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RESEARCH ARTICLE

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Xiaotansanjiefang inhibits the viability of colorectal cancer cells via Jagged 1/Notch 3/Snail signaling pathway

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Abstract

The purpose of this study is to explore the anti-colorectal cancer of Xiaotansanjiefang, a famous traditional Chinese medicine, and its potential anti-cancer mechanism. In this study, the HCT116 cell spheres were prepared as in vitro study model. We found the Xiaotansanjiefang medication was able to inhibit the proliferation of HCT116 cell spheres in a dose-dependent manner, especially in 3 and 6 mg/ml Xiaotansanjiefang medication treated groups. We also found the high concentration of Xiaotansanjiefang medication could suppress the migration and promote the apoptosis of HCT116 cell spheres. Moreover, we found the expression of Jagged 1, Notch 3, Snail, and Hes 1 were decreased in HCT116 cell spheres treated with Xiaotansanjiefang medication. Furthermore, the proliferation and apoptosis behaviors of HCT116 cell spheres treated with Xiaotansanjiefang medication were reversed with the addition of Jagged 1 Fc chimera protein. The expression of Jagged 1, Notch 3, Snail, and Hes 1 were also increased again in HCT116 cells treated with Xiaotansanjiefang medication plus with Jagged 1 Fc chimera protein. The presented study may provide a promising strategy to treat and prevent colorectal cancer.

KEYWORDS
apoptosis, colorectal cancer, Jagged 1/Notch signaling, proliferation, traditional Chinese medicine

1 | INTRODUCTION

Colorectal cancer (CCR) is the third most common cancer worldwide in men and women,[1] the second largest cause of death related to cancer,[2] and the leading cause of death in gastrointestinal cancer.[3] The risk of developing this cancer is related to bad dietary habits, smoking, intestinal inflammatory disease, polyps, genetic factors, and aging.[4] Of the patients diagnosed with colorectal cancer, 90% are older than 50, with a median age of 64 years.[5] However, the disease is more aggressive in patients that are diagnosed at younger generations. According to the American Cancer Association, it was accounted for more than 49 700 deaths in 2015. Colorectal cancer has threatened the lives and health of people. New therapeutic drugs are urgently needed.

Traditional Chinese medicine has been discovered as a novel or additional treatment method for cancer treatment.[6–8] For example, Xiaotansanjiefang, a Chinese famous herbal prescription, it consists of Ban-xia (12.7%), Nan-xing (12.7%), Fu-ling (12.7%), Zhi-shi (8.5%),

Min Ye, Jiaqi Du, and Xiaowei Wang contributed equally to this work.

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Chen-pi (7.6%), Quan-xie (5.1%), Wu-gong (7.6%), Ji-nei-jin (12.7%), Bei-mu (7.6%), Bai-jie-zi (7.6%), and Gan-cao (5.1%). It is effective in gastric cancer treatment in China.[9,10] Previous observations indicated that it is effective in advanced gastric cancer patients.[11] Animal studies demonstrated that it could suppress tumor growth and inhibit angiogenesis.[12] It inspires us that the Xiaotansanjiefang medication may be effective against colorectal cancer. Herein, we will try to explore the anti-colorectal cancer effects of Xiaotansanjiefang medication in this study. In order to mimic the microenvironment of colorectal cancer tissues as much as possible, the colorectal cancer cell spheres were made as in vitro model. The potential mechanism was also explored in this study.

2 | METHODS AND MATERIALS

2.1 | HCT116 cell culture and cell spheres formation

The human colon cancer cell line HCT116 was obtained from the ATCC. The cells were maintained in DMEM/F12 medium plus with 10% FBS and 1% streptomycin/penicillin at 37°C. The medium was refreshed every 2 days. For the formation of HCT116 cell spheres, the inducing medium was prepared, the DMEM/F12 supporting with EGF (20 ng/ml), FGF (20 ng/ml), LIF (10 ng/ml), and B27 (1×). The HCT116 cell spheres were ready to use after 4 days of inducing.

2.2 | Proliferation assay

The HCT116 cell spheres were incubated with EdU working solution for 4 h, then fixed for 15 min and permeabilized (0.1% Triton X-100 in PBS solution) for 15 min, and treated with blocking buffer (5% BSA in 0.1 M PBS) for 30 min. Following, the EdU additive solution reaction was performed according to the protocol. Finally, the cell nucleuses were co-stained with DAPI for 5 min. The detection of EdU was performed by fluorescence microscopy (Ex/Em = 491/520 nm).

2.3 | Migration assay

HCT116 cell spheres were placed in inserts and cultured with 100 μl of serum-free DMEM/F12 medium. Then, the lower chamber of culture inserts was refreshed with 20% FBS containing DMEM/F12 medium. In addition, cells were continuously cultured for 24 h. Then membranes were obtained and washed with PBS, fixed with methanol for 15 min, finally stained with Hematoxylin and Eosin (HE) according to the standard procedures. The cells without any treatment as base line control, the cells treated with 5-FU as positive control. Finally, the migrated cells were photographed and counted in five randomly selected areas under an inverted microscope (20×).

2.4 | Apoptosis assay

After the HCT116 cell spheres treated with different concentration of Xiaotansanjiefang medication, cells spheres were treated with 0.25% trypsin, then were centrifugated and stained with annexin V-FITC and propidium iodide (PI) (Sigma, St. Louis, USA) and for 20 min at room temperature. Finally, the apoptosis of cells was evaluated by flow cytometry. The apoptosis test was repeated three times.

2.5 | Western blot assay

The HCT116 cell spheres were directly lysed for 5 min in the Lysis Buffer. Lysates were separated by centrifugation (13 000 *g*, 30 min, 4°C) and 50 μg of total proteins were electrophoresed on a 10% SDS-PAGE, which was transferred onto polyvinylidene difluoride (PVDF) membranes in a transfer tank using transfer buffer. The first stained membrane was confirmed the transfer efficiency with ponceau S. Then the PVDF membranes were blocked for 1 h at room temperature with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween 20 (TBS-T). Membranes were incubated by primary antibodies, including Hes1, Notch 1, Snail, Notch 3, Jagged 1, then followed by secondary antibody conjugated with horseradish peroxidase. Positive band intensities were shown by utilizing a gel documentation system.

2.6 | Statistical analysis

Data are presented as the mean ± SD, and statistical analysis was performed using SPSS 13.0 software (IBM Corporation, Armonk, NY). Differences among groups were assessed using one-way ANOVA followed by post hoc tests. The values of $p < .05$ were considered statistically significant.

3 | RESULTS

3.1 | The formation of HCT116 cell spheres

For the cancer study, the study of cancer cell spheres makes more sense than the study of cancer cell, which is able to mimic tumor tissues as much as possible. Thus, in this study, we used HCT116 cell spheres as in vitro model to explore the anti-colorectal cancer ability of Xiaotansanjiefang medication. As shown in Figure 1A, the HCT116 cell gradually formed cell spheres under the inducing medium, and all the HCT116 cells aggregated together to form HCT116 cell spheres after 4 days of inducing. In addition, the formed HCT116 cell spheres highly expressed CD26/CD44, CD26/CD326 (Figure 1B). In addition, the HCT116 cell spheres highly expressed Hes1 and Notch1 compared with HCT116 cells (Figure 1C).

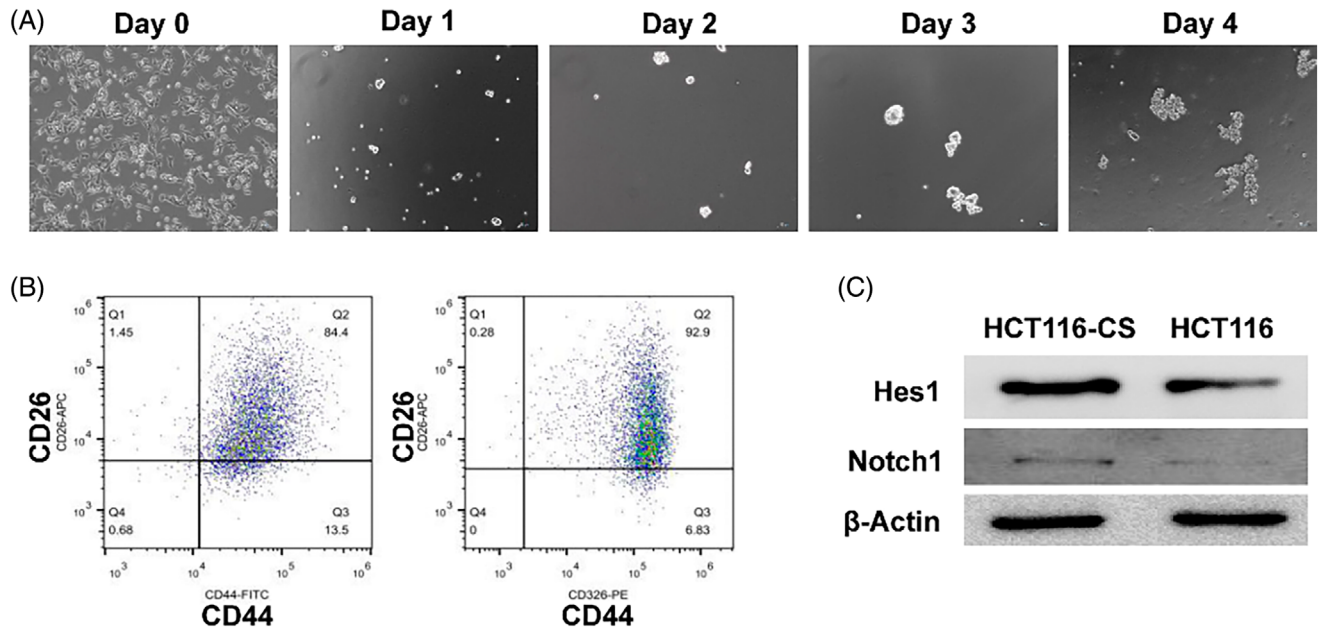

FIGURE 1 The formation and identification of HCT116 cell spheres. (A) The photographs show the formation processes of HCT116 cell spheres from day 0 to day 4. (B) The expression of CD26/CD44, CD26/CD326 of HCT116 cell spheres. (C) The expression of Hes1 and Notch1 on HCT116 cells and HCT116 cell spheres. HCT116-CS, HCT116 cell spheres

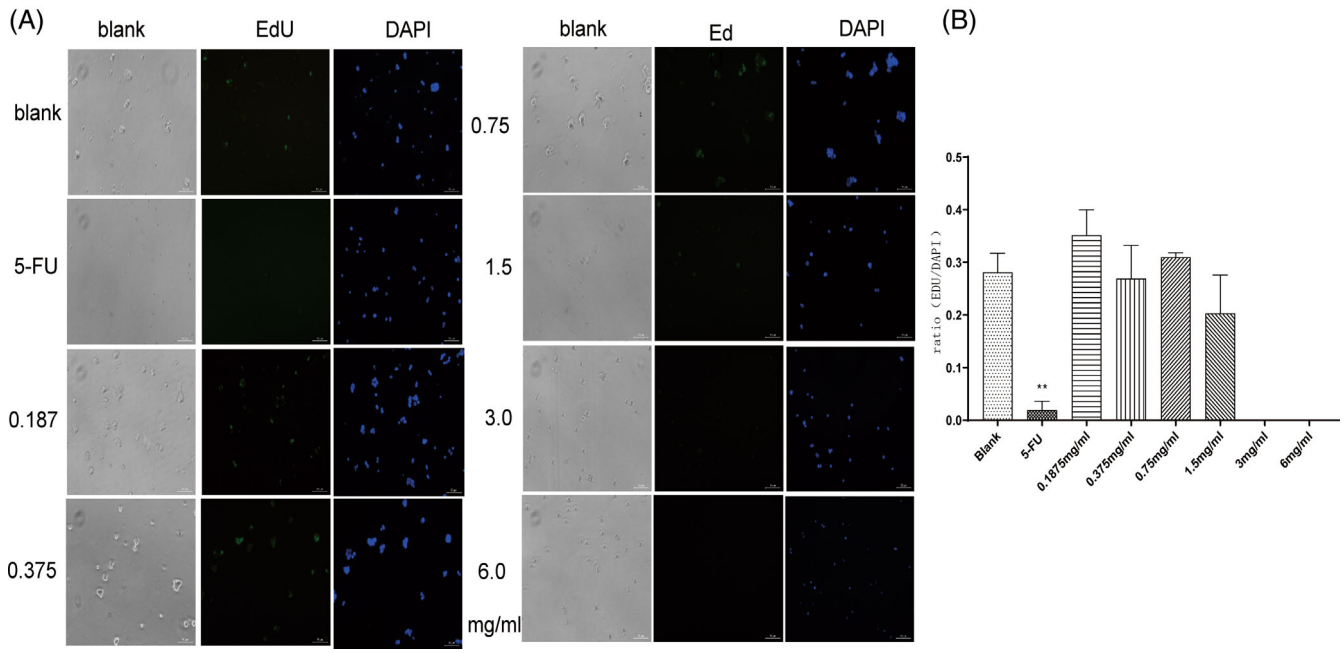

FIGURE 2 The effects of Xiaotansanjiefang medication on the proliferation of HCT116 cell spheres. EDU/DAPI staining of HCT116 cell spheres treated with different concentration of Xiaotansanjiefang. The HCT116 cell spheres treated with 5-FU as control. Relative to the blank group, $^{**}p < .01$

3.2 | Xiaotansanjiefang medication inhibits the proliferation of HCT116 cell spheres

We first examined the effects of Xiaotansanjiefang medication on the proliferation of HCT116 cell spheres. As shown in Figure 2A, highly expressed EdU was detected in the HCT116 cell spheres without any treatment. In addition, 5-FU significantly reduced the expression of EdU in the HCT116 cell spheres. Suggesting the positive control drug 5-FU could inhibit the proliferation of HCT116 cell spheres. Following, we found the Xiaotansanjiefang medication also could suppress the proliferation HCT116 cell spheres in a dose dependent manner, the inhibitory effects 3 and 6 mg/ml Xiaotansanjiefang medication is

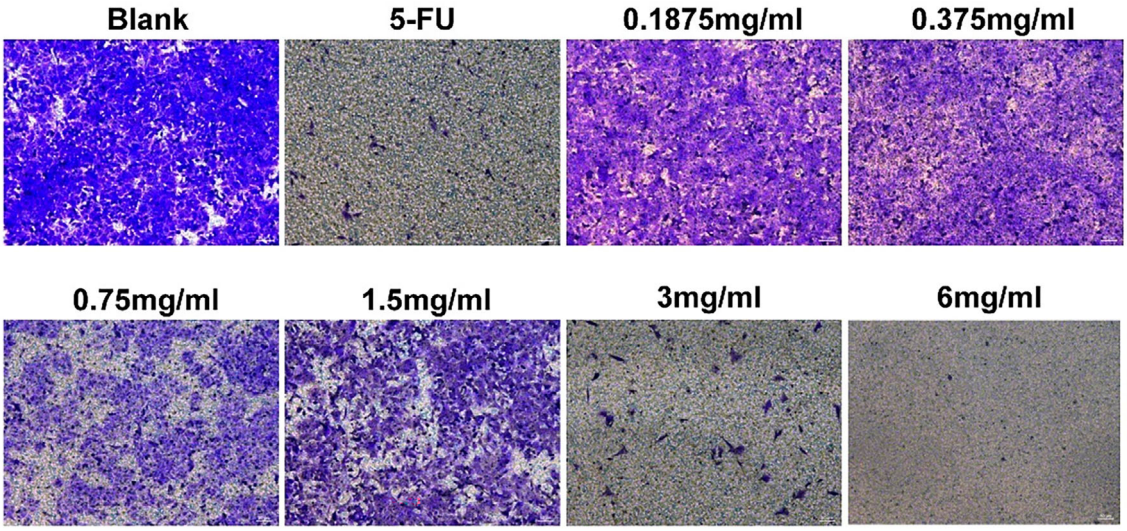

FIGURE 3 The effects of Xiaotansanjiefang medication on the migration of HCT116 cell spheres. H&E staining of HCT116 cells treated with different concentration of Xiaotansanjiefang. The HCT116 cells treated with 5-FU as control

better than the 5-FU treatment (Figure 2B). It reveals that the Xiaotansanjiefang medication with proper concentration also can inhibit the proliferation of HCT116 cell spheres.

3.3 | Xiaotansanjiefang medication prevents the migration of HCT116 cell spheres

Following, we also explored the effects of Xiaotansanjiefang medication on the migration of HCT116 cell spheres. As shown in Figure 3, The HCT116 cells from the HCT116 cell spheres without any treatment could migrate very well. While, no significant migrated HCT116 cells were detected in 5-FU treated HCT116 cell spheres. In the different concentration of Xiaotansanjiefang medication treated HCT116 cell spheres groups, no significant migrated HCT116 cells were detected in 3 and 6 mg/ml Xiaotansanjiefang medication treated groups, especially in 6 mg/ml Xiaotansanjiefang medication treated group. Suggesting, high concentration of Xiaotansanjiefang medication can significantly prevent the migration of HCT116 cells.

3.4 | Xiaotansanjiefang medication promotes the apoptosis of HCT116 cell spheres

Furthermore, we also explored the effects of Xiaotansanjiefang medication on apoptosis of HCT116 cell spheres. As shown in Figure 4A,B, the positive control drug 5-FU could definitely accelerate the apoptosis of HCT116 cell spheres. We also found the Xiaotansanjiefang medication also could promote the apoptosis of HCT116 cell spheres in a dose dependent manner. The apoptotic rate was increased with the increased concentration of Xiaotansanjiefang medication. Moreover, we found the apoptotic rate of HCT116 cell spheres was higher in 3 and 6 mg/ml Xiaotansanjiefang medication treated groups compared the 5-FU treated group. These results discovered that Xiaotansanjiefang medication is able to promote the apoptosis of HCT116 cell spheres.

3.5 | Xiaotansanjiefang medication inhibits the viability of HCT116 cell spheres via Jagged 1 medicated Notch/Snail signaling pathway

Finally, we explored the potential inhibitory mechanism of Xiaotansanjiefang medication on colorectal cancer. As shown in Figure 5, the expression of Jagged1 of HCT116 cell spheres were gradually reduced with the increased concentration of Xiaotansanjiefang medication. It is well known that Jagged1 mediates the Notch/Snail signaling pathway, in this study, we found the expression of Notch 3 and Snail were also gradually decreased with the increased concentration of Xiaotansanjiefang medication. Especially in 3 and 6 mg/ml Xiaotansanjiefang medication treated groups, which was very close to positive control group (5-FU treated group). In addition, we also found the expression of Hes1 had a similar trend with the Jagged1, Notch 3, and Snail. In order to further verify whether the Jagged 1 medicated Notch/Snail signaling pathway involved in this process, we did further verification work. The recombinant human Jagged 1 Fc chimera protein (FC) and DAPT (GSI-IX, LY-374973) is a novel γ-secretase inhibitor were selected for deeply study. As shown in the Figure 6, the treatment of DAPI could significantly inhibit the migration of HCT116 cell spheres, a similar inhibitory result was found in the Xiaotansanjiefang medication treated group. However, the migration of HCT116 cell spheres treated with Xiaotansanjiefang medication + FC reversed the migration inhibitory effects. In addition, The DAPI showed an apoptotic promotion effect, which is similar with the Xiaotansanjiefang medication. We also found the apoptotic promotion role of Xiaotansanjiefang medication could also been reversed by the addition of FC (Figure 7).

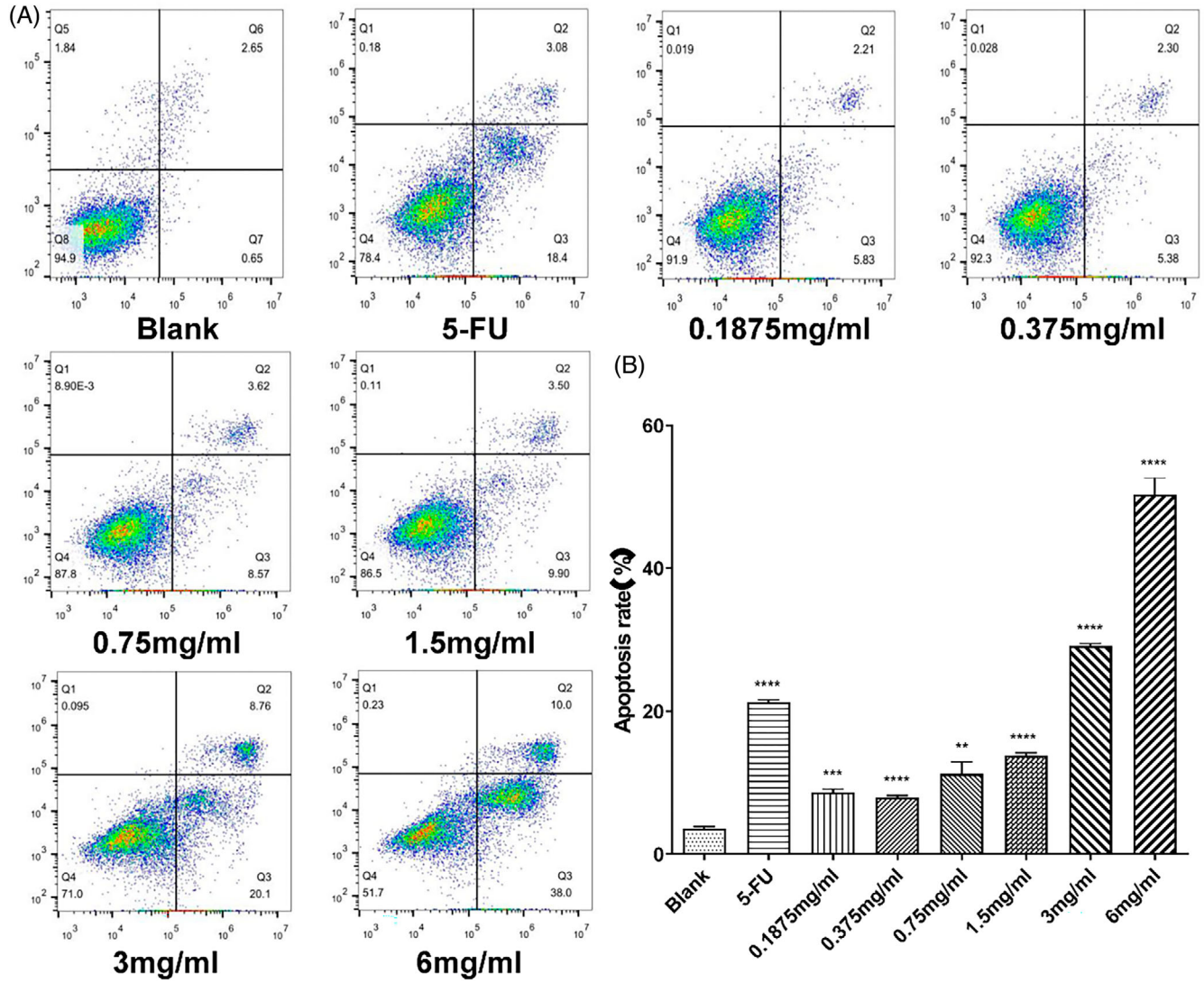

FIGURE 4 The effects of Xiaotansanjiefang medication on the apoptosis of HCT116 cell spheres. The flow cytometry assay of HCT116 cell spheres treated with different concentration of Xiaotansanjiefang. The HCT116 cell spheres treated with 5-FU as control. Relative to the blank group, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$

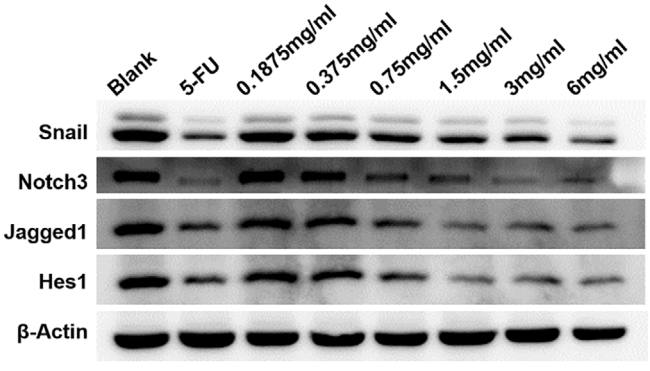

FIGURE 5 The effects of different concentration of Xiaotansanjiefang medication on the expression of Snail, Notch3, Jagged1, and Hes1 on HCT116 cell spheres

Furthermore, the expression of Jagged 1, Notch 3, Snail, and Hes1 could also be reversed in the Xiaotansanjiefang + FC group compared to the Xiaotansanjiefang medication alone group (Figure 8).

4 | DISCUSSION

Jagged1, also known as JAG1, is one of five cell surface ligands that interact with four receptors in the mammalian Notch signaling pathway.[13] Jagged 1 has been reported that abnormal Jagged 1 expression in many types of cancer, including breast cancer, brain cancer, cervical cancer, colorectal cancer, gastric cancer and so on.[14] In the colorectal cancer, it plays very important role on tumor angiogenesis, cancer cell proliferation and apoptosis, function and phenotype of cancer stem

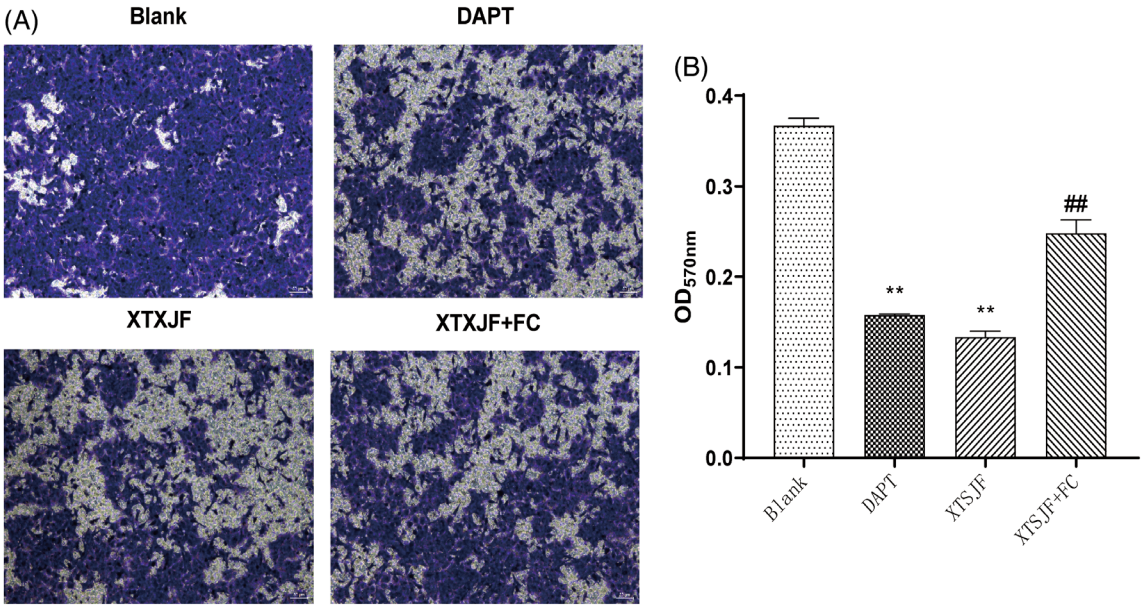

FIGURE 6 (A) The effects of DAPT, Xiaotansanjiefang, Xiaotansanjiefang + FC on the migration of HCT116 cell spheres. (B) The quantification of migration of HCT116 cells treated with DAPT, Xiaotansanjiefang, Xiaotansanjiefang + FC. XTXJF, Xiaotansanjiefang medication. FC, Jagged 1 Fc chimera protein. Relative to the blank group, $^{**}p < .01$; Relative to the DAPT group, $^{\#\#}p < .01$

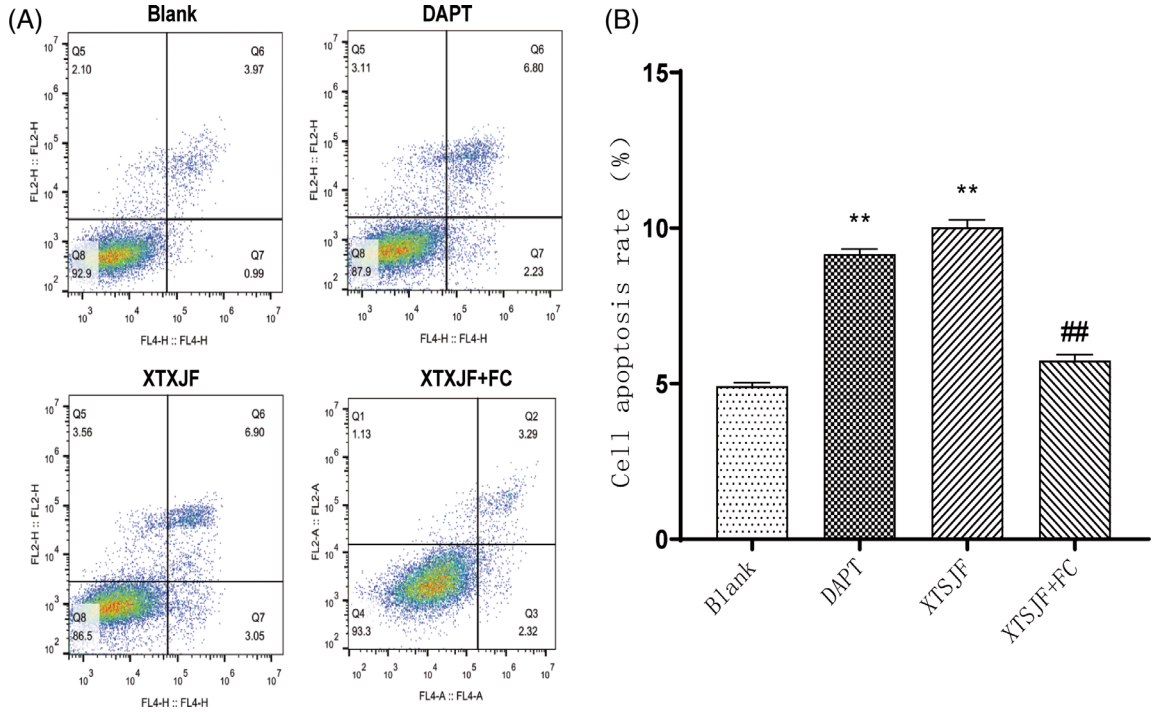

FIGURE 7 (A) The effects of DAPT, Xiaotansanjiefang, Xiaotansanjiefang + FC on the apoptosis of HCT116 cell spheres. (B) The quantification of apoptotic rate of HCT116 cells treated with DAPT, Xiaotansanjiefang, Xiaotansanjiefang + FC. XTXJF, Xiaotansanjiefang medication. FC, Jagged 1 Fc chimera protein. Relative to the blank group, $^{**}p < .01$; Relative to the DAPT group, $^{\#\#}p < .01$

cells, and epithelial-mesenchymal transition.[14,15] Herein, the targeting of Jagged1 could a promising strategy for treating colorectal cancer. In this study, we found the Chinese traditional medicine, Xiaotangshanjiefang medication could inhibit the viability of colorectal cancer cells via suppressing the expression of Jagged 1.

Lots of studies have revealed the role of Jagged 1/Notch signaling pathway on different types of cancer. For example, high expression of Jagged-1/Notch-1 expression in poor prognosis of patients with head–neck cancer.[16] In our study, we found the Xiaotansanjiefang medication inhibited the viability of HCT116 cell spheres via

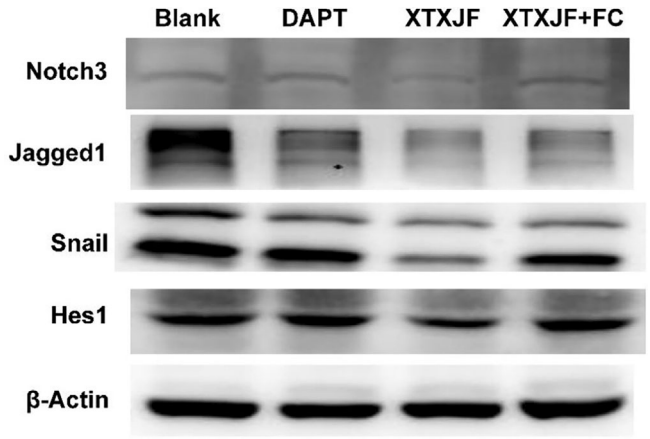

FIGURE 8 The effects of DAPT, Xiaotansanjiefang, Xiaotansanjiefang + FC on the expression of Notch3, Jagged1, Snail, and Hes1 on HCT116 cell spheres. XTXJF, Xiaotansanjiefang medication. FC, Jagged 1 Fc chimera protein

the Jagged 1/ Notch 3 signaling pathway. Wang et al.[17] reported that the expression of Notch3 and Jagged genes were upregulated in many ovarian serous, and there was a positive regulatory loop between Jagged1 and Notch3, which is consistent with our results. γ-Secretase as a key regulator of Notch signaling pathway, the inhibition of γ-secretase could suppress the activity of Jagged 1/Notch signaling pathway,[18] DAPI is a novel γ-secretase inhibitor, the γ-Secretase inhibitor was able to prevent Notch3 activation and inhibit cancer cell proliferation.[19] These findings were also consistent with our results.

The Snail family of Zinc-finger-containing transcriptional repressors, which is, play a significant role in the process of EMT,[20] and Notch has been shown to promote EMT via Snail induction during the oncogenic transformation.[21] In the presented study, the Xiaotansanjiefang medication could inhibit the expression of Notch 3, subsequently inhibit the expression of Snail to suppress the EMT of colorectal cancer. Furthermore, we found the reduced expression of Jagged 1, Notch 3, and Snail on HCT116 cell spheres treated with Xiaotansanjiefang medication could be reversed by the addition of Jagged 1 Fc chimera protein, which proved our results from the other side.

5 | CONCLUSION

In summary, the anti-colorectal cancer effects of Xiaotansanjiefang, a famous traditional Chinese medicine, was explored in this study. We found the Xiaotansanjiefang medication was able to inhibit the proliferation of HCT116 cell spheres in a dose-dependent manner, especially in 3 and 6 mg/ml Xiaotansanjiefang medication treated groups. We also found the high concentration of Xiaotansanjiefang medication could suppress the migration and promote the apoptosis of HCT116 cell spheres. The mechanism studies results discovered that the Jagged1/Notch 3/Snail signaling pathway was involved in this process. The presented study may provide a promising strategy to treat and prevent colorectal cancer.

AUTHOR CONTRIBUTIONS

Xiaoqiang Yue and Dashi Sun conceived and designed the experiments; Min Ye, Jiaqi Du, Xiaowei Wang, Lijuan Xiu, Xuan Liu, Yufang Gu, and Bei Pei performed the experiments; Min Ye, Jiaqi Du, and Xiaowei Wang analyzed the data; Min Ye, Jiaqi Du, Xiaowei Wang, Dashi Sun, and Xiaoqiang Yue wrote the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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