analysis showed that ZBTB12 mRNA expression was also elevated by over 50 % in CAFs than that in NFs ($p < 0.001$) (Fig. 3f), and Western blot analysis indicated that the protein expression of ZBTB12 in the CAFs group was nearly two folds higher than that in the NFs ($p < 0.001$) (Fig. 3g). The treatment of astragaloside IV significantly downregulated HOXA6 and ZBTB12 expression in a concentration-dependent manner in CAFs ($p < 0.001$) (Fig. 3h,i).

HOXA6-silenced CAFs inhibit gastric cancer cell malignancy

HOXA6 is reported as an oncogene that promotes tumor cell proliferative and migration ability in multiple cancers such as glioma, clear cell renal cell carcinoma, and colorectal cancer (35–37). HOXA6 also promotes the malignant progression of gastric cancer by interacting with PBX2 (24). ZBTB12 is a BTB (BR-C, ttk, and bab) domain-containing zinc finger protein present in human embryonic stem cell nuclei and can balance self-renewal and differentiation (38). We then explored the role of HOXA6 in CAF-mediated gastric cancer progression. HOXA6 was silenced by shRNA-1/-2, and RT-qPCR analysis showed that the expression of HOXA6 was identified to be significantly reduced by over 70 % in CAFs transfected with sh-HOXA6-1/-2 relative to the sh-NC group ($p < 0.001$) (Fig. 4a). Then

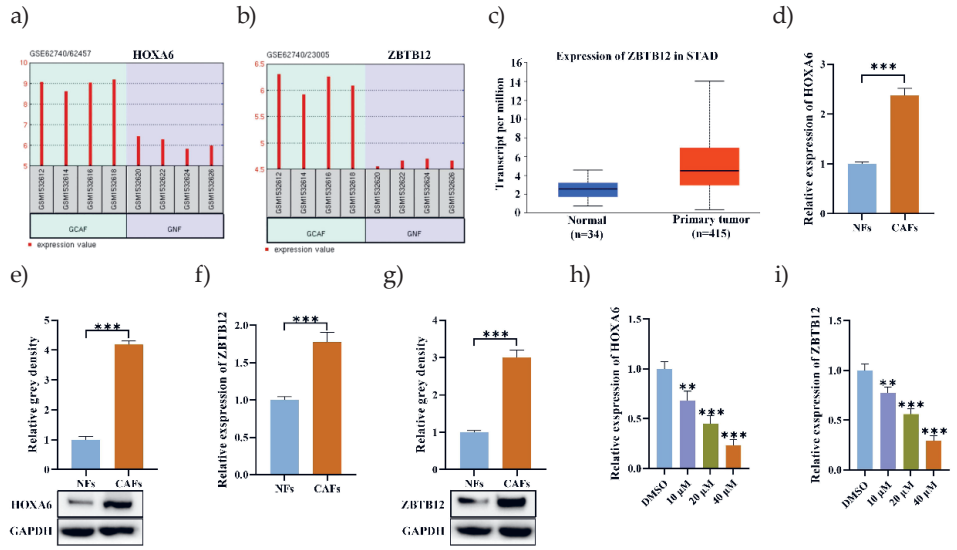

Fig. 3. Astragaloside IV downregulates HOXA6 and ZBTB12 in CAFs in gastric cancer. Expression profile of a) HOXA6 and b) ZBTB12 based on the GSE62740 dataset from the GEO database (https://www.ncbi.nlm.nih.gov/geo/); c) expression pattern of ZBTB12 in the gastric cancer tissues (STAD, stomach adenocarcinoma) and adjacent normal tissues based on the UALCAN database (http://ualcan.path.uab.edu/); d) RT-qPCR was used to detect the expression of HOXA6 in NFs and CAFs. e) Western blot was conducted to examine the protein levels of HOXA6 in NFs and CAFs; f) RT-qPCR was used to detect the expression of ZBTB12 in NFs and CAFs; g) the protein expression of ZBTB12 in NFs and CAFs was examined by Western blot. h) RT-qPCR was used to detect the expression of HOXA6 in CAFs treated with 0.2 % DMSO or different concentrations of astragaloside IV (10, 20, 40 μM); g) i) RT-qPCR was used to detect the expression of ZBTB12 in CAFs under indicated treatment; $^{**}p < 0.01$.

we cocultured the HOXA6-silenced CAFs with BGC-823 and MGC803 cells. The results of CCK-8 and colony formation assays revealed that the viability and proliferative ability of gastric cancer cells were significantly inhibited after coculturing with HOXA6-silenced CAFs ($p < 0.001$) (Fig. 4b,c). Moreover, Transwell assays were used to detect the impact of HOXA6-silenced CAFs on migration and invasiveness of BGC-823 and MGC803 cells, and the results showed that the number of migrated and invaded gastric cancer cells in the sh-NC group was over 4 folds higher than that in the sh-HOXA6-1/-2 groups ($p < 0.001$), which uncovered that the promoting impact of CAFs on gastric cancer cell migration and invasiveness was inhibited by HOXA6 knockdown (Fig. 4d,e). Overall, the results indicated that HOXA6 silencing counteracted the oncogenic impact of CAFs on gastric cancer.

Astragaloside IV suppressed the oncogenic impact of CAFs on gastric cancer cell malignancy by regulating HOXA6

Since we revealed that HOXA6 silencing counteracted the CAF-mediated oncogenic effects on gastric cancer cells, whether HOXA6 was implicated in the astragaloside IV-

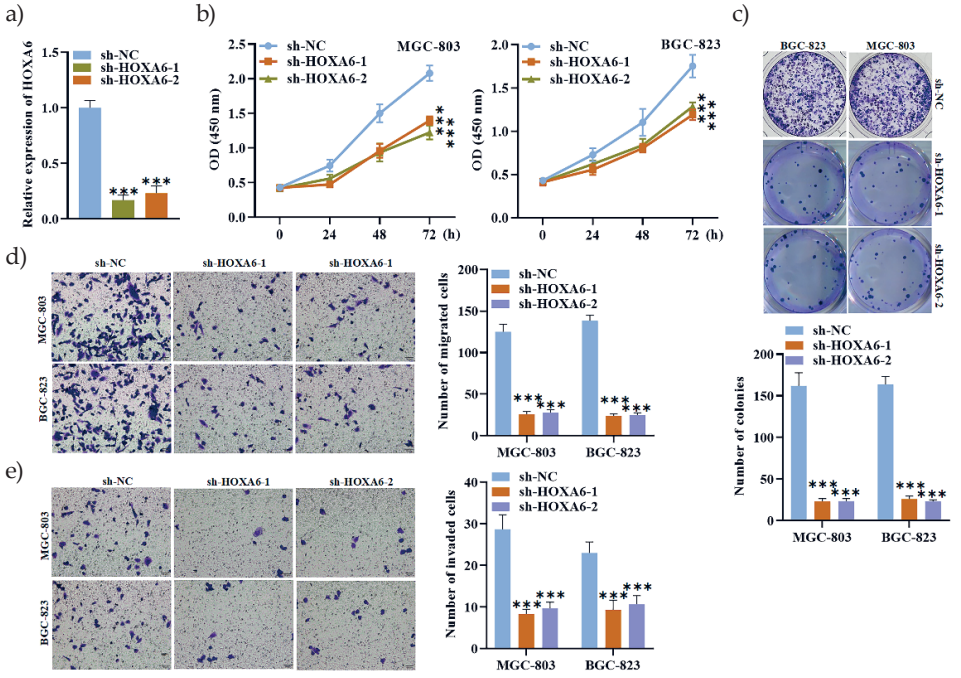

Fig. 4. HOXA6-silenced CAFs inhibits malignant phenotypes of gastric cancer cells. a) RT-qPCR was used to detect the expression of HOXA6 in CAFs transfected with shRNA-1/-2; b) CCK-8 assays were performed to detect the viability of gastric cancer cells cocultured with HOXA6-silenced CAFs; c) colony formation assays were used to detect the proliferation ability of gastric cancer cells under indicated treatments; d) Transwell assays were conducted to detect the impact of HOXA6-silenced CAFs on gastric cancer cell migration; e) Transwell assays were performed to determine the invasion ability of gastric cancer cells in each group; $^{**}p < 0.01$, $^{***}p < 0.001$.

mediated inhibitory effects on CAFs and consequent gastric cancer cell malignancy was explored. The overexpression efficacy of HOXA6 was identified by RT-qPCR analysis in the CAFs, and the expression of HOXA6 in the pcDNA3.1-HOXA6 group was over 10 folds higher than that in the pcDNA3.1 group ($p < 0.001$) (Fig. 5a). The effects of HOXA6 overexpression on cell malignant behaviors in gastric cancer were investigated. We demonstrated that HOXA6 overexpression in CAFs dramatically facilitated the viability and proliferation of cocultured gastric cancer cell lines ($p < 0.001$), and the treatment of astragaloside IV on CAFs was demonstrated to reverse this increase induced by HOXA6-overexpressed CAFs ($p < 0.001$) (Fig. 5b,c). Moreover, we also found that the number of migrated and invaded gastric cancer cells showed a significant increase by nearly two folds after overexpressing HOXA6 in the cocultured CAFs ($p < 0.001$), which was evidently counteracted by the treatment of astragaloside IV on CAFs ($p < 0.001$) (Fig. 5d,e). Overall, the results indicated that astragaloside IV inhibited the effects CAFs on gastric cancer cell malignancy by downregulating HOXA6.

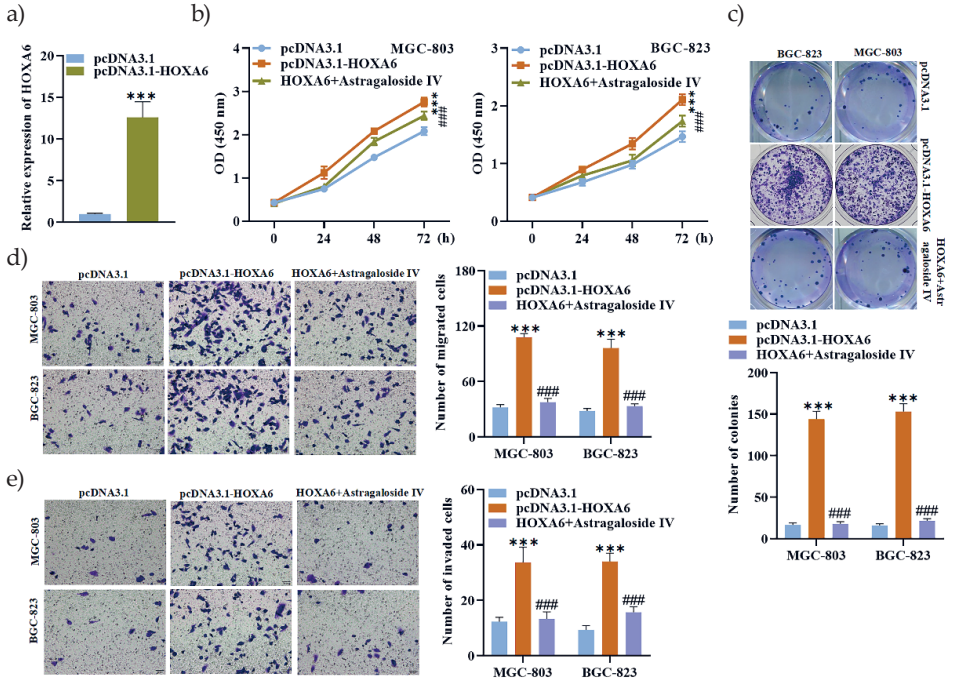

Fig. 5. HOXA6 mediated the suppression of astragaloside IV on oncogenic effects of CAFs on gastric cancer cell malignancy. a) RT-qPCR was used to detect the expression of HOXA6 in CAFs after indicated transfection; b) CCK-8 assays were used to detect the viability of gastric cancer cells in each group; c) colony formation assays were performed to detect the proliferation of gastric cancer cells after indicated treatments; d) Transwell assays were performed to assess the migration ability of gastric cancer cells in each group; e) Transwell assays were used to detect gastric cancer cell invasion in each group; $^{**}p < 0.01$.

ZBTB12-silenced CAFs inhibited the malignant phenotypes of gastric cancer cells

The role of ZBTB12 in cancer progression is rarely reported. A study has found that ZBTB12 chr6_31975605 variants are associated with ER-positive breast cancer in the Chinese Han population (39). In this study, we further investigated the role of ZBTB12 in CAF-mediated gastric cancer cell malignancy. RT-qPCR analysis revealed that the expression of ZBTB12 was evidently reduced by nearly 80 % using the shRNAs targeting ZBTB12 in CAFs relative to the sh-NC group ($p < 0.001$) (Fig. 6a). After coculturing the ZBTB12--silenced CAFs with BGC-823 and MGC803 cells, CCK-8 and colony formation assays indicated that the viability and proliferation potential of gastric cancer cells exhibited significant reduction compared with those cultured with the CAFs transfected with sh-NC ($p < 0.001$) (Fig. 6b,c). Additionally, Transwell assays showed that ZBTB12-silenced CAFs also decrease the number of invaded and migrated gastric cancer cells by nearly 70 % compared with the sh-NC group ($p < 0.001$) (Fig. 6d,e). Therefore, it was indicated that CAFs promoted gastric cancer malignancy by modulating ZBTB12.

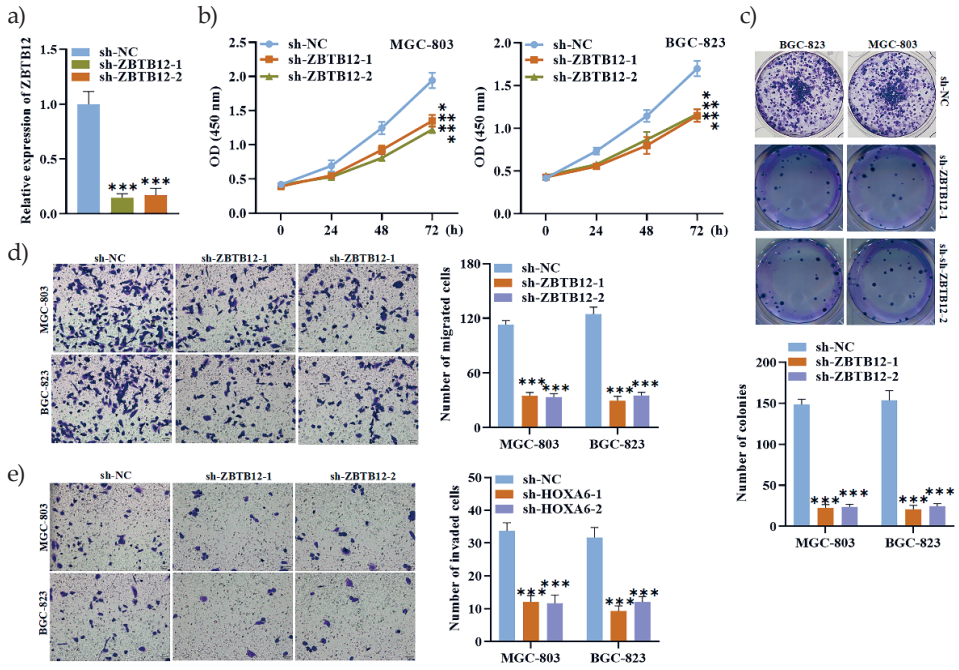

Fig. 6. ZBTB12-silenced CAFs inhibited the malignant phenotypes of gastric cancer cells. a) RT-qPCR was used to detect the expression of ZBTB12 in CAFs transfected with sh-ZBTB12-1/-2; b) CCK-8 assays were used to detect the effects of ZBTB12-silenced CAFs on the viability of gastric cancer cells; c) colony formation assays were performed to detect the proliferation of gastric cancer cells after coculturing with ZBTB12-silenced CAFs; d) Transwell assays were used to examine gastric cancer migration in each group; e) gastric cancer cell invasion in each group was detected using Transwell assays; $^{**}p < 0.01$, $^{***}p < 0.001$.

ZBTB12 was involved in astragaloside IV-mediated suppression on the promoting impact of CAFs on gastric cancer cell malignancy

As ZBTB12 silencing inhibited the promoting effects of CAFs on gastric cancer cells, the role of ZBTB12 in astragaloside IV-induced suppression of the oncogenic effects of CAFs was further analyzed. ZBTB12 was successfully overexpressed by pcDNA3.1/ZBTB12 in CAFs, and qRT-PCR analysis revealed that the expression of ZBTB12 in the pcDNA3.1-ZBTB12 group was over 15 folds higher than the pcDNA3.1 group ($p < 0.001$) (Fig. 7a). CCK-8 and colony formation assays showed that ZBTB12-overexpressed CAFs significantly facilitated the growth of gastric cancer cells ($p < 0.001$), which was restored by the stimulation of astragaloside IV on CAFs ($p < 0.001$) (Fig. 7b,c). Additionally, Transwell assays indicated that the number of migrated gastric cancer cells was elevated by over 2 folds by ZBTB12 overexpression in cocultured CAFs ($p < 0.001$), which was demonstrated to be rescued by astragaloside IV treatment on CAFs ($p < 0.001$) (Fig. 7d). Similarly, gastric cancer cell invasion enhanced by ZBTB12-overexpressed CAFs was counteracted after treatment of astragaloside IV ($p < 0.001$) (Fig. 7e).

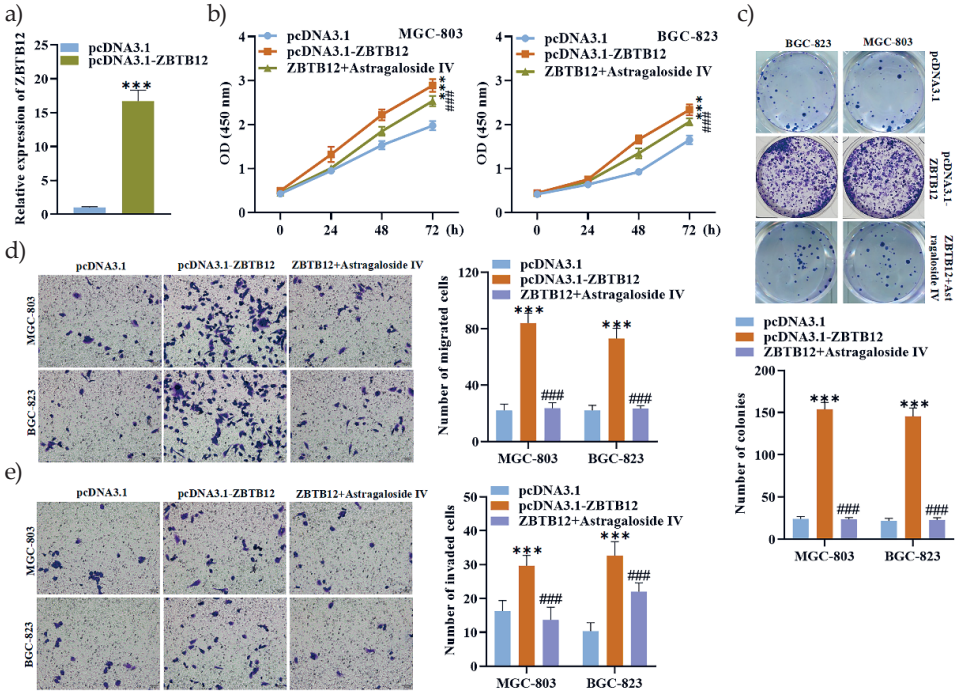

Fig. 7. Astragaloside IV reversed the promoting effects of CAFs on gastric cancer by mediating ZBTB12. a) RT-qPCR was used to detect the expression of ZBTB12 in CAFs transfected with pcDNA3.1/ZBTB12 or empty pcDNA3.1 vector; b) the viability of gastric cancer cells cocultured with transfected CAFs in the presence or absence of astragaloside IV was detected using CCK-8 assays; c) colony formation assays were performed to examine the proliferation of gastric cancer cells under indicated treatments; d) Transwell assays were performed to examine the migration ability of gastric cancer cells in each group; e) Transwell assays were used to detect the invasion ability of gastric cancer cells under indicated treatments; $^{**}p < 0.01$.

Astragaloside IV downregulated ZBTB12 by inhibiting HOXA6 transcriptional activity

The regulatory mechanism of astragaloside IV was further explored. Previous studies have suggested that HOXA6 belongs to the homeobox (HOX) transcription factor family (24, 40). We then examined whether HOXA6 transcriptionally regulated ZBTB12. Based on the hTFtarget database, HOXA6 was predicted to be a transcriptional factor for ZBTB12 (Fig. 8a). Then we examined the mRNA expression of ZBTB12 in CAFs, and the results of RT-qPCR analysis showed that ZBTB12 was significantly downregulated nearly half by silencing HOXA6 in CAFs relative to the sh-NC group ($p < 0.001$) (Fig. 8b). Similarly, Western blot analysis showed that ZBTB12 protein levels also exhibited significant decrease by nearly 50 % in CAFs transfected with sh-HOXA6 compared with the sh-NC group ($p < 0.001$) (Fig. 8c). Moreover, luciferase reporter assays revealed that the luciferase activities of ZBTB12 promoter showed evident elevation after HOXA6 overexpression in 293T cells and CAFs ($p < 0.001$), which indicated the binding between ZBTB12 promoter and

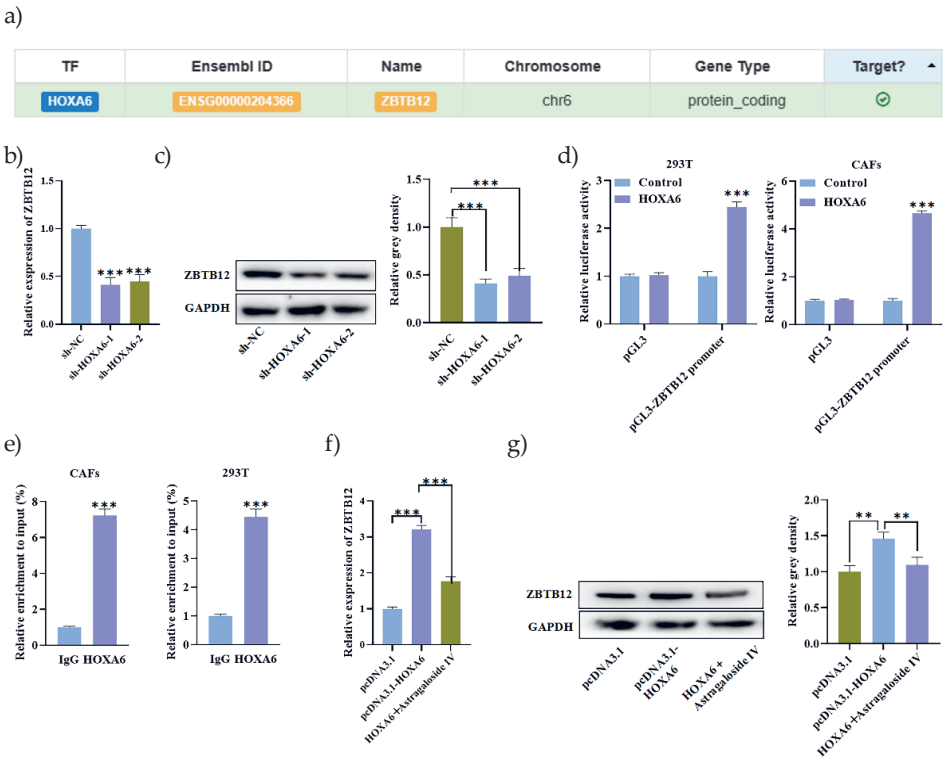

Fig. 8. Astragaloside IV downregulated ZBTB12 by inhibiting the transcriptional activity of HOXA6. a) The hTFtarget database (http://bioinfo.life.hust.edu.cn/hTFtarget#!/) was used to explore whether ZBTB12 was transcriptionally regulated by HOXA6; b) RT-qPCR and c) Western blot was used to examine the mRNA and protein expression of ZBTB12 in HOXA6-silenced CAFs; d) Luciferase reporter assays were performed to detect the transcriptional regulation of HOXA6 on ZBTB12 in 293T cells and CAFs; e) ChIP assays were conducted to explore the binding of ZBTB12 promoter and HOXA6 in 293T cells and CAFs; f) RT-qPCR and g) Western blot were used to detect the mRNA and protein expression of ZBTB12 in CAFs under indicated treatments; $^{**}p < 0.01$.

HOXA6 (Fig. 8d). Similarly, the results of ChIP assays demonstrated the enrichment of ZBTB12 promoter was 3–5 folds higher in the precipitates of HOXA6 than that in the IgG group ($p < 0.001$), which verified that HOXA6 bound with ZBTB12 promoter in 293T cells and CAFs (Fig. 8e). The results of RT-qPCR and Western blot analyses showed that ZBTB12 mRNA expression was upregulated by HOXA6 overexpression by nearly two folds ($p < 0.001$) and its protein levels were increased by nearly a half of the pcDNA3.1 group ($p < 0.01$), which were both significantly reduced by the treatment of astragaloside IV ($p < 0.001$, $p < 0.01$), suggesting that astragaloside IV suppressed the transcriptional activity of HOXA6 to downregulate ZBTB12 in CAFs (Fig. 8f,g).

CONCLUSIONS

Astragaloside IV inhibited cell proliferative potential, migration as well as invasiveness in gastric cancer by inhibiting the tumor-promoting effects of CAFs *via* the HOXA6 / ZBTB12 axis. The results of our work might provide clues to the therapeutic value of astragaloside IV for gastric cancer and its underlying molecular mechanism.

Conflicts of interest. – The authors declare no conflict of interest.

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Authors contributions. – Conceptualization, Z.C. and D.Y.; methodology, H.L.; analysis X.S.; investigation, H.L., S.L., and X.S.; writing, original draft preparation, H.L.; writing, review and editing, Z.C. and D.Y. All authors have read and agreed to the published version of the manuscript.

Ethics statement. – The Ethics Committee of the Hongze District People's Hospital of Huai'an City Hospital has approved this study involving human gastric tissues. Written informed consent had been signed by all participants.

Data availability. – Original data are available from corresponding authors by reasonable requirements.

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