Effect of tetrandrine combined with arsenic trioxide on stem cells of triple negative breast cancer

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Abstract

Background Although the triple negative breast cancer is sensitive to chemotherapy, breast cancer stem cells (BCSCs) is the origin of tumor chemotherapy resistance, tumor recurrence and tumor invasion and metastasis. This study aims to examine the effect of tetrandrine combine with arsenic trioxide on BCSCs and potential mechanism of anti- triple negative breast cancer metastasis. **Methods** We cultured the triple negative breast cancer cell MDA-MB-231 and induced BCSCs sphere formation by serum-free medium for 5 days. In the MDA-MB-231 cell and MDA-MB-231 stem cell, we compared the ratio of CD44+/CD24- and sorted stem cells by flow cytometry, the expression of Oct4 and Sox2mRNA were by rt-PCR, invasion ability were by Transwell assay. We subsequently measured the effect of tetrandrine combine with arsenic trioxide on BCSCs proliferation by CCK8 method. The stem cell morphology observation was by trypan blue staining. Stem cell cycle and apoptosis were evaluated by flow cytometry. Western Blot was used to measure the protein levels of Hedgehog, Notch1 and PTEN signaling of BCSCs. **Results** The ratio of CD44+/CD24- in MDA-MB-231 stem cells was 95.0%, while MDA-MB-231 cell was 89.3%. The invasion number of MDA-MB-231 stem cell was significantly higher than that of MDA-MB-231 cells ($p<0.01$). Furthermore, we demonstrated that tetrandrine and arsenic trioxide could inhibit the BCSCs proliferation. Tetrandrine combine with arsenic trioxide could significantly promote the apoptosis ($p<0.01$) and increase the percentage of G0/G1 phase and decrease the G2/M phase ($p<0.01$) of BCSCs. Compared with the control group, arsenic trioxide, tetrandrine and the combined group could significantly reduce the expression of GLI1 and SMO and increase the expression of PTEN protein ($P<0.05$). **Conclusions** These findings revealed that tetrandrine combined with arsenic trioxide could suppress the proliferation and induce apoptosis of BCSCs by decreased Gli and SMO expression and increased PTEN expression. Targeting BCSCs treatment, this study provides potential therapeutic drugs against triple negative breast cancer metastasis.

Background

With the using of conventional therapeutic surgery, radiotherapy, chemotherapy, hormones and color Doppler ultrasound, mammography, MRI imaging, breast cancer achieved early diagnosis and early treatment, and improved the 5-year survival rate of breast cancer patients. However, breast cancer is still the second leading cause of cancer death in women, which is due to the tumor recurrence and metastasis^[1]. Breast cancer is divided into luminal A, luminal B, HER2, and triple negative breast cancer (TNBC). Among them, TNBC is the most invasive subtype with high metastatic potential. It may be due to a large number of breast cancer stem cell (BCSC) populations present in TNBC tumors^[2]. Breast cancer stem cells (BCSCs) are derived from human breast tumors and mainly with the ability to regenerate breast malignant cells and force the growth of the breast cancer. BCSCs are with the characteristics of resistance to radiotherapy and chemotherapy, hypoxia tolerance, high tumorigenicity, high invasion and metastasis, contributing to tumor recurrence and metastasis. Traditional treatments only kill most differentiated tumor cells, and a few remaining cancer stem cells are the source of tumor recurrence and metastasis^[3]. BCSCs has established with a minimum cell surface markers CD44⁺/CD24⁻ and

CD44⁺/CD24⁻ phenotype cell population enhancement showing increased tumorigenicity and invasion^[4]. It's found that a large number of CD44⁺/CD24⁻ and ALDH-positive cells and microsphere formation were observed in basal-like breast cancer cells, and is more resistant to chemotherapy, hormone therapy, and radiation therapy, indicating that triple-negative breast cancer cells have a higher incidence of cancer than other subtypes^[5, 6].

Arsenic trioxide (As₂O₃, ATO) is an effective active ingredient of arsenic. In modern research, arsenic trioxide has widely applied to treat acute promyelocytic leukemia^[7], for other malignant tumors such as lung cancer^[8], colon cancer^[9], liver cancer^[10], gastric cancer^[11], pancreatic cancer^[12], ovarian cancer^[13] and breast cancer^[14]. ATO, in vitro, could induce human breast cancer MCF-7 cells to arrest in G1 phase and induce MDA-MB-231 and MDA-MB-468 cells to arrest in G2/M phase^[15]. Besides, ATO could inhibit tumor growth in vivo after inoculation of MCF-7 cells into nude mice, induce apoptosis and inhibit growth in MCF-7 breast cancer cells in vitro^[16]. Elevation of the cancer stem cells (CSCs) like properties is associated with the onset of human cancer, tumorigenesis, metastasis and recurrence, through the stem cell processes of self-renewal and differentiation into multiple cell types. ATO attenuated the CSCs-like properties in human hepatocellular carcinoma^[17]. Tetrandrine (Tet), a bisbenzylisoquinoline alkaloid extracted from *Stephania tetrandra* S.Moore, has been reported to treat patients with tumors, such as prostate cancer^[18], lung cancer^[19], breast cancer^[20]. Our group had demonstrated the apparent cytotoxic effect of As₂O₃ on ER-positive human breast cancer MCF-7 cell, and further elucidated that tetrandrine synergized with arsenic trioxide to significantly enhance toxicity to ER-positive human breast cancer cells^[21]. However, whether ATO and Tet could inhibit the human triple negative breast cancer MDA-MB-231 cell and the associated mechanism is unclear.

Notch signaling, Hedgehog signaling cascade and PTEN, the important CSC signaling pathways, have been related to the regulating proliferation, self-renewal, differentiation, maintenance of CSCs^[22]. However, whether ATO combined with Tet could decrease the metastatic characteristics of BCSCs and the mechanism is not clear. Stem cell invasion and metastasis is strong, so we take BCSCs as research objects. We hypothesized that stem cells can be isolated from breast cancer cells. The combination of arsenic trioxide and tetrandrine could inhibit stem cell proliferation and induce apoptosis, thereby inhibiting stem cell metastasis. Furthermore, we aim to investigate whether the inhibition is related to the Hedgehog, Notch, and PTEN signaling pathways to explore the anti-breast cancer metastasis mechanisms of tetrandrine combine with arsenic trioxide targeting BCSCs.

In this study, serum-free enrichment of breast cancer stem cells was carried out by adding growth factors to the culture medium. In order to identify the characteristics of the isolated mammary stem cells, the ratio of CD44⁺/CD24⁻ in stem cells was detected by flow cytometry, and the expression of Sox2 and Oct4 mRNA of stem cells were detected by QPCR, invasive of breast cancer stem cells with transwell assay. Furthermore, we measured the effect of tetrandrine and arsenic trioxide on the morphology, proliferation, cell apoptosis and cycle of breast cancer stem cell. In addition, we investigate its potential association with the Hedgehog signaling, PTEN and Notch pathways. This study is expected to provide a preliminary

basis for the study of anti-metastasis of arsenic trioxide combined with tetrandrine against breast cancer stem cells and experimental evidence for arsenic trioxide combined with tetrandrine clinically anti-breast cancer metastasis.

Methods

- **Chemicals and Reagents.**

B27 (Life Technologies,USA,NY14072), DMEM/F12 (Gibco,USA,AC10238685), bFGF (novoprotein,Shanghai,China,C046), EGF(novoprotein,Shanghai,C029), 0.4% Trypan blue dye solution (Solarbio,Beijing,China,Cat#C0040), CD44-PE(BD,USA,550989), CD24-FITC(BD,USA,555427), PE (BD,USA,555749), FITC(BD,USA,553456), Agarose (ABI-invitrogen,USA,16500100), TRIZOL(Invitrogen,USA,10296028), SuperScript III RT Reverse Transcription Kit (ABI-invitrogen,USA,11752050), Sybr QPCR mix (ABI-invitrogen,USA,4472920), DAPI (Solarbio, Beijing,China,C0065), Matrigel Matrix Basement Membrane(Corning,USA,356234), CCK-8(Dojindo,Japan,LB633), Matrigel Matrix Basement Membrane (Corning,USA,356234). All other reagents were obtained from Sigma-Adrich Co. (St. Louis, MO, USA). The epidermal growth factor (EGF) was prepared into a mother liquor at a concentration of 100µg/mL, and basic fibroblast growth factor (bFGF) was prepared to prepare a mother liquor having a concentration of 100µg/mL, and stored at -20°C. The stem cell medium was serum-free medium DMEM/F12, supplemented with bFGF (10ng/mL), EGF (20ng/mL) and 2% B27, stored at 4°C and protected from light.

Tetrandrine standard was purchased from China Food and Drug Administration Institute, cat. No. 110711-201609. Tetrandrine solution was added DMSO to help dissolve and the final concentration of DMSO is less than 0.1%. The Tetrandrine mother solution was mixed with the DMEM culture to a final concentration of 36µg/mL, filtered through a 0.22µm filter, stored at -20 °C and protected from light. Arsenic acid injection was purchased from Heilongjiang Harbin Medical University Pharmaceutical Co., Ltd. Cat. No. H19990191. Arsenic trioxide mother solution was mixed with DMEM culture solution to a final concentration of 100µg/mL, stored at -20 °C and protected from light.

- **Breast cancer stem cell culture**

The MDA-MB-231 single cells were plated at a density of 1×10^4 cells in serum-free medium DMEM/F12, supplemented with commercial hormone mix 2%B27, EGF (20ng/mL), bFGF (10ng/mL) and inoculated into a low-adsorption 6-well plate cultured for 5 days when cells started to grow forming floating aggregates. Then, the MDA-MB-231 stem cells were resuspended in DMEM medium supplemented with 10% fetal bovine cultured in a 37°C, 5% CO₂ incubator. Cells were counted after 0.4% trypan blue staining and the growth curve was drawn by averaging. The cell growth curve was plotted with time as the horizontal axis and cell number as the vertical axis.

- **Flowcytometric analysis of CD44 and CD24 content**

Logarithmic growth phase MDA-MB-231 cells and 5 days of stem cells were suspended in DMEM complete medium and DMEM/F12 serum-free medium to prepare single cell suspension. The number of cells was adjusted to 1×10^6 cells/ml. The cells were washed with PBS. Then, the cells were resuspended in 100 μ l of PBS, and labeled with CD44-PE and CD24-FITC, respectively. Each fluorescent antibody was set up with an isotype control. Incubate the cells in the dark for 15 min at room temperature, centrifuge at 1000rpm for 5 min and then test with 200 μ l PBS on the flow cytometry to detect the expression of CD44 and CD24.

- **Assay for the detection of apoptosis and cell cycle**

MDA-MB-231 stem cells were cultured for 5 days, the cell density was adjusted to 1×10^5 cells/mL to prepare a single cell suspension to seed in a 6-well plate, at a 37°C, 5% CO₂ incubator for 24h, then add the different drugs (0, tetrandrine (1.5 μ g/ml), arsenic trioxide (2.5 μ g/ml) and tetrandrine combined with arsenic trioxide), to culture for 48h. After the end of the culture, collect the culture solution and cells in each well. Follow the steps below to detect cell apoptosis using flow cytometry. PBS wash once, discard the supernatant, add 1 $\times$ Binding Buffer 100 μ L to resuspend the cells; add 5 μ L FITC Annexin V and 5 μ L PI to each tube, protect from light, and incubate for 15 min at room temperature. 200 μ L of 1 $\times$ Binding Buffer was added to each tube. The cell apoptosis was detected by flow cytometry within 1 hour. Then, follow the steps below to detect cell cycle using flow cytometry as well. The supernatant was discarded after washing with PBS, 500 μ L of pre-cooled 70% ethanol was added, and placed in a refrigerator at 4°C overnight; PBS washed once and 200 μ L of RNase A-PI was added to each tube protected from light and incubated for 30 minutes at room temperature, the cell cycle was measured by a flow cytometer within 1 hour.

- **CCK-8 assay**

The MDA-MB-231 stem cells cultured for 5 days in the second generation were adjusted to a cell density of 8×10^4 cells/mL to prepare a single cell suspension; 100 μ L of single cell suspension was added to each well. After routine incubation for 24 hours at 37°C in a 5%CO₂ incubator, the drug-containing culture solutions were separately added with tetrandrine (0, 0.375, 0.75, 1.5, 2, 3, 6 μ g/ml) and arsenic trioxide (0, 0.625, 1.25, 2.5, 5, 10 μ g/ml). Then, 10 μ L of CCK-8 solution was added to each well for 4 hours, and the absorbance OD value at a wavelength of 450nm was measured with a microplate reader. The cell inhibition rate was calculated using the OD value, and the experiment was repeated three times or more to calculate the cell inhibition rate. The formula is as follows: Cell inhibition rate (%) = (1-average OD value of the experimental group / average OD value of the control group) $\times$ 100%

- **Transwell invasion assay**

Add the diluted Matrigel gel (1:3) to the bottom of the Transwell chamber at 50 μ l/well, gently shake the 24-well plate to distribute the added liquid evenly and place it in a 37°C incubator to gel it into a solid. After two hours, take out, carefully aspirate the liquid culture solution in the small chamber, and air dry for

use. MDA-MB-231 cells in logarithmic growth phase and stem cells cultured for 5 days were adjusted to a cell density of 1×10^6 cells/ml. The cells were added to the upper chamber, 150 μ L of cell suspension per well, and 600 μ L of serum-containing medium was added to the lower chamber at 37°C for 48 hours. Gently wipe off the unmigrated cells and Matrigel gel in the upper chamber, dry it, add methanol for 10 min, add 10 μ g/mL DAPI 100 μ L, protect from light, and incubate for 30 min at room temperature. The underlying cells were observed under an inverted microscope. Each chamber was photographed and the relative invasion index (V_2/V_1 , V_2 refers to the number of transmembrane cells of tumor stem cells, and V_1 refers to the number of transmembrane cells of MDA-MB-231 cells) were counted.

- **Western blot analysis**

The MDA-MB-231 stem cells cultured for 5 days were washed with pre-cooled PBS, collected into a centrifuge tube, and add the cell lysate (1 mL of RIPA lysate was added to 10 μ L of PMSF). Measure the cell protein concentration with BCA protein concentration kit, prepare 10% separation gel, 5% concentrated gel, electrophoresis, PVDF transfer, and 5% skim milk for 1 hour. The first antibody was diluted 1:500, overnight at 4°C. To wash the membrane three times with TBST. With the secondary antibody 1:3000, incubated for 1 hour and washed three times with TBST. Add ECL chemiluminescence solution and expose it with gel imager.

- **RNA isolation, preparation of cDNA and Real time PCR analysis**

RNA isolation. MDA-MB-231 cells and 5 days of stem cells were lysed by adding 1 ml of Trizol (Invitrogen), allowed to stand at room temperature for 5 min, collected into a 1.5 mL EP tube, and then 200 μ L of chloroform was added, followed by vigorous shaking 15s immediately. After standing for 5 min, it was placed in a centrifuge at 12000 rpm for 15 min at 4 °C. Carefully pipe the upper liquid into another new centrifuge tube, add 500 μ L of isopropanol, let stand for 10 min, centrifuge at 12000 rpm for 10 min at 4°C, and discard the supernatant. Rinse once with 75% ethanol, air dry, dissolve in 50 μ L DEPC water.

Reverse transcription cDNA. Take a mixture of template RNA (2 μ g) and oligo (dT) 1 μ L, mix gently, centrifuge for 30 seconds, incubate in a 65°C incubator for 5 min and cool on ice. And then it was mixed 5 $\times$ Reaction Buffer 4 μ L, RiboLock™ RNase Inhibitor 1 μ L, 10 mM dNTP Mix 2 μ L, with RevertAid™ M-MuLV Reverse Transcriptase 1 μ L. Incubate for 60 min in a 42°C incubator. Next, it was placed at 85°C and reacted for 10 min to inactivate reverse transcriptase, cooled at 4°C and stored at -20°C until use.

RT-PCR amplification. Mix cDNA 2 μ L, qPCR mix 10 μ L, primer F 1 μ L, primer R 1 μ L with RNase-free water 6 μ L. Each sample repeated 3 times. β -actin is an internal reference. Primer sequence is shown in the table below:

- **Statistical Analysis**

All results were expressed as mean $\pm$ standard deviation (SD). One-way ANOVA was performed between multiple groups using SPSS 20.0 software when homogeneity of variance and normality were met.

Otherwise, Dunnett's T3 and nonparametric tests were conducted between multiple groups. $p < 0.05$ was considered statistically difference. Drawing with GraphPad Prism 5 software.

Results

3.1 Enrichment and differentiation of breast cancer cell MDA-MB-231 stem cells

MDA-MB-231 stem cells were enriched by serum-free medium, and the growth of the cells was observed at different time points. Under serum-free medium, MDA-MB-231 stem cells grew in suspension. After 24h enrichment, MDA-MB-231 stem cells began to aggregate. As the culture time prolonged, the number of tumor stem cells became more and more and the bigger the volume (Fig1(a)-1(d)). Serum-containing medium was added, and after 24 hours, it was showed a small amount of adherence appeared in the cells. After 72 hours, the adherent cells were gradually increased and began to fuse with the surrounding cells (Fig1(e)-1(h)). There was no statistically difference in morphology between adherent stem cells and MDA-MB-231 cells (Fig 2(i)-2(j)). The results of growth curve showed that suspension cultured MDA-MB-231 stem cells were transferred to serum-containing medium and began to differentiate and proliferate on the 1st day, enter to the logarithmic growth period on the 3rd day, and to the platform period on the 7th day (Fig 2(k)).

3.2 Expression of $CD44^+/CD24^-$, Oct4mRNA and Sox2mRNA and invasive ability in MDA-MB-231 cells and MDA-MB-231 stem cells

Flow cytometry results showed that the ratio of $CD44^+/CD24^-$ in the MDA-MB-231 cell line was 89.3%, while in the serum-free enriched MDA-MB-231 stem cells was 95.0% (Fig 3(a)-3(d)). The expression of Oct4 and Sox2mRNA in MDA-MB-231 cells and MDA-MB-231 stem cells was detected by real-time fluorescent quantitative PCR. The results showed that the expression of Oct4 and Sox2mRNA increased after enrichment in serum-free medium, but the difference is not statistically significant ($p > 0.05$) (Fig 3(e)). The Transwell assay results showed that the number of transmembrane cells of tumor stem cells was 758.11 ± 49.67 , and the number of transmembrane cells of MDA-MB-231 cells was 273.33 ± 58.41 . The number of MDA-MB-231 stem cell transmembrane cells was significantly higher than that of MDA-MB-231 cells ($p < 0.01$) (Fig 3(f)-(h)). MDA-MB-231 stem cell relative invasion index $V2/V1$ was 2.88 ± 0.79 .

3.5 Effects of tetrandrine and arsenic trioxide on proliferation inhibition of MDA-MB-231 stem cells

The CCK-8 assay results showed that the proliferation of MDA-MB-231 stem cells was inhibited by tetrandrine (0.375, 0.75, 1.5, 3.0, 6.0 μ g/mL) (Fig 4(a)) and arsenic trioxide (0.625, 1.25, 2.5, 5.0, 10.0 μ g/mL) (Fig 4(b)). The inhibition effect had taken on time and dose dependence.

3.6 Effects of arsenic trioxide and tetrandrine on the morphology of MDA-MB-231 stem cells

In trypan blue staining, live cells are not stained blue, and dead cells are dyed light blue. In the control group, MDA-MB-231 stem cells grew in a round shape, and some cells aggregated into a cluster. The number of normal stem cells was large, and the blue-stained cells were rarely seen after trypan blue staining. Compared with the control group, intervention with tetrandrine (1.5 µg/ml) and arsenic trioxide (2.5 µg/ml) or combined, the blue cells increased, cell volume shrank, cell debris increased in culture medium, and a large number of cell collapses and died (Fig 5(a)-(d)).

3.7 Effects of arsenic trioxide and tetrandrine on cell apoptosis and cell cycle distribution of MDA-MB-231 stem cells

The results of flow cytometry showed that the effects of tetrandrine and arsenic trioxide on the cell apoptosis and cell cycle of MDA-MB-231 stem cells. The results showed that arsenic trioxide group and the combination of tetrandrine and arsenic trioxide group could significantly promote the apoptosis of MDA-MB-231 stem cells ($p < 0.01$). There was no statistically difference between arsenic trioxide with the combined group to treat BCSCs ($p > 0.05$) (Fig 6 (a)-(d) and Table2). Besides, compared with the control group, the percentage of G0/G1 phase cells in the cell cycle was significantly increased ($p < 0.05$, $p < 0.01$) and the G2/M phase was decreased ($p < 0.01$) in the combination group and tetrandrine group, which could lead to the cell DNA synthesis and cell proliferation reduced. Compared with the control group, there was no statistically difference in the arsenic trioxide group ($p > 0.05$) (Fig 6 (e)-(h) and Table3).

3.9 Effects of arsenic trioxide and tetrandrine on the expression of GLI1, SMO, PTCH, PTEN and Notch1 proteins in MDA-MB-231 stem cells

Western blot analysis results (Fig 7(a)-(f)) showed that compared with the control group, arsenic trioxide, tetrandrine and the combined group could significantly reduce the expression of GLI1 and SMO and increase the expression of PTEN protein ($P < 0.05$). The expression of PTCH and Notch1 protein had a decreasing trend than the control group, but there was no statistically difference ($P > 0.05$).

Discussion

Using the serum-free suspension culture, we made MDA-MB-231 stem cells enriched. The ration of the serum-free enriched MDA-MB-231 stem cells was 95.0%. The expression of Oct4 and Sox2mRNA increased and the invasion ability was stronger than the MDA-MB-231 cell. Further, it's revealed that tetrandrine, arsenic trioxide, tetrandrine and the combination could inhibit breast cancer stem cell proliferation, block stem cell cycle, induce stem cell apoptosis and necrosis, and the mechanism may be related to decreasing the expression of GLI1 protein and SMO protein and increasing the expression of PTEN protein.

In 2003, Al-Hajj et al.^[23] firstly isolated a group of cells with CD44⁺/CD24^{-/low}/ESA⁺/Lin⁻ phenotype from breast cancer patients and proved that compared with other cells, the tumorigenicity of the cells with CD44⁺/CD24^{-/low}/ESA⁺/Lin⁻ phenotype is increased by 50 times, and with the potential of multi-directional differentiation and self-renewal, confirming the existence of BCSCs. It is believed that CSC could form microspheres in serum-free medium. CD44⁺CD24⁻ cells could sort from these spheres and possess higher ability of sphere formation^[24]. In this experiment, we enriched the stem spheres with serum-free medium containing DMEM/F12, supplemented with bFGF, EGF and B27. Fillmore et al.^[25] found that in the breast cancer cell lines MDA-MB-231, SUM159, SUM315, the proportion of CD44⁺CD24⁻ phenotype was above 90%. In our study, it was found that the ratio of CD44⁺CD24⁻ was found highly expressed in MDA-MB-231 cells (89.3%) and stem cells (95%), which was consistent with previous findings.

Embryonic stem cells transcription factors such as octamer-binding transcription factor 4 (Oct4) and sex determining region Y-box 2 (Sox2) are thought to be involved in the regulatory of stem cells and contribute to tumorigenesis and progression of human breast cancer^[26]. While other studies reported that overexpression of OCT4 could inhibit the metastasis of breast cancer cells^[27]. Besides, Sox2 is considered to be one of the important stem cell transcription factors. Al-Joudi et al.^[28] retrospectively analyzed 382 cases of invasive breast cancer. The expression of Sox2 was detected by immunohistochemistry and the positive rate was 68.1%. Mimeault et al.^[29] found that Sox2, a transcription factor with differentiation potential, affects epigenetic recombination and stem cell characteristics during tumor formation and progression, and is expected to be an important molecular marker for predicting the risk of tumor metastasis and disease recurrence. In this study, real-time quantitative PCR was used to detect the expression of Oct4 and Sox2 genes in MDA-MB-231 cells and MDA-MB-231 stem cells. We found that the expression of Oct4 and Sox2mRNA of BCSCs increased, but the difference is not statistically significant ($p>0.05$). The reason may be that the MDA-MB-231 cell and its serum-free enriched MDA-MB-231 stem cells have a relatively high proportion of CD44⁺CD24⁻ phenotype cells, and the content is relatively high, so there was no statistically difference in the expression of Oct4 and Sox2mRNA. Besides, our results showed that human triple negative breast cancer MDA-MB-231 cells had certain invasive ability, which might due to the high proportion of CD44⁺CD24⁻ phenotype cells and expression of Oct4 and Sox2mRNA. However, the invasiveness ability of BCSCs was significantly higher than that of MDA-MB-231 cells ($p<0.01$).

Our previous study found that tetrandrine could induce autophagy in triple negative breast cancer MDA-MB-231 cells by inhibiting PI3K/AKT/mTOR signaling pathway^[30]. Besides, As₂O₃ combined with tetrandrine inhibited proliferation of breast cancer MDA-MB-231 cells, its mechanism might relate to the cell G2/M phase arrest, bcl-2 protein level decreased to promote apoptosis, GSK3 β protein and PARP protein levels up-regulated^[31]. Chen et al.^[32] also indicated that tetrandrine could enhance the anti-cancer effect induced by As₂O₃. As to the anti-cancer effect of As₂O₃ and tetrandrine on MDA-MB-231 cell, we further study their function on BCSCs and explore the mechanism of anti-breast cancer metastasis. In

this study, after isolation and identification of MDA-MB-231 stem cell characteristics, we further study the effect of tetrandrine and arsenic trioxide on BCSCs. We found that different concentrations of tetrandrine, arsenic trioxide, and two drugs combined could significantly inhibit the proliferation of BCSCs in a time-dose dependent manner. In addition, tetrandrine (1.5µg/mL) and arsenic trioxide (2.5µg/mL) for 48h had a high synergy degree on BCSCs. In the morphology of BCSCs, compared with the control group, the combination of Tet and ATO for 48h had taken on more cell death and smaller cell size of BCSCs. In addition, the effects of arsenic trioxide and tetrandrine on the apoptosis and cycle distribution of MDA-MB-231 stem cells were detected by flow cytometry. The results show that the combination of tetrandrine and arsenic trioxide could induce the apoptosis of MDA-MB-231 stem cells, and the ability of combined group is better than that of tetrandrine alone. For the cell cycle, tetrandrine alone or in combination with arsenic trioxide could increase the G0/G1 phase, arrest the G2/M phase to reduce DNA synthesis and cell proliferation, while arsenic trioxide alone was not.

Abnormalities and dysregulation in the signaling pathways such as Notch, Hedgehog, PTEN/PI3K/Akt, may lead to malignant transformation, which are important ways to participate in cancer stem cell formation, transformation and to ensure tumor viability^[33]. The Hedgehog signal transduction pathway mainly includes Hedgehog (HH) ligands and their receptors Patched (PTCH) and Smoothened (SMO)^[34]. In ER-positive breast cancer cells, E₂ directly binds to the promoter region of the GLI1 gene, activates Hh signaling through non-canonical pathways, increases GLI1 expression, promotes GLI1 target gene activity, and increases CSC self-renewal^[35]. Besides, emerging preclinical data also implicated Hh signaling in TNBC pathogenesis. The high expression level of GLI1, the key downstream effector of the Hedgehog signaling pathway, is associated with triple-negative breast cancer stem cells and TNBC patients' unfavorable overall survival rate^[36]. Activation of the PI3K/AKT pathway is essential for maintaining the dryness and chemoresistance of breast CSCs. PTEN is a tumor suppressor in human breast cancer, which can negatively regulate the role of PI3K/AKT signaling pathway in tumor suppressor genes^[37, 38]. In the present study, compared with the control group, arsenic trioxide, tetrandrine and the combination could significantly reduce the expression of GLI1 and SMO and increase the expression of PTEN protein (P<0.05), which might be the anti-BCSCs mechanism of arsenic trioxide and tetrandrine.

However, there are some limits should be noted in the present study. We provided a preliminary experimental basis for the study of the mechanism of tetrandrine in combination with arsenic trioxide in the treatment of triple-negative breast cancer stem cells, but no gene knockout was performed, and no pathway inhibitors were used for further validation. Therefore, although this study provides an effective drug for the treatment of triple-negative breast cancer stem cells, it is necessary to further study the mechanism of tetrandrine combined with arsenic trioxide in the treatment of triple-negative breast cancer stem cells to better serve the clinic.

Conclusions

We have proposed a new approach to treat triple-negative breast cancer and metastasis targeting breast cancer stem cells (BCSCs). The high expression of CD44⁺/CD24⁻, Oct4, Sox2 and high invasive ability were characteristics of MDA-MB-231 stem cells. In this study, breast cancer stem cells were enriched by serum-free growth factor-containing medium and their characteristics have been verified. Intervention with tetrandrine and arsenic trioxide on BCSCs induced the apoptosis, increased the G0/G1 phase cells and reduced the G2/M phase cells, which leading to inhibition of the DNA synthesis and cell proliferation. The mechanism might be due to the expression of GLI and SMO were decreased and PTEN expression was increased. This study provided experimental basis for the clinical treatment of anti-triple-negative breast cancer metastasis targeting BCSCs by arsenic trioxide combined with tetrandrine.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

YG was a major contributor in writing the manuscript. XP contributed to the design of the study and analytic strategy. YF and RW performed the experiment and analyzed the data. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, the tables have been placed in the supplementary files section.

Figures

Figure 1

Microscopic morphology of MDA-MB-231 stem cell microspheres enriched by serum-free medium (a-d) and cultured by serum-containing medium (e-h) (10 $\times$).

Figure 2

Morphology comparison of MDA-MB-231 cells and MDA-MB-231 stem cells (i-j, 10 $\times$) and the growth curve of MDA-MB-231 stem cells (k) with serum-containing medium.

Figure 3

Stem characteristics measurement of CD44+CD24-proportion by flow cytometry (Fig a-d); Oct4 and Sox2 mRNA expression of BCSCs by rt-PCR (Fig e); Invasion ability by Transwell assay (Fig f-g) and data analysis Fig (h) of MDA-MB-231 cells and BCSCs.

Figure 4

The inhibition rate of Tetrandrine and Arsenic trioxide on BCSCs for 24, 48,72hour. 3.6 Effects of arsenic trioxide and tetrandrine on the morphology of MDA-MB-231 stem cells

Figure 5

Trypan blue staining to observe the effect of arsenic trioxide combined with tetrandrine on the morphology of MDA-MB-231 stem cells after 48 hours (Fig (a)-(d), magnification 10×, blue cells represent dead cells). 3.7 Effects of arsenic trioxide and tetrandrine on cell apoptosis and cell cycle distribution of MDA-MB-231 stem cells

Figure 6

Effect of arsenic trioxide and tetrandrine on apoptosis percentage (Fig (a)-(d)) and cell cycle percentage (Fig (e)-(h)) of MDA-MB-231 stem cells by flow cytometry.

Figure 7

Effect of arsenic trioxide and tetrandrine on apoptosis percentage (Fig (a)-(d)) and cell cycle percentage (Fig (e)-(h)) of MDA-MB-231 stem cells by flow cytometry.

Supplementary Files

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