Apatinib Suppresses Gastric Cancer Stem Cells Properties by Inhibiting Sonic Hedgehog Pathway

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Research

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Abstract

Background Gastric cancer stem cells (GCSCs) are considered as the basis of gastric carcinoma onset. Sonic Hedgehog (SHH) pathway plays a crucial role in maintaining GCSCs characteristics. Apatinib, a highly selective vascular endothelial growth factor receptor 2 (VEGFR-2) inhibitor, has been approved in China for the treatment of advanced gastric cancer after the failure of second-line therapy. To data, the effects of apatinib on GCSCs and the underlying mechanisms have not been elucidated yet.

Methods Tumorsphere formation assay was used to enrich GCSCs from BGC-823 and SGC-7901 cells and the number of CD133-positive cells was examined by flow cytometry. Western blotting, immunofluorescence and immunohistochemistry assays were used to determine protein expression. Cell proliferation and apoptosis of GCSCs after apatinib treatment were evaluated by CCK8, colony formation, flow cytometry and Hoechst 33258 assays. Transient transfection assay was used to upregulate Gli1 expression in GCSCs. Nude mouse xenograft was used to investigate the suppressive effects of apatinib in vivo.

Results We revealed that the levels of GCSCs markers and the number of CD133-positive cells were significantly elevated in the sphere-forming cells. We further illustrated that apatinib efficiently abolished GCSCs traits, as manifested by inhibition of tumorsphere formation, decreased expression of GCSCs markers, reduced number of CD133-positive cells, drug resistance protein suppression as well as proliferation inhibition and apoptosis induction. Furthermore, we found that apatinib downregulated the activation of SHH pathway, while upregulation of SHH pathway attenuated the inhibitory effects of apatinib on GCSCs. Furthermore, apatinib treatment significantly delayed tumor growth and inhibited GCSCs characteristics in xenograft model.

Conclusions Taken together, our data suggested that apatinib exhibited its inhibitory effects on GCSCs via suppression of SHH pathway. These findings could provide new insights into the therapeutic application of apatinib in GCSCs suppression and advanced gastric cancer treatment.

Background

Gastric cancer is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide, with half of all cases occurring in eastern Asia[1]. Almost two thirds of patients present with locally advanced or metastatic disease at diagnosis because the early stages are clinically silent[2]. The palliative chemotherapy can induce tumor regression in some patients, while most therapies are ultimately followed by relapse and the recurrent cancer being highly resistant to further chemotherapy[3]. As a result, a 5-year survival rate of gastric cancer is less than 10% and a median overall survival limits to 12 months[2]. Hence, there is a real need for more effective therapies that counteract cancer relapse in advanced gastric cancer.

Apatinib is an oral small-molecule tyrosine kinase inhibitor that highly selectively binds to VEGFR-2, which is a critical player in the progress of tumor angiogenesis[4]. Based on the phase III trial in China[5],

apatinib significantly prolonged median progression-free survival and overall survival compared with placebo in patients with advanced gastric cancer or gastroesophageal junction adenocarcinoma for whom at least two lines of prior chemotherapy had failed. Besides, apatinib also exhibits promising therapeutic effects against epithelial ovarian cancer[6], glioma[7], colorectal cancer[8] and hepatocellular carcinoma[9] in clinical trials. However, the antitumor activity and potential molecular mechanism of apatinib remain to be elucidated.

Cancer stem cells (CSCs) are defined as subpopulations of cells within a tumor that possess the capacity for self-renewal and differentiation[10]. It is well acknowledged that CSCs are responsible for the initiation, metastasis, drug resistance and recurrence of tumors[11]. GCSCs were first described in 2007 by Yang et al[12]. Accumulating evidence indicates that GCSCs are the root of gastric cancer development and progression [13–17]. Moreover, chemotherapy has been found to induce cancer cell stemness[18]. Resistance to chemotherapy may result in CSCs enrichment during anti-cancer treatments[19]. Thus, targeting GCSCs could be an effective therapeutic strategy for advanced gastric cancer treatment, especially in the relapse and recurrence of gastric cancer.

The aberrant activation of SHH pathway has been implicated in contributing to tumorigenesis through the maintenance of CSCs, including GCSCs[20–22]. SHH pathway is initiated by the binding of SHH protein to its receptor Patched (PTCH). When inactive, PTCH suppresses the signal transduction by integrating the transmembrane protein Smoothed (Smo). Upon SHH binding, the inhibitory effect of PTCH on Smo is relieved, resulting in the nuclear translocation of glioma-associated oncogenes (Gli) transcription factors, ultimately regulating the downstream genes related to cell stemness, proliferation and apoptosis.

Although the inhibitory effect of apatinib on angiogenesis has been determined, whether apatinib could affect GCSCs in gastric cancer remains unclear. Therefore, the present study aimed to investigate the suppressive action of apatinib on targeting GCSCs and SHH pathway in this process.

Materials And Methods

Cell culture and reagents

Gastric cancer cell lines BGC-823 and SGC-7901 were purchased from Chinese Academy of Typical Culture Collection Cell Bank (Shanghai, China). Both cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in the condition of 37 °C with 5% CO₂.

Apatinib was obtained from Jiangsu Hengrui Medicine Co., Ltd (Lianyungang, China). Purmorphamine was acquired from Selleckchem (Houston, TX, USA). Vismodegib was purchased from MCE (Junction, New Jersey, USA). The growth factors, epidermal growth factor (EGF), basic fibroblast growth factor

(bFGF) and insulin were obtained from Peprotech (Rocky Hill, NJ, USA). 2% B27 was obtained from Gibco.

Tumor sphere-formation assay

Tumorspheres were generated by seeding BGC-823 and SGC-7901 cells in 6-well ultralow attachment plates with a serum-free DMEM/F12 medium (SFM) (Gibco). The SFM of BGC-823 cells was supplemented with 5 ng/mL EGF and 2% B27, and the medium of SGC-7901 included 20 ng/mL bFGF, 20 ng/mL EGF, 2% B27 and 5 µg/mL insulin. The spheroids were grown for 6 days and fed every 48 h. The images of representative fields were acquired under a microscope (Nikon, Japan).

To analyze the effect of apatinib on GCSCs, both sphere-forming cells were treated with different concentrations of apatinib (0, 1, 2, 5, 10 µM). Six days after treatment, the tumorspheres of each group were imaged and examined.

Western blotting

Cells were collected and followed by lysing in RIPA buffer (Beyotime, Shanghai, China) containing protease inhibitors. Protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, WI, USA). Total protein (40–60 µg per sample) was then loaded for sodium dodecyl sulfate gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with relevant primary antibodies overnight at 4 °C, followed by the horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO, Beijing, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as the loading control. Antibodies for CD133, CD44, Oct4, Sox2, Nanog, epithelial cellular adhesion molecule (EpCAM), P glycoprotein(P-gp), ABCC1, VEGFR-2, SHH, Smo, Gli1, Gli2, PCNA, Cyclin D1, Bcl-2, Bax, Cleaved Caspase (8,9,3) and GAPDH were purchased from Proteintech (Rosemont, IL, USA).

Immunofluorescence staining

The cell spheroids were washed with phosphate buffer saline supplemented with 0.5% Tween20 (PBST) and fixed in methyl alcohol. Subsequently, the spheroids were blocked with 5% bovine serum albumin (BSA) for 2 hours at room temperature. Cells were then incubated with rabbit-anti CD133 and EpCAM antibodies (1:100) at 4 °C overnight. After being washed with PBST, cells were stained with cy3-conjugated goat anti-rabbit IgG (1:200) (Beyotime, Shanghai, China). 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) used to stain the nucleus for 15 min. The fluorescent images were captured with a fluorescence microscope (Olympus IX-70, Tokyo, Japan).

Transient transfection

BGC-823 and SGC-7901 cells were seeded into 6-well plates at a density of 2×10^5 cells in RPMI-1640 medium containing 10% FBS without antibiotics. Transfection of EX-NEG-M29-Gli1 (2 µg) and the corresponding control vector EX-NEG-M29 (2 µg) was performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's procedures. Following 6 h of

transfection, cells were trypsinized and then cultivated in SFM overnight. Subsequently, cells were treated with or without apatinib for another 4 days. Cell lysates were used for the measurements of the indicated protein levels.

Colony formation assay

The sphere-forming cells were suspended and separated to single cells. Cells were seeded into 6-well plates (500 cells per well) and cultured in RPMI-1640 medium containing 10% FBS and antibiotics for 13 days after treatment. Colonies were fixed with 10% cold formaldehyde for 10 min, followed by incubating with crystal violet at room temperature for 10 min. Stained cells were then photographed under a microscope (Olympus, Tokyo, Japan).

Cell viability

The cell spheroids were treated with indicated concentrations of apatinib. After 6 days of incubation, cell viability was evaluated by using Cell Counting Kit 8 (CCK-8) (Beyotime, Shanghai, China) according to the manufacturer's instructions. The absorbance at 450 nm was quantified by a multimode reader Infinite M200 Pro (Tecan, Männedorf, Switzerland).

Detection of CD133 positive cells by flow cytometry

After collected by centrifuge, the adherent cells and cell spheroids were washed twice with PBS. A 1×10^6 single-cell suspension was stained with 1 μ L APC-conjugated human monoclonal CD133 antibody (Miltenyi Biotec, Teterow, Germany) at 4 °C for 10 min in the dark. Immunoglobulin G (IgG) isotype antibody (Miltenyi Biotec, Teterow, Germany) was used as a negative control. The stained samples were analyzed using the FACS Aria III system (Becton-Dickinson, San Jose, CA, USA).

Detection of apoptotic cells by flow cytometry

After exposure to different concentrations of apatinib for 4 days, the sphere-forming cells were harvested and resuspended in 400 μ L binding buffer. The cells were then stained with 5 μ L Annexin V-FITC at 4 °C in the dark for 15 min, followed by incubating with 5 μ L propidium iodide (PI) at room temperature in the dark for 5 min. Subsequently, stained cells were subjected to flow cytometry analysis within 1 h. Both PI negative/Annexin V positive and PI positive/Annexin V positive cells were defined as apoptotic cells.

Hoechst 33258 staining

Sphere-forming cells were treated with various concentrations of apatinib. After treatment, the fixed cells were stained with 5 μ g/mL Hoechst 33258 solution (Beyotime, Shanghai, China) according to the manufacturer's instructions. The tumorspheres were then imaged using a reverse fluorescence microscope with excitation at 350 nm and emission at 460 nm.

Apatinib treatment in xenograft model

Fifteen male BALB/c nude mice aged 4 weeks and weighed 17–22 g were purchased from the Animal Core Facility of Nanjing Medical University. Animals received humane care and all experiments were

performed in accordance with the guidelines of the Animal Care and Welfare Committee of Nanjing Medical University (IACUC-1907002). 5×10^6 BGC-823 cells were suspended in 0.1 mL PBS and implanted subcutaneously into the right flank region of nude mice. Body weight and tumor volume were measured every 2 days. The tumor volume (V) was calculated according to the following formula: $V = 0.5 \times \text{length} \times \text{width}^2$. To determine the effects of apatinib on tumor growth, fifteen mice bearing gastric tumor of approximately 140mm^3 (7 days after implantation) were randomized into three groups: (a) vehicle-only solution; (b) 50 mg/kg body weight Apatinib; (c) 100 mg/kg body weight Apatinib. Vehicle-only solution or doses of apatinib suspended in carboxymethylcellulose sodium salt were administered via oral gavage once daily for 14 days. At harvest, all mice were sacrificed and tumor tissues were collected and fixed in 4% formalin or stored at -80°C for further analysis.

Immunohistochemistry

A series of 4 μm sections were obtained from each paraffin block. Incubation with rabbit anti-CD44, Sox2, EpCAM, VEGFR-2, Ki67, PCNA, P-gp (1:200) antibodies, and mouse anti-Gli1 (1:100) antibody overnight at 4°C was conducted. The above primary antibodies were purchased from Proteintech (Rosemont, IL, USA). The sections were then incubated with goat anti-mouse/rabbit-biotin at room temperature for one hour. Signals were amplified with Vectastain Elite ABC Kit. Finally, the slides were scanned with a Panoramic MIDI scanner. Three tumor sections for each animal were analyzed.

Statistical Analysis

Data were presented as Mean $\pm$ standard deviation (SD), and all experiments were performed in triplicate. Statistical differences were determined by a two-tailed student t-test between two groups. One-way ANOVA analysis of variance was carried out among multiple groups. GraphPad Prism 6.0 software was used for statistical analysis. Statistical significance was set at * $p < 0.05$ or ** $p < 0.01$.

Results

Enrichment of GCSCs by SFM culture in vitro

Tumor sphere-formation assay is a practical method for the isolation and enrichment of CSCs. In the present study, both BGC-823 and SGC-7901 cells formed stable tumorspheres with SFM culture (Fig. 1a). As shown in Fig. 1b, 6 days post SFM culture, the protein levels of GCSCs markers, including CD133, CD44, Oct4, Sox2, Nanog and EpCAM, were obviously upregulated compared with the adherent counterparts culturing in serum supplied medium (SSM). In addition, flow cytometry analysis showed an increased percentage of CD133⁺ cells in those sphere-forming cells (Fig. 1c). Collectively, these results suggested the characteristics of CSCs in BGC-823 and SGC-7901 sphere-forming cells.

Apatinib suppressed the stemness of GCSCs

We then examined the effects of apatinib on gastric cancer cell spheroids. It was shown that apatinib treatment led to a decrease of spheroids size in a dose-dependent manner (Fig. 2a). With increasing apatinib concentrations, the protein expression of GCSCs markers was gradually reduced in both cell lines spheroids. Meanwhile, apatinib treatment resulted in the downregulation of P-gp, ABCC1 and VEGFR-2 (Fig. 2b). The percentage of CD133⁺ cells was decreased from 67.14–38.26% in BGC-823 spheroids (Fig. 2c, d). Besides, immunofluorescence staining also showed similar results for the protein levels of CD133 and EpCAM (Fig. 2e, f). Together, these data revealed that apatinib diminished the traits of GCSCs.

Apatinib inhibited the proliferation of GCSCs

To further determine the inhibitory effects of apatinib, we examined the proliferation of GCSCs following apatinib treatment. As shown in Fig. 3a, apatinib markedly reduced the expression of cell proliferation proteins PCNA and Cyclin D1. Apatinib suppressed the formation of colonies in BGC-823 and SGC-7901 sphere-forming cells (Fig. 3b). Moreover, cell viability of sphere-forming cells was decreased to $35.04 \pm 2.92\%$ and $48.64 \pm 6.38\%$ at 10 μM apatinib treatment (Fig. 3c). These results suggested that apatinib suppressed the proliferation of GCSCs.

Apatinib induced apoptosis of GCSCs

We further examined the effect of apatinib on GCSCs apoptosis. Figure 4a showed that decreased level of anti-apoptosis protein Bcl2, and elevated pro-apoptotic proteins Bax and Caspases was observed. Hoechst 33258 staining confirmed the execution of apoptosis in sphere-forming cells following apatinib treatment (Fig. 4b). In addition, flow cytometry analysis indicated that apatinib increased the rate of cell apoptosis in both spheroids (Fig. 4c, d). Collectively, our data illustrated that apatinib induced apoptosis of GCSCs.

Apatinib downregulated SHH pathway in GCSCs

Given that SHH pathway plays a critical role in the stemness of CSCs, we then sought to examine whether apatinib affected the activation of SHH pathway. Our results showed that the SHH pathway components, including SHH, Smo, Gli1 and Gli2, were markedly upregulated in the sphere-forming cells (Fig. 5a). We also found that downregulation of SHH activation by vismodegib, a Smo inhibitor, led to the suppression of GCSCs markers' expression (Fig. 5b). Furthermore, apatinib significantly decreased the protein expression of SHH pathway molecules in the sphere-forming cells (Fig. 5c). These results suggested that apatinib inhibited the activation of SHH pathway in GCSCs.

Apatinib diminished GCSCs properties through suppression of SHH pathway

Next, we explored the role of SHH pathway in the inhibitory effects of apatinib on GCSCs. Purmorphamine, an activator of Smo, was used to activate SHH pathway. It was shown that purmorphamine facilitated tumor sphere formation and upregulated the levels of GCSCs markers. The suppressive effects of apatinib on tumor sphere formation, SHH pathway and GCSCs markers were attenuated by the combination treatment of apatinib with purmorphamine (Fig. 6a, b). Furthermore, transfection of Gli1 plasmids enhanced GCSCs activities as well as abolished apatinib-induced downregulation of CSCs markers in sphere-forming cells (Fig. 6c). Taken together, these results indicated that SHH pathway played an essential role in the inhibitory effects of apatinib on GCSCs.

Apatinib inhibited GCSCs traits in xenograft model

Finally, xenograft model was established to determine the effects of apatinib on gastric tumor in vivo. As shown in Fig. 7a-7c, apatinib markedly retarded gastric tumor growth in a dose-dependent manner. After 14 days apatinib treatment, the terminal tumor weight was statistically reduced (Fig. 7b). Compared with the control group, the tumor volume of 100 mg/kg apatinib group was remarkably decreased by 1.3 folds (from 2299.51 mm³ to 979.34 mm³) (Fig. 7c). Meanwhile, no significant difference was observed in mice body weight during the process of apatinib treatment (Fig. 7d). Western blotting further revealed that the protein levels of GCSCs markers, VEGFR-2 and drug resistance proteins were down-regulated in apatinib treatment, along with suppressed cell proliferation related proteins, increased apoptosis related proteins, and inhibition of the SHH pathway in gastric tumors (Fig. 7e). Moreover, immunohistochemistry assays (Fig. 7f) indicated that the expression levels of GCSCs markers as well as drug-resistant proteins were suppressed. Both VEGFR-2 and proliferation antigens (Ki67 and PCNA) were reduced after apatinib treatment. Meanwhile, the expression of Gli, the SHH pathway key molecule, was dramatically decreased in 100 mg/kg apatinib group compared with the control. These data were consistent with the in vitro results, which further depicted that apatinib repressed GCSCs traits and SHH pathway in vivo.

Discussion

Despite advances in chemotherapy and surgery, the prognosis of patients with advanced gastric cancer remains poor. Emerging evidence has shown that chemotherapy induces the enrichment of GCSCs which are highly associated with the degree of malignancy and drug resistance[23]. Apatinib has been approved as third-line treatment for metastatic gastric cancer in China. Regarding the anti-cancer mechanisms of apatinib, most researches focused on antiangiogenesis. In the present study, we revealed that apatinib suppressed GCSCs properties and downregulation of SHH pathway in vitro and vivo.

SFM culture, which is based on the capability of CSCs to form three-dimensional spheres under serum-free culture condition in vitro, has been widely used for the isolation and enrichment of CSCs [23]. Combined detection of CD133 and CD44 expression is found to be a tool for the clinical prediction and diagnosis of gastric cancer [15, 24]. It is reported that EpCAM(+)/CD44(+) cells grow exponentially in vitro as cancer spheres in SFM [25]. Furthermore, pluripotent stem cell factors like Oct4, Sox2 and Nanog also regulate GCSCs properties[13, 26]. In the present study, we showed significantly elevated tumorsphere

formation capacity of both BGC-823 and SGC-7901 cells cultured in SFM, as well as markedly increased expression levels of GCSCs markers in the sphere-forming cells, which depicted the enrichment of GCSCs.

Increasing evidence indicates that non-stem cancer cells can acquire stemness under some conditions, including chemotherapy[3, 18]. CSCs are subpopulations of cells that are capable of maintaining tumor growth, metastasis, chemotherapy resistance and recurrence [16]. As the first generation of oral antiangiogenesis drug approved in China, apatinib has demonstrated acceptable safety, tolerability and efficacy in the treatment of advanced gastric carcinoma[5, 27], which is consistent with the results from our xenograft model. It has been reported that apatinib could enhance the efficacy of conventional chemotherapeutical drugs in cancer stem-like leukemia cells[28]. However, no study has been conducted yet to examine the anti-GCSCs properties of apatinib. Therefore, we determined the effects of apatinib on GCSCs characteristics. Our data illustrated that apatinib effectively diminished GCSCs properties by inhibiting tumorsphere formation capacity, GCSCs markers expression, drug-resistant proteins expression and VEGFR-2 expression, along with a decreased number of CD133⁺ cells. Besides, apatinib also suppressed the proliferation and induced apoptosis of GCSCs in vitro and in vivo.

The SHH signaling plays a pivotal role in stemness maintenance and tumorigenesis[29]. Once SHH pathway activated, transcription factors Gli transmit the cytoplasmic signal and enhance the transcription of downstream target genes[30]. Dysregulation of SHH pathway is frequently identified in gastric cancers[31, 32]. Moreover, it has been revealed that SHH is critically involved in maintaining the characteristics of GCSCs[21, 22, 33]. In line with these findings, we showed that SHH pathway was highly activated in GCSCs, while SHH suppression abolished GCSCs traits, suggesting the essential role of SHH pathway in the stemness of GCSCs.

To data, the role of SHH pathway in the suppressive effects of apatinib on GCSCs has not been defined. We showed that apatinib impeded the activity of SHH pathway in GCSCs. Moreover, our data illustrated that activation of SHH by purmorphamine and Gli1-plasmid transfection abolished the effects of apatinib on tumorsphere formation, GCSCs markers' expression and SHH pathway activity in GCSCs. Collectively, these results revealed the interventional action of apatinib on GCSCs via SHH pathway inhibition. Moreover, in nude mice xenograft model, the SHH pathway components were significantly repressed after apatinib treatment. Further studies are warranted to investigate the detailed mechanisms implicated in apatinib regulation of SHH signaling.

Conclusion

In summary, our study demonstrated that apatinib inhibited the properties of GCSCs through downregulation of SHH pathway. These novel findings could provide important information for the therapeutic application of apatinib in GCSCs suppression and gastric cancer treatment.

Abbreviations

CSCs: Cancer stem cells; GCSCs: Gastric cancer stem cells; SHH: Sonic Hedgehog; PTCH: Patched; Smo: Smoothed; Gli: Glioma-associated oncogene; VEGFR-2: Vascular endothelial growth factor receptor 2; FBS: Fetal bovine serum; EGF: Epidermal growth factor; bFGF: basic fibroblast growth factor; SFM: Serum-free medium; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; EpCAM: Epithelial cellular adhesion molecule; P-gp: P glycoprotein; PBST: Phosphate buffer saline with Tween20; BSA: Bovine serum albumin; DAPI: 4', 6-diamidino-2-phenylindole; CCK8: Cell Counting Kit 8; PI: Propidium iodide; V: Volume; SD: standard deviation; SSM: Serum supplied medium

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Author contributions

C.Z., H.H. and G.G. conceived and designed the study and revised the manuscript; W.C. and Y.L. carried out the majority of the experiments, analyzed the data and wrote the manuscript; H.S., J. Z., C.X., X.L., J. W., S.G., L.W. and L.S. participated in conducting the experiments and analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animals were received humane care and all experiments were performed in accordance with the guidelines of the Animal Care and Welfare Committee of Nanjing Medical University (IACUC-1907002).

Consent for publication

All contributing authors agree to the publication of this article.

Competing interests

L.W., L.S., and G.G. are employees of Jiangsu Hengrui Medicine Co.,Ltd The other authors declare no competing interests.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin*. 2016;66(1):7–30.
2. Lordick F, Allum W, Carneiro F, Mitry E, Tabernero J, Tan P, et al. Unmet needs and challenges in gastric cancer: the way forward. *Cancer Treat Rev*. 2014;40(6):692–700.
3. Li Y, Rogoff HA, Keates S, Gao Y, Murikipudi S, Mikule K, et al. Suppression of cancer relapse and metastasis by inhibiting cancer stemness. *Proc Natl Acad Sci USA*. 2015;112(6):1839–44.
4. Jayson GC, Kerbel R, Ellis LM, Harris AL. Antiangiogenic therapy in oncology: current status and future directions. *Lancet*. 2016;388(10043):518–29.
5. Li J, Qin S, Xu J, Xiong J, Wu C, Bai Y, et al. Randomized, Double-Blind, Placebo-Controlled Phase III Trial of Apatinib in Patients With Chemotherapy-Refractory Advanced or Metastatic Adenocarcinoma of the Stomach or Gastroesophageal Junction. *J Clin Oncol*. 2016;34(13):1448–54.
6. Miao M, Deng G, Luo S, Zhou J, Chen L, Yang J, et al. A phase II study of apatinib in patients with recurrent epithelial ovarian cancer. *Gynecol Oncol*. 2018;148(2):286–90.
7. Wang L, Liang L, Yang T, Qiao Y, Xia Y, Liu L, et al. A pilot clinical study of apatinib plus irinotecan in patients with recurrent high-grade glioma: Clinical Trial/Experimental Study. *Med (Baltim)*. 2017;96(49):e9053.
8. Chen X, Qiu T, Zhu Y, Sun J, Li P, Wang B, et al. A Single-Arm, Phase II Study of Apatinib in Refractory Metastatic Colorectal Cancer. *Oncologist*. 2019;24(7):883-e407.
9. Lu W, Jin XL, Yang C, Du P, Jiang FQ, Ma JP, et al. Comparison of efficacy between TACE combined with apatinib and TACE alone in the treatment of intermediate and advanced hepatocellular carcinoma: A single-center randomized controlled trial. *Cancer Biol Ther*. 2017;18(6):433–8.
10. Batlle E, Clevers H. Cancer stem cells revisited. *Nat Med*. 2017;23(10):1124–34.
11. Tang QL, Zhao ZQ, Li JC, Liang Y, Yin JQ, Zou CY, et al. Salinomycin inhibits osteosarcoma by targeting its tumor stem cells. *Cancer letters*. 2011;311(1):113–21.
12. Yang YC, Wang SW, Hung HY, Chang CC, Wu IC, Huang YL, et al. Isolation and characterization of human gastric cell lines with stem cell phenotypes. *J Gastroenterol Hepatol*. 2007;22(9):1460–8.

13. Molina-Castro S, Pereira-Marques J, Figueiredo C, Machado JC, Varon C. Gastric cancer: Basic aspects. *Helicobacter*. 2017;22 Suppl 1.
14. Li K, Dan Z, Nie YQ. Gastric cancer stem cells in gastric carcinogenesis, progression, prevention and treatment. *World J Gastroenterol*. 2014;20(18):5420–6.
15. Brungs D, Aghmesheh M, Vine KL, Becker TM, Carolan MG, Ranson M. Gastric cancer stem cells: evidence, potential markers, and clinical implications. *J Gastroenterol*. 2016;51(4):313–26.
16. Singh SR. Gastric cancer stem cells: a novel therapeutic target. *Cancer letters*. 2013;338(1):110–9.
17. Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, et al. Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells*. 2009;27(5):1006–20.
18. Hu X, Ghisolfi L, Keates AC, Zhang J, Xiang S, Lee DK, et al. Induction of cancer cell stemness by chemotherapy. *Cell cycle (Georgetown Tex)*. 2012;11(14):2691–8.
19. Stojnev S, Krstic M, Ristic-Petrovic A, Stefanovic V, Hattori T. Gastric cancer stem cells: therapeutic targets. *Gastric cancer: official journal of the International Gastric Cancer Association the Japanese Gastric Cancer Association*. 2014;17(1):13–25.
20. Ruiz i Altaba A, Mas C, Stecca B. The Gli code: an information nexus regulating cell fate, stemness and cancer. *Trends Cell Biol*. 2007;17(9):438–47.
21. Kim YS, Lee HJ, Park JM, Han YM, Kangwan N, Oh JY, et al. Targeted molecular ablation of cancer stem cells for curing gastrointestinal cancers. *Expert Rev Gastroenterol Hepatol*. 2017;11(11):1059–70.
22. Song Z, Yue W, Wei B, Wang N, Li T, Guan L, et al. Sonic hedgehog pathway is essential for maintenance of cancer stem-like cells in human gastric cancer. *PLoS One*. 2011;6(3):e17687.
23. Shaheen S, Ahmed M, Lorenzi F, Nateri AS. Spheroid-Formation (Colonosphere) Assay for in Vitro Assessment and Expansion of Stem Cells in Colon Cancer. *Stem Cell Rev Rep*. 2016;12(4):492–9.
24. Chen S, Hou JH, Feng XY, Zhang XS, Zhou ZW, Yun JP, et al. Clinicopathologic significance of putative stem cell marker, CD44 and CD133, in human gastric carcinoma. *J Surg Oncol*. 2013;107(8):799–806.
25. Han ME, Jeon TY, Hwang SH, Lee YS, Kim HJ, Shim HE, et al. Cancer spheres from gastric cancer patients provide an ideal model system for cancer stem cell research. *Cell Mol Life Sci*. 2011;68(21):3589–605.
26. Yong X, Tang B, Xiao YF, Xie R, Qin Y, Luo G, et al. *Helicobacter pylori* upregulates Nanog and Oct4 via Wnt/ β -catenin signaling pathway to promote cancer stem cell-like properties in human gastric cancer. *Cancer letters*. 2016;374(2):292–303.
27. Zhang Y, Han C, Li J, Zhang L, Wang L, Ye S, et al. Efficacy and safety for Apatinib treatment in advanced gastric cancer: a real world study. *Sci Rep*. 2017;7(1):13208.
28. Tong XZ, Wang F, Liang S, Zhang X, He JH, Chen XG, et al. Apatinib (YN968D1) enhances the efficacy of conventional chemotherapeutical drugs in side population cells and ABCB1-overexpressing leukemia cells. *Biochem Pharmacol*. 2012;83(5):586–97.

29. Cochrane CR, Szczepny A, Watkins DN, Cain JE. Hedgehog Signaling in the Maintenance of Cancer Stem Cells. *Cancers*. 2015;7(3):1554–85.
30. Kinzler KW, Ruppert JM, Bigner SH, Vogelstein B. The GLI gene is a member of the Kruppel family of zinc finger proteins. *Nature*. 1988;332(6162):371–4.
31. Akyala AI, Peppelenbosch MP. Gastric cancer and Hedgehog signaling pathway: emerging new paradigms. *Genes Cancer*. 2018;9(1–2):1–10.
32. Wan J, Zhou J, Zhao H, Wang M, Wei Z, Gao H, et al. Sonic hedgehog pathway contributes to gastric cancer cell growth and proliferation. *Biores Open Access*. 2014;3(2):53–9.
33. Samadani AA, Akhavan-Niaki H. Interaction of sonic hedgehog (SHH) pathway with cancer stem cell genes in gastric cancer. *Med Oncol*. 2015;32(3):48.

Figures

Figure 1

Tumorsphere formation of GCSCs by SFM culture. BGC-823 and SGC-7901 cells were separately cultured in SSM and SFM for 6 days. a Cell morphology was imaged under a light microscope. Bar 100 μ m. b Protein expression levels of GCSC markers, including CD133, CD44, Oct4, Sox2, Nanog and EpCAM were determined by western blotting. c Detection of CD133-positive cells in both cells by flow cytometry assay.

Data are presented as the mean $\pm$ SD of three independent experiments. ** $P < 0.01$ compared with SSM group.

Figure 2

Apatinib diminishes the traits of GCSCs. Tumorsphere-forming cells were treated with different concentrations of apatinib for 6 days. **a** The representative images of sphere-forming cells were acquired. Bar 100 μ m. **b** The proteins level of GCSC markers (CD133, CD44, Oct4, Sox2, Nanog and EpCAM), P-gp, ABCC1 and VEGFR-2 were determined by Western blotting. **c**, **d** Detection of CD133-positive cells in BGC-823 spheroids by flow cytometry assay. **c** Representative images. **d** Percentage of CD133-positive cells. **e**, **f** Immunofluorescence staining images of BGC-823 spheroids were obtained to determine the expression of CD133 and EpCAM. Data are presented as the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with control group.

Figure 3

Apatinib suppresses GCSCs proliferation. BGC-823 and SGC-7901 spheroids were incubated with appropriate concentrations of apatinib for 6 days. a Protein levels of PCNA and Cyclin D1 were measured by western blotting. b Abilities of colonies formation were detected with colony formation assay. c Cell viability was measured by CCK-8 assay. Data are presented as the mean $\pm$ SD of three independent experiments. ** $P < 0.01$ compared with control group.

Figure 4

Apatinib induces GCSCs apoptosis. Cell spheroids were treated with apatinib (1-10 μM) for 4 days and fed every 24 h. a The protein levels of apoptosis-related proteins were measured by western blotting. b Hoechst 33258 staining of the tumorspheres. Bar 100 μm . c, d The percentage of apoptotic cells was analyzed by flow cytometry. c Representative images. d Percentage of apoptotic cells. Data are presented as the mean $\pm$ SD of three independent experiments. ** P < 0.01 compared with control group.

Figure 5

Apatinib suppresses SHH pathway in GCSCs. **a** Cells cultured in SSM and SFM were collected for the measurements of changes in SHH, Smo, Gli1 and Gli2 by western blotting. **b** The spheroids were incubated with vismodegib (15 μ M) for 6 days, and the protein levels of critical molecules in SHH pathway and GCSCs markers were detected using western blotting. **c** The tumorspheres were treated with 1-10 μ M apatinib for 6 days, and the expression of SHH pathway components was analyzed by western blotting.

Figure 6

Downregulation of SHH pathway mediates the inhibitory effects of apatinib on GCSCs. a, b Sphere-forming cells were incubated with 5μM apatinib with/without 1μM purmorphamine for six days. a The protein levels of SHH pathway molecules were measured by western blotting. b Representative images. Bar 100 μm. c Both cells were transfected with control vector or Gli1 plasmid (2 μg). Following 24 h of transfection, cells were then treated with or without apatinib (5μM) for another 4 days. The levels of GCSCs markers were detected by western blotting. Pur, purmorphamine.

Figure 7

Apatinib inhibits GCSCs traits in vivo. xenograft model exerted anti-tumor efficacy in vivo. a Tumor size. The picture was taken on the 22nd day after implantation. b Changes in tumor weight. Data are presented as the mean $\pm$ SD. c Changes in tumor volume. Data are presented as mean $\pm$ SD. d Changes in body weight. Data are presented as the mean $\pm$ SD. e The protein levels of GCSCs markers, drug-resistant proteins, VEGFR-2, proliferation related proteins, apoptosis related proteins and SHH pathway molecules were determined by western blotting. f Expression of GCSCs markers, drug-resistant proteins, VEGFR-2, proliferation related proteins, apoptosis related proteins and Gli1 was measured using immunohistochemistry (400X). ** $P < 0.01$ compared with control group.