Biochemical and Biophysical Research Communications 450 (2014) 353–359

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Atractylenolide I-mediated Notch pathway inhibition attenuates gastric cancer stem cell traits

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ARTICLE INFO

Article history:
Received 22 May 2014
Available online 2 June 2014

Keywords:
Gastric cancer
Cancer stem cells
Atractylenolide I
Notch
CD44

ABSTRACT

Atractylenolide I (AT-I), one of the main naturally occurring compounds of *Rhizoma Atractylodis Macrocephalae*, has remarkable anti-cancer effects on various cancers. However, its effects on the treatment of gastric cancer remain unclear. Via multiple cellular and molecular approaches, we demonstrated that AT-I could potently inhibit cancer cell proliferation and induce apoptosis through inactivating Notch pathway. AT-I treatment led to the reduction of expressions of Notch1, Jagged1, and its downstream Hes1/ Hey1. Our results showed that AT-I inhibited the self-renewal capacity of gastric stem-like cells (GCSLCs) by suppression of their sphere formation capacity and cell viability. AT-I attenuated gastric cancer stem cell (GCSC) traits partly through inactivating Notch1, leading to reducing the expressions of its downstream target Hes1, Hey1 and CD44 *in vitro*. Collectively, our results suggest that AT-I might develop as a potential therapeutic drug for the treatment of gastric cancer.

1. Introduction

Gastric cancer is one of the most common malignancies Worldwide, more than 70% of new cases occur in economically backward countries, the incidence rate of gastric cancer in males is about twice as high as in females [1]. Although standard treatments including surgical resection and chemotherapy are currently most effective for gastric cancer, the overall survival rate is still low [2,3]. Hence, it is very essential to focus on innovative therapeutic research.

Rhizoma Atractylodis Macrocephalae (composite family), one of the traditional Chinese crude materials, has remarkable effects on digestive disorders, stomach diseases and anorexia [4]. Recent findings have also proved that there exists other activities, including anti-inflammatory, anti-tumor and anti-bacterial properties [5,6]. Atractylenolide I (AT-I) (Fig. 1A), a eudesmane-type sesquiterpenoid lactone and one of the main naturally occurring compounds of *Rhizoma Atractylodis Macrocephalae*, has drawn more and more attention because of its multiple therapeutic activities, such as anti-inflammatory [7,8], anti-atopic [9] and especially anti-tumor effects [10–13]. For instance, previous study demonstrated that AT-I induced apoptosis in A549 lung cancer cells through a mitochondria-mediated apoptosis pathway [10]. AT-I exerted its cytotoxicity by pro-oxidant activity and the inhibition of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) activity in human promyeloleukemic HL-60 cells [11]. In addition, AT-I exerted anti-proliferative activities, induced cell differentiation and inhibited cell migration via Ras/extracellular regulated protein kinases (ERK) and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways in B16 melanoma cells [13].However, few systematic studies have been performed to evaluate the effects of AT-I on human gastric cancer.

Notch signaling, which is an evolutionarily conserved pathway, emerges as a mediator of short-range cell–cell interactions and associates with a series of cellular processes, involving cell-fate determination, cell differentiation, proliferation and death [14]. Notch signaling is initiated when membrane-bound ligands including Jagged1 bind to the Notch receptor, γ-secretase-mediated cleavage subsequently liberates intracellular of Notch (NIC) that can translocate into the nucleus to activate the transcription of target genes of Notch signaling, such as Hes1/Hey1 [15]. Accumulating evidences suggest that deregulation of Notch has been implicated in the development, progression and survival of gastric cancer [16,17].

It is considered that the existence of cancer stem cells (CSCs), which are defined as the special subpopulation in the tumor tissues with potential for tumorigenesis, self-renewal and metastasis, partly contributes to cancer relapse and metastasis, major challenges for improving overall cancer survival [18]. Notch

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http://dx.doi.org/10.1016/j.bbrc.2014.05.110
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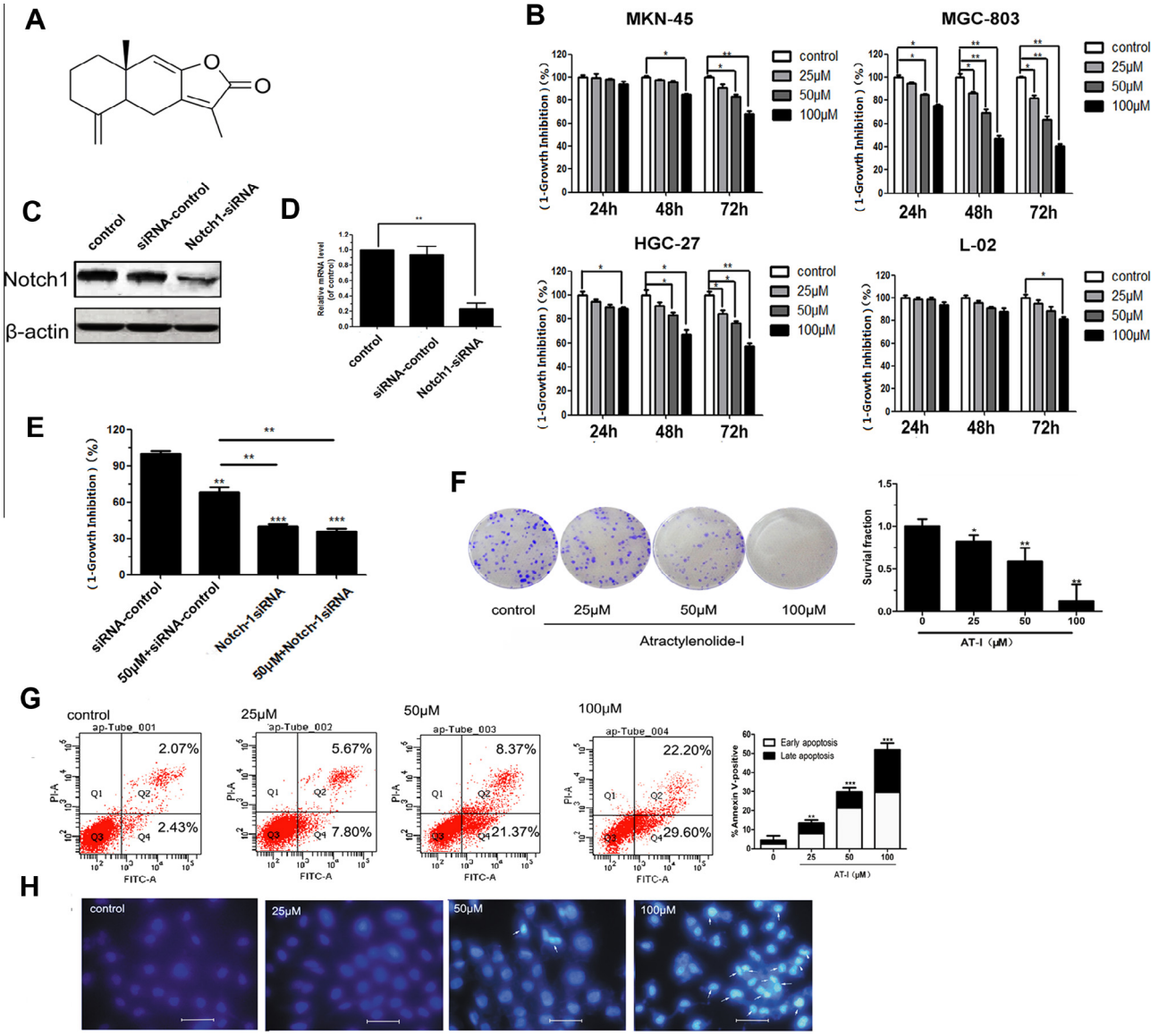

Fig. 1. AT-I potently inhibits cancer cell growth and induces apoptosis in gastric cancer cells. (A) The structural formula of AT-I. (B) AT-I treatment inhibited cell viability in a dose- and time-dependent manner. Gastric cancer cells (MKN-45, HGC-27, MGC-803) and normal cells (L-02) were treated with indicated concentrations (range from 0 to 100 μM) of AT-I for different times (range from 24 to 72 h). (C and D) The mRNA and protein expression levels of Notch1 were determined by real-time PCR and Western blot analysis in MGC-803 cells. β-Actin served as control group. (E) Notch1-siRNA transfection or AT-I significantly suppressed the cell growth. Cells treated with AT-I alone, or Notch1-siRNA, or AT-I plus Notch1-siRNA for 48 h. (F) AT-I inhibited colony formation in MGC-803 cell line. Cells treated with various concentrations (range from 0 to 100 μM) of AT-I for 7 d were evaluated by the colony formation assay. Data were representative of three independent experiments. (G) Flow cytometry analysis of AT-I induced apoptosis in MGC-803 cells using Annexin V-FITC/PI apoptosis assay. Cells (1×10^6/2 mL) were seeded in a 6-well plate and incubated with AT-I in different concentrations (range from 0 to 100 μM) for 48 h. The data in the second quadrant represented early apoptosis and those in the fourth quadrant represented late apoptosis. Image data was a representative of three individual experiments. (H) Fluorescent staining of nuclei in AT-I-treated and untreated cells by Hoechst 33342. Cells untreated, cells treated with AT-I for 48 h were observed by a fluorescence microscope. Scale bars: 20 μm. All the data above are presented as mean ± S.D. ($n = 3$ per group). Significant differences are considered as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

signaling is implicated in the self-renewal of various cancer stem cells, including breast cancer, medulloblastoma and pancreatic cancer [19].

The main purposes of this study were to examine the anti-tumor effect of AT-I on gastric cell lines and to elucidate its underlying mechanism.

2. Materials and methods

2.1. Reagents

AT-I (HPLC grade) was purchased from Must Bio-technology (Chengdu, China). Solid powder was dissolved in dimethyl sulphoxide (DMSO), then made into a stock solution (100 mM) and stored in the dark at −20 °C. The final DMSO concentration never exceeded 0.1% (v/v), in either control or treated samples. Human recombinant basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were from R&D systems Inc. Anti-CD44, anti-Hey1, anti-Jagged1 were from GeneTex Inc. Anti-Notch1 was from Epitomics company. Anti-Hes1 was from Aviva Systems Biology. β-Actin was from Santa Cruz Technology.

2.2. Cell culture

Human cell lines (MGC-803, HGC-27, MKN-45, L-02) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in

monolayer using RPMI 1640 medium supplemented with 10% (vol/vol) FBS (Hyclone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, CA, USA). Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.3. MTT assay

Cell viability was accessed by MTT assay [20]. The half inhibition concentration (IC_{50}) means the concentration of the drug when survival rate of cells reaches 50%.

2.4. SiRNAs and transfection

Cells (5×10^3 or 1×10^4 per well) seeded in 24- or 6-well cell culture plates were transfected with 50 nM scrambled small interfering RNA (siRNA)(sequence: 5′-CCTACGCCACCAATTTCGT-3′) or Notch1-specific siRNA (sequence: 5′-AAGTGTCTGAGGCCAGCAAGA-3′) from Biotend Biological technology (Shanghai, China) using Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the supplier's instructions. Subsequently, the efficiency of siRNA was analyzed by Western blotting and quantitative real-time PCR.

2.5. Colony formation assay

For colony formation assays, cells (200–300 per well) were plated into a 24-well cell culture plate with or without treatment. After 7 d, cells were fixed by ethyl alcohol for 15 min, and then stained with 0.1% (wt/vol) crystal violet. After incubation for 10 min, the cells were washed with cold PBS for three times, and then photographed.

2.6. Hoechst 33342 staining

Briefly, cells (5×10^3 per well) were plated into a 24-well culture plate. Adherent cells were treated with AT-I in various concentrations (range from 0 to 100 μM) for 48 h. Cells were stained with Hoechst 33342 (Sigma, USA). After incubation for 10 min in the darkness, the cells were washed with cold PBS for three times, observed and photographed under a fluorescence microscope (Nikon, Japan).

2.7. Apoptosis measurement

The extent of apoptosis was determined with flow cytometer (Becton Dickinson, USA) of 1×10^4 cells using the Annexin V-FITC apoptosis detection kit (Invitrogen, CA, USA) as described by the manufacturers' instructions.

2.8. Flow cytometry analysis and cell sorting

Cells were trypsinized. PBS buffer containing 2% BSA (Beyotime, Shanghai, China) was used to resuspend cells. Cells were set at a 37 °C incubator for 30 min so as to inhibit non-specific binding and then washed with PBS for three times. Approximately 1×10^6 cells were labeled with anti-human CD44-FITC (eBioscience, USA) conjugated. After incubation at 4 °C for 30 min, cells were washed for three times, resuspended in 500 μL of PBS buffer with 2% FBS, analyzed with flow cytometer.

To cell sorting by flow cytometer, all the steps recommended were carried out under a sterile condition.

2.9. Cell growth assay

Cells were seeded into 96-well cell culture plates at a density of 200 per well. At 1, 2, 3, 4, 5, 6 and 7 d post-plating, the 20 μL of MTT solution was added to each well and incubated for 4 h, and the formed reduction product was dissolved in 100 μL of DMSO and measured at a test wavelength of 490 nm using EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA).

2.10. Soft-agar sphere formation assay

For the study on the proliferation capacity of GCSLCs and total cells, a first layer (1 mL) was made by mixing 1.2% soft agar (Bio-Rad, USA) and 2× culture media and placed into a 24-well cell culture plate, and then refrigerated at 4 °C for 30 min. Cells were harvested, suspended in medium containing 0.3% soft agar (1 mL), and placed on top of the first layer at a density of 2×10^3 cells per well, and then refrigerated at 4 °C for 30 min. Plates were incubated at 37 °C in humidified incubator for 2 weeks. Plates were fed with 0.2 mL of medium. Medium was changed three times a week. At last, sphere colonies were photographed, and colonies with one diameter of 100 μm within photographic fields were chosen.

For the investigation about the effect of AT-I on self-renewal capacity of GCSLCs, the basal layer (0.5 mL) was made by mixing 1.2% soft agar and 2× serum-free culture media with 20 ng/ml bFGF and EGF supplementation, and then placed into a 24-well cell culture plate. The second layer (0.5 mL) was made by mixing 0.6% soft agar and 2× serum-free culture media with 20 ng/ml bFGF and EGF supplementation at a density of 4×10^3 cells per well, and placed onto the basal layer. Plates were fed with 0.1 mL of serum-free medium with bFGF and EGF supplementation twice a week.

2.11. Western blots

In brief, lysates from AT-I-treated cells were resolved in an SDS/PAGE gel and Western blot analyses were performed as previously described [20]. β-Actin was used as a loading control.

2.12. Total RNA isolation and cDNA synthesis

Total RNA of the un-treated and treated cells was extracted by TRIZOL™ reagent (Invitrogen, CA, USA) according to the instruction. RNA was quantitated by optical density measurement for an A260/A280 ratio by a Nucleic Acid and Protein Analyzer (BioPhotometer plus, Eppendorf, German) (The A260/A280 ratio of every RNA sample is more than 1.8), and integrity was confirmed by running an aliquot on a 1.0% agarose gel. cDNA was synthesized by the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan).

2.13. Real-time PCR analysis

Quantitative real-time PCR was performed in triplicate for each gene of interest by SYBR Green Supermix (Takara, Japan) with an iCycler thermal cycler (Bio-Rad, USA). Relative quantification of target genes was calculated by the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$) with genes normalized to GAPDH. The primer pairs of the expected products were as follows (Notch1: F: 5′-GTC AA CGCCGTAGATGACCT-3′; R: 5′-TCTCC TCCCTGTTGTTCTGC-3′. Hes1: F: 5′-AAGGCGGACATTCTGGAAAT-3′; R: 5′-GTC ACCTCGTTCATGCACTC-3′. Hey1: F: 5′-TGGATCACCTGAAAATGCTG-3′; R: 5′-TTGTTG AGATGCGAAACCAG-3′. Jagged1: F: 5′-GATCCT GTCCATGCAGAACG-3′; R: 5′-GGATCTGATACTCAAAGTGG-3′. GAPDH: F: 5′-CAGGGCTGC TTTTAACTC-3′; R: 5′-GGAAGATGGTGATGGGAT-3′). All quantifications were normalized to the mRNA expression of GAPDH.

2.14. Statistical analysis

All data are described as mean ± standard deviation (S.D.) and performed triplicates. Two-tailed Student's *t* tests are used to assess differences between groups. Statistical analysis is performed by GraphPad Prism 5.0 software (GraphPad Software Inc., CA, USA) and Excel (Microsoft). Significant differences are considered as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. AT-I exerts inhibitory effect on cell viability in human gastric cancer cells

To assess the anti-cancer effect of AT-I, MTT assay was performed on three kinds of gastric cancer cells, namely HGC-27, MGC-803 and MKN-45. The data were presented in Fig. 1B. The experiments demonstrated that the cytotoxicity of AT-I was in a dose- and time-dependent manner in three cell lines, among which, MGC-803 cell line was the most sensitive. The IC_{50} of AT-I at 48 h and 72 h in MGC-803 cells were found to be 94.3 ± 1.8 μM and 77.6 ± 2.2 μM, respectively. However, when the concentrations up to 100 μM, AT-I had less cytotoxicity on human normal L-02 cells. Thus, we chose MGC-803 cell line for further investigation.

As Notch pathway plays an important role in cell proliferation and apoptosis [14], we investigated Notch1 specific activity in response to AT-I. At first, we evaluated the knockdown efficiency of Notch1-siRNA by real-time PCR and Western blot analysis. The protein (Fig. 1C) and mRNA (Fig. 1D) expression levels of Notch1 in cells transfected with Notch1-siRNA significantly decreased, as compared with the cells transfected by siRNA-control, indicating that Notch1-siRNA can effectively suppress Notch1 expression at both transcriptional and translational levels. As shown in Fig. 1E, knockdown of Notch1 expression by Notch1-siRNA restrained the cell growth in AT-I-treated cells. We also tested the effects of AT-I on cell viability by colony formation assay as shown below (Fig. 1F). There was a dramatic inhibition of colony formation of AT-I-treated cells in comparison to the control group, suggesting that AT-I inhibits cell proliferation in MGC-803 cells.

3.2. Apoptotic response is induced by AT-I

We determined to test whether apoptosis contributed to the cell death emerging in AT-I-treated cells. As the results obtained by flow cytometry showed, the apoptotic population (the sum of the population in second and the fourth quadrant) in the control group was 4.5 ± 2.1%, which increased to 51.8 ± 3.6% at 100 μM (Fig. 1G). It was found to be dose-dependent in the induction of apoptosis. To further ensure the induction of apoptosis by AT-I in MGC-803 cells, the treated cells were analyzed by Hoechst 33342 staining. We observed nuclear condensation and fragmentation, and perinuclear apoptotic bodies in the AT-I-treated cells (Fig. 1H).

3.3. AT-I inactivates Notch signaling in human gastric cancer cells

Next, we investigated whether AT-I could regulate Notch signaling pathway, including the expressions of Notch1, Hey1, Jagged1 and Hes1 at mRNA or protein expression level by quantitative real-time PCR or Western blot analysis. Compared with the untreated group, AT-I resulted in a reduction of Notch1, Hes1, Hey1 and Jagged1 at both protein and mRNA expression levels, indicating that AT-I treatment leads to the reduction of Jagged1 expression and the transcriptional inactivation of Notch signaling in gastric cancer cells (Fig. 2A and B).

3.4. AT-I inhibits cell proliferation in gastric cancer stem-like cells (GCSLCs)

Here, we examined the effects of AT-I on GCSLCs. In Fig. 3A, the average CD44-positive percent in MGC-803 cell lines was 90.1 ± 2.4%. By fluorescent cell sorting, we obtained the subpopulation of CD44-positive cells in gastric MGC-803 cells. Cell growth and sphere formation assay showed that CD44-positive cells have stronger proliferation ability and higher sphere forming efficiency compared with total cells without sorting (Fig. 3B and C). The experiments above confirmed that CD44-positive cells enriched for GCSCs. Strictly, CD44-positive cells should be termed as "GCSLCs". As shown in Fig. 3D, the results of Western blotting proved that the expression of Notch1 were significantly increased in GCSLCs compared with total cells without sorting at protein level.

By colony formation assay (Fig. 3E), we demonstrated that the down-regulation of Notch1 by siRNA or AT-I restrained proliferation capacity in GCSLCs.

3.5. AT-I attenuates gastric cancer stem cell (GCSC) traits in vitro

One of the typical characteristics of CSCs is the self-renewal ability. Therefore, we examined whether AT-I could inhibit the self-renewal capacity of GCSLCs by sphere formation and cell viability assay. Our results showed that AT-I treatment significantly reduced sphere formation capacity and decreased cell viability in a dose-dependent manner (Fig. 4A and B).

Furthermore, we examined the molecular mechanism of the inhibitory effect of AT-I on GCSLCs. We found that AT-I also inactivated Notch1 and suppressed its downstream target Hes1, Hey1 and CD44 by Western blotting (Fig. 4C). CD44, a multifunctional transmembrane protein involved in cell proliferation,

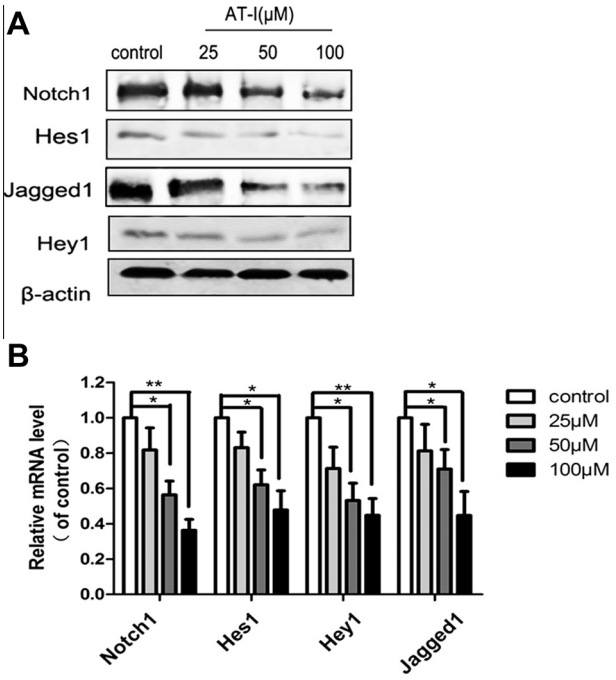

Fig. 2. AT-I inactivates Notch signaling pathway. (A) Lysates from AT-I-treated cells were analyzed by Western blotting for Notch1, Jagged1, Hey1 and Hes1 with specific primary antibody. β-Actin was used as a loading control. (B) RNA from AT-I-treated cells was subjected to quantitative real-time PCR for Notch1, Jagged1, Hey1 and Hes1 mRNA expression. Data are representative of three independent experiments. Significant differences are considered as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

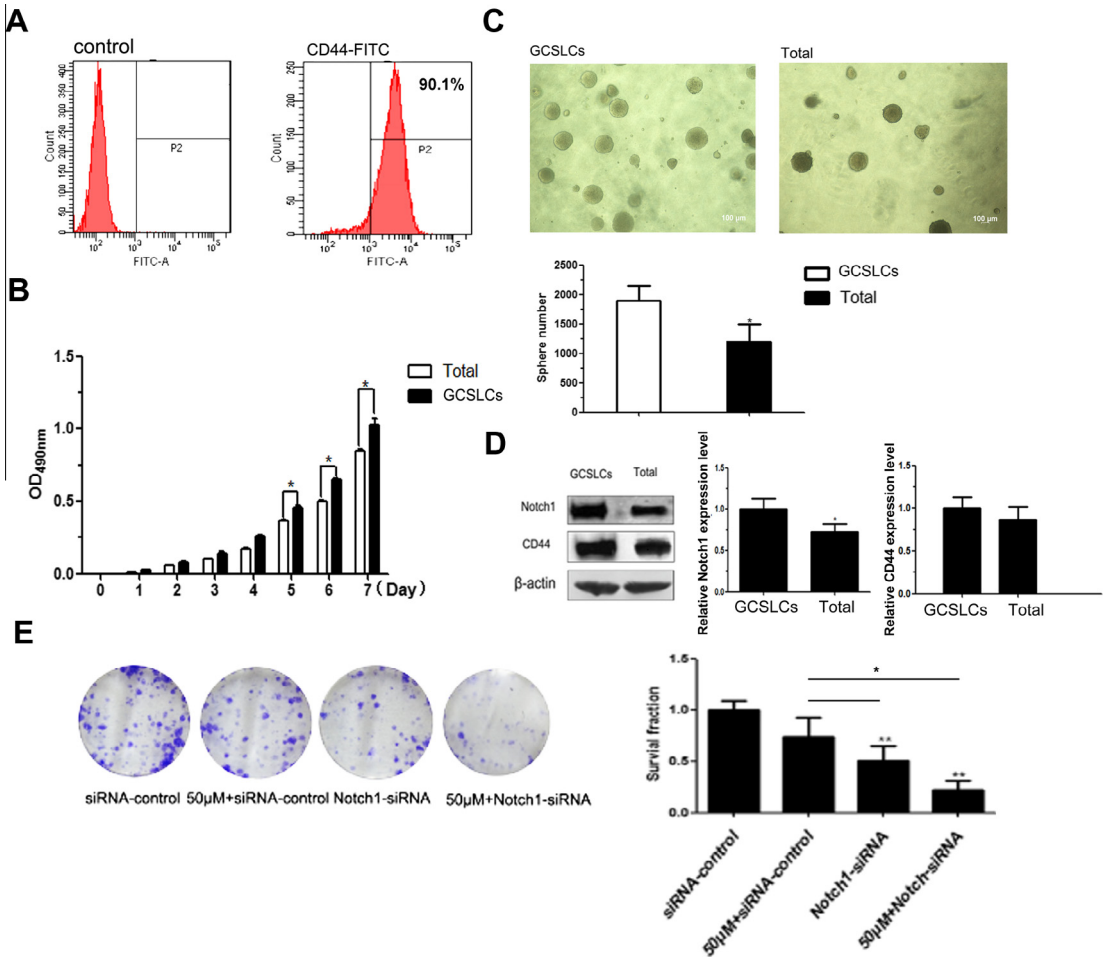

Fig. 3. AT-I inhibits cell proliferation in GCSLCs. (A) Flow cytometry analysis of candidate surface marker CD44 for GCSLCs. (B) GCSLCs had an enhanced proliferation capacity compared with total cells. Equal numbers of GCSLCs and total cells incubated for 1, 2, 3, 4, 5, 6 and 7 d were evaluated by MTT assay. (C) Soft agar formation assay of GCSLCs and total cells. Equal numbers of GCSLCs and total cells were seeded into soft agar as described in Section 2. After 2 weeks of culture, the number of microspheres was counted and microscopic fields were photographed. (D) Western blot analysis of Notch1 and CD44 expression of GCSLCs after FACS sorting and total cells. (E) Notch1-siRNA alone or AT-I reduced the number of colonies in GCSLCs. Colonies were stained with crystal violet. The number of colonies was counted after 1 week. All the data above are presented as mean ± S.D. ($n = 3$ per group). Significant differences are considered as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

angiogenesis, invasion and metastasis[21], is not only a reliable marker for the identification of cells enriched for GCSCs[22], but also a transcriptional target gene of Notch1[23,24]. The ability of expressing of cancer stem cell marker is also a characteristic of CSCs. Collectively, these data suggested that AT-I might be a potent inhibitor of GCSC traits and suppresses GCSLCs traits at least partly through the Notch pathway.

4. Discussion

The experimental evidence strongly supports the anti-tumor effects of AT-I on gastric cancer *in vitro*. Therefore, we believe that AT-I can be an effective and potential therapeutic agent that can inactivate Notch signaling, then leading to the inhibition of cell proliferation and induction of apoptosis.

Notch signaling plays an important role in a series of cellular processes, including cell proliferation and apoptosis [14]. Accumulating evidences have also proved that Notch activity is implicated in maintaining "CSC" phenotype in many cancers, such as gastrointestinal cancer, breast cancer, embryonal brain tumors, glioma, hepatocellular carcinoma, pancreatic and prostate carcinomas [19,25]. Besides, high-level of Notch1 was associated with poorer overall survival in cancer patients. Notch1 also served as a diagnostic and prognostic marker in cancer diagnosis and treatment [26,27].

In our study, we used MGC-803 as the experimental cell line. The data revealed that AT-I had a remarkable effect on proliferative inhibition of MGC-803 gastric cell line by MTT and colony formation assay. The process of cell apoptosis is usually characterized by biochemical and morphological changes. The combined results indicated that AT-I induced apoptosis in gastric cancer cells. To clarify the molecular mechanisms of growth inhibition by AT-I, we investigated whether AT-I could regulate the Notch signaling. The data showed that AT-I suppressed Notch signaling, including Notch1, Hes1, Hey1 and Jagged1 activity. Therefore, AT-I-mediated cell growth inhibition and apoptosis could be at least partly owing to inactivation of Notch signaling.

By a series of cellular approaches, we demonstrated that CD44-positive cells, which were termed as "GCSLCs", had some CSC-like characteristics. AT-I restrained cell proliferation in GCSLCs. Meanwhile, we found that down-regulation of Notch1 by siRNA combined with AT-I treatment suppressed colony formation to a greater degree in GCSLCs compared with AT-I or Notch1-siRNA

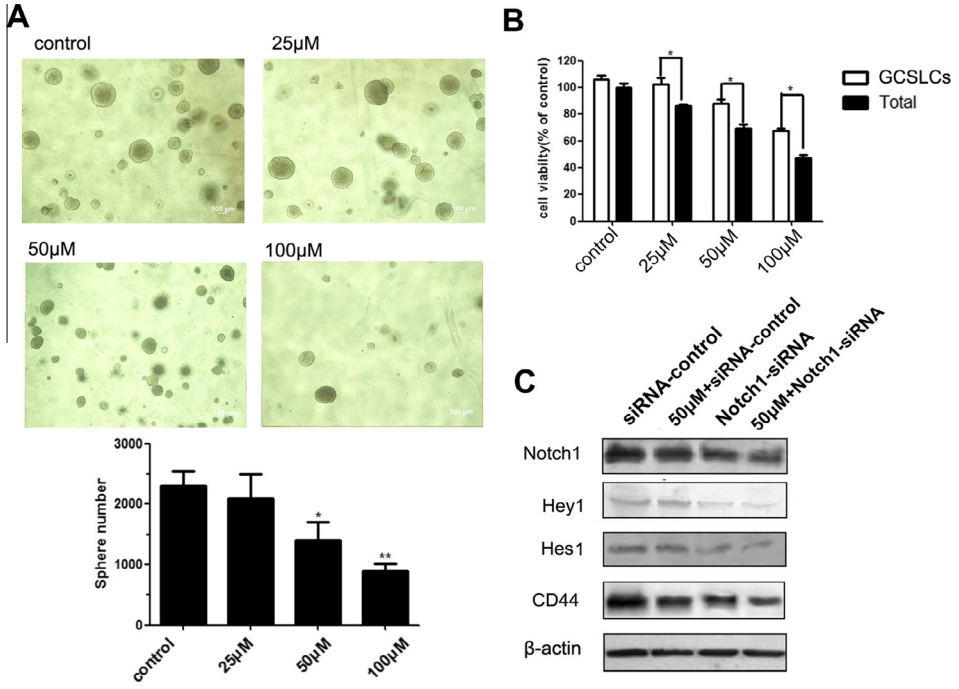

Fig. 4. AT-I attenuates GCSC traits *in vitro*. (A) Sphere formation capacity of GCSLCs treated with various concentrations (range from 0 to 100 μM) of AT-I. The number of microspheres was counted after 2 weeks. (B) Cell growth inhibition induced by AT-I with various concentrations (range from 0 to 100 μM) in GCSLCs and total cells. Cells were treated for 48 h. (C) Lysates from AT-I- or Notch1-siRNA-treated cells were subjected to Western blot analysis for Notch1, Hes1, Hey1 and CD44 expression. β-Actin was used as a loading control. All the data above are presented as mean ± S.D. ($n = 3$ per group). Significant differences are considered as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

treatment alone. Furthermore, AT-I attenuated GCSC traits, which mainly reflected in inhibiting cell growth and self-renewal capacity of GCSLCs in a dose-dependent manner and expression of surface marker of GCSCs, partly through inactivating Notch signaling.

CD44 is the recognized cell-surface marker of gastric cancer. However, as accessed by flow cytometer, the positive expression rate of CD44 in MGC-803 cells was high. To solve this matter, we attempted to add another potential GCSC biomarker CD24 [28] or CD133 [29], which were reported previously. Unfortunately, neither showed any positive rate in MGC-803 cells (data were not shown). CD44 appeared to be the most useful marker for prospective purification of GCSCs in MGC-803 cell line. Hence, it will be important in the future to identify additional markers that may further narrow and define GCSCs, which likely account for a minority of the GCSC population.

To our knowledge, this is the systematic study regarding AT-I suppression of Notch pathway in gastric cancer cells and GCSLCs. However, further *in vivo* researches are warranted to be performed, which can intensively dissect this molecular mechanism of AT-I on gastric cancer models.

Disclosure summary

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Shanghai Committee of Science and Technology (No. 13140902300), Nano Science and Technology Special Funding of Shanghai Committee of Science and Technology (No. 11nm0503700), the Shanghai Committee of Science and Technology [grant 11DZ2260600] and the Fundamental Research Funds for the Central Universities.

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