FOLFOX Combined with Mithramycin-A Has A Cytotoxic Effect On The Primary Colorectal Cancer Stem Cells

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1. Abstract

1.1. Objectives: To screen out sensitive chemotherapy regimens for cancer stem cells (CSCs) from colorectal cancer (CRC) patients in northeast Sichuan of China and provide the basis for "regional precision" targeted therapy.

1.2. Methods: Primary colorectal CSCs from the northeast Sichuan of China were extracted using the CRC CSCs protocol developed previously. The chemotherapeutic agents, FOLFOX, Curcumin, Salinomycin, and Mithramycin-A (Mit-A) were used to assess changes in the proportion of CD133+, CD44+ and CD133+CD44+ cells in each group using flow cytometry. Colony Formation Assay, Sphere-Forming Assay, Cell migration and cell invasion assay, and CCK-8 were utilized to detect the proliferation, migration and invasion ability of primary CRC CSCs in vitro. QT-PCR detected expression of the core CSC genes, Oct-4, Sox2, Nanog, and KLF4, CRC CSCs marker genes, and Wnt/β-catenin pathway related genes. By comparing the effectiveness of the three regimens on primary CRC CSCs in northeast Sichuan of China, chemotherapy regimens that are sensitive to CSCs in CRC patients from this area could be screened out.

1.3. Results: The proportion of CD133+CD44+ cells in the DLD-1 cell line reached 54.42%, and the proportion of CD133+CD44+ cells in primary CRC cells was 13.72%. In response to the C4 regimen, the proportions of CD133+, CD44+ and CD133+CD44+ cells were significantly reduced, the volume and number of cell spherules were suppressed, cell survival rate was significantly re-duced, and almost no cells were able to migrated or invade. In addition, there was a reduction in mRNA expression of the CSC related genes, Oct-4, Nanog, SOX2 and KLF4, the CRC CSCs-related gene, Lgr5, the mesenchymal genes, Vimentin and Snail, and Wnt/β-catenin pathway related genes.

Conclusion: The combination of FOLFOX and Mithramycin A can effectively inhibit the occurrence, metastasis and recurrence of colorectal cancer CSCs and inhibit the dryness and mesenchymal properties using the Wnt/β-catenin and TGF-β1/snail pathways.

2. Introduction

Colorectal cancer (CRC) is second only to lung cancer as the most common malignancy, with more than 1.9 million new diagnoses made each year in developed countries [1-3]. CRC incidence is increasing worldwide likely as a result of changes in human behavior, including reduced exercise, increased consumption of animal-derived foods, and excessive alcohol intake [4]. The incidence of CRC in transitioning countries is four times that of other areas, in particular North America and Europe [5,6]. CRC incidence in Asia is increasing in countries such as China that are becoming more westernized [7-9]. Of note, CRC accounts for nearly 45% of all cancer-related deaths due to late diagnoses in China [10,11]. FOLFOX is one of the most common first-line chemotherapeutic drugs used to treat a variety of cancers [12,13]. CRC patients are frequently resistant to chemotherapy regimens using FOLFOX, however, and often require additional conventional chemotherapeutic agents such as Capecitabine, Irinotecan, angiogenesis inhib-

itors and anti–epidermal growth factor receptor (EGFR) antibodies, to enhance tumor cell chemosensitivity [14-18]. As a result, CRC treatment remains unsatisfactory. Cancer stem cells (CSCs) are a subpopulation of cancer cells identified in multiple malignancies, including breast cancer and CRC, that have the ability to self-renew and give rise to new tumors and metastases [19-25]. Thus, there is an urgent need for safer and more effective chemotherapeutic treatments that target CSC activity.

3. Materials and Methods

3.1 Ethics Statement

The informed written consent was obtained from patients. This study was conducted in accordance with the Declaration of Helsinki and approved by the institution's ethics board (Permit Number#S255).

3.2. Cell Lines and Culture

DLD-1 human CRC cell lines were obtained from the American Type Culture Collection (ATCC) and authenticated by short tandem repeat PCR profiling at ATCC. Cell lines were obtained in March 2016 and cultured in ATCC medium in a humidified incubator with 5% CO_2 at 37°C.

3.3. Collection

: and treatment of CRC tissues and enrichment of primary tumor stem cells Samples from patients receiving surgical therapy at Affiliated Hospital of North Sichuan Medical College were histologically diagnosed as CRC. Surgically resected tumor tissues were stored in PBS containing 1000 units/mL penicillin, 1000 μg/mL streptomycin, and 3 μg/mL fungizone. Samples were repeatedly washed with PBS after transportation to the laboratory to remove blood, fat, and feces, cut into 1×1×1 mm pieces, soaked for 15 min in high concentration antibiotics independently developed by our research group, and then digested with 2 mg/ml collagenase II (Invitrogen) on an oscillator for 3 h at 37°C. Cells were triturated, filtered through 100 μm and 50 μm filters to obtain a single-cell suspension, submerged in high concentration antibiotics for 30 min at 37°C, and washed three times with sterile PBS [29]. Primary human CRC stem cells were collected by adding serum-free medium (SFM) (described below). Cultures were maintained in an incubator with 5% CO_2 at 37°C during the whole culturing period. The cultures were treated every other day with 2 ml SFM, digested every ten days, cultured until the thirtieth day, and relevant experiments were carried out.

3.4. Enrichment of Tumor Atem Cells

DLD-1 cells were grown at a density of 2×106 cells/ml in serum-free DMEM/F12 medium containing 20 ng/ml epidermal growth factor (EGF, PeproTech), 10 ng/ml basic Fibroblast Growth Factor (bFGF, PeproTech), 5 μg/ml insulin (Sigma), 0.4% BSA (Amresco), and 2% B27 (Invitrogen). All cells were maintained in a humidified incubator at 37°C and 5% CO_2.

3.5. Chemotherapy Regimens

Oxaliplatin, 5-FU, Salinomycin, and Curcumin were purchased

from Sigma Pharmaceuticals, North Liberty, IA, USA. Mithramycin-A was purchased from Merck & Co., Kenilworth, NJ, USA. After every thirty days of culturing the cells, the solution was changed and the following chemotherapeutic drugs were added for 48 h: C1(5.4 μM/m of Oxaliplatin, 220 μM/ml of 5-Fu), C2(5.4 μM/m of Oxaliplatin, 220 μM/ml of 5-Fu,17.82 μM/ml of Curcumin), C3(5.4 μM/m of Oxaliplatin, 220 μM/ml of 5-Fu, 8 μM/ml of Salinomycin) and C4(5.4 μM/m of Oxaliplatin, 220 μM/ml of 5-Fu, 0.5 μM/ml of Mithramycin-A) [31-34].

3.6. Flow Cytometry

Surface expression of CD133 + CD44 + on DLD-1 cells was detected using flow cytometry. After the spheroid cells are trypsinized and resuspended in PBS, 5×105 cells were added to the blocking solution, blocked at 4°C for 1 hour, and the supernatant was discarded following centrifugation (1500 r/min 5min). Twenty-five microliters of blocking solution and 1.25μl of the corresponding CD133+CD44+ antibodies were added. The cells were then blocked for 0.5 h at 4℃ in the dark, centrifuged and suspended in 500 μl PBS. Surface expression was detected using a BD FACSVerse flow cytometer.

3.7. Colony Formation Assay

After conventional trypsin digestion and resuspension in PBS, 5×103 cells were taken from each group and cultured in a 6-well plate with 1640 medium containing 10% FBS. After 14 days, the cells were stained with crystal violet, the number of clones were counted and statistical analysis was performed.

3.8. Sphere-Forming Assay

After conventional trypsin digestion and resuspension in PBS, 1×103 cells were removed from each group and seeded in a 24-well plate with 1ml/well of the SFM medium described above. After the culture reached day 14, the number of cell spheres formed in each well were counted and statistical analysis was performed.

3.9. Cell Migration and Cell Invasion Assay

Matrigel glue was diluted to a working concentration of 200 μg/ml and 100 μl was placed in the upper chamber of the Transwell cell at 37°C for 2 h to solidify. The spheroid cells were routinely trypsinized and resuspended in serum-free 1640 medium. The cell density was adjusted to 5×105 cells/ml, and 200 μl was added to the upper chamber of the Transwell chamber. Six-hundred microliters of 1640 medium containing 10% FBS was added to the lower chamber of the Transwell cell and cultivated in a 37°C, 5% CO_2 incubator. After 48 h, the cells were removed, fixed with 4% paraformaldehyde for 30 min, stained with crystal violet solution for 30 min, observed and photographed under a microscope, and counted.

3.10. Cell Proliferation Assay

After routine trypsin digestion, the cells were resuspended in PBS, and 5×103 cells from each group were innoculated into 96-well plates and placed in an incubator for 24 h. Ten microliters of CCK-

8 solution was added to each well, the culture plate was placed in an incubator for 4 h, and the absorbance was measured at 450 nm using a microplate reader. The expression of cell viability was measured using the following forumla: cell viability (%) = [A (sample)−A (blank)]/[A (control)−A (blank)] ×100%.

3.11 Real-time-PCR

Total mRNA was extracted from the cells and reverse transcribed into cDNA using Prime Script RT Master Mix (Takara, Japan) according to the manufacturer's instructions. To measure mRNA levels in each sample, real-time PCR based on intercalators was used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. SYBR Green Master Mix (Takara, Japan) was used for real-time PCR in the StepOnePlus™ real-time PCR system (Applied Biosystems, Inc.). PCR amplification was performed as follows: 45 cycles of denaturation at 95°C for 30s, annealing at 60°C for 60s, and extension at 95°C for 5 s. The reaction was repeated for each sample and the 2-ΔΔCt method was used to assess the average mRNA level of each gene. The primer pairs used to measure the level of each gene are listed in Table S1.

3.12 Statistical Methods

The GraphPad Prism 8.0 was used to conduct the Student's t test or ANOVA analysis, as appropriate. Compared with untreated ("control") or treated cells, the difference was considered statistically significant, p<0.05. Results are expressed as the mean ± standard error of the mean (SEM) for at least three independent experiments.

4. Results

3.1 The combination of FOLFOX regimen and Mithramycin A can effectively reduce the ratio of CD133+CD44+ in CSCs. Studies show that CSCs exist in various solid tumors; however, their numbers are scarce and they are thus difficult to obtain [19-22]. At present, CSCs are mainly obtained through flow cytometry sorting (FACS) [35-36], magnetic activated cell sorting (MAC) [37], or serum-free medium (SFM) suspension culture method [38]. However, due to the small number of cells obtained by flow sorting and immunomagnetic bead separation, it is difficult to meet the experimental requirements. Thus, a self-developed serum-free suspension culture method was used to enrich and label the colorectal cancer CSCs with CD133+CD44+ in this study (Figure 1). Findings indicate that the proportion of CD133+CD44+ cells in the DLD-1 cell line was as high as 54.42%, and the proportion of CD133+CD44+ cells in the primary CRC cells was 13.72% in serum-free suspension culture (Figure 1). After 48 hours, in both the DLD-1 cell line and primary cells, the ratio of CD133+CD44+ cells exposed to the C3 regimen were higher than those exposed to the other regimens, with a higher ratio of CD133+ cells and a lower ratio of CD44+ cells (Figure 2). After exposure to the C4 regimen, the proportions of CD133+, CD44+ and CD133+CD44+ were significantly reduced. These results indicate that the FOLF-

OX combined with Mithramycin-A regimen is more effective than other regimens at inhibiting CD44+ and CD133+CD44+ in primary cells. 3.2 The combination of FOLFOX and Mithramycin-A can effectively inhibit the proliferation, invasion, and metastasis of CRC CSCs. To further study the effect of the combined program on the proliferation, invasion, and metastasis of primary CSCs, we performed a cell migration and cell invasion assay, a Sphere-Forming Assay, a Colony Formation Assay and CCK-8 on cells treated with the different chemotherapy groups. Primary CRC CSCs formed the largest number of spheroids after 10 days. The spheroid cells were stable in shape, the cells were tightly arranged, and the surface of the spheres was smooth (Figure 3A). A-B: As assessed by the spheroidization experiment, the growth of spheroid cells was restricted and the number decreased after chemotherapy; C-F: According to the assessment of cell invasion and migration experiments, the migration of tumor stem cells after FOLFOX combined with Mithramycin-A regimen chemotherapy. And invasion ability is significantly slower than other group programs. n=3, *P＜0.05, **P ＜0.01, ns: no statistical significance. After chemotherapy, scattered cell particles appeared around the spheroid cells, the cell structure was damaged to varying degrees, and the cell spheroid volume was reduced (Figure 3A). At the same time, the ability of the CSCs to proliferate, invade, and metastasize was significantly weakened, indicating that the FOLFOX and combination regimens can effectively inhibit the malignant behavior of primary CSCs in vitro (Figure 3,4A). After the addition of Mithramycin-A, cell sphere volume and number and survival rate were significantly reduced, and few cells were able to migrate or invade. These findings indicate that FOLFOX combined with Mithramycin-A regimen is more effective than other regimens in inhibiting the proliferation, migration, and invasion of colorectal CSCs. 3.3 FOLFOX combined with Mithramycin-A can effectively inhibit the stemness and mesenchymal properties of primary colorectal cancer CSCs. The effect of combined chemotherapy on the stemness of primary CRC CSCs was assessed by studying expression of CSCs Oct-4, Sox2, Nanog, KLF4 and CRC CSC marker genes. Results showed no significant differences between expression of the cancer stem cell-related stem genes, Oct-4, Sox2 and Nanog following the C1 and C2 chemotherapy regimens (Figure 5). Mesenchymal-related gene Vimentin mRNA expression was higher following C2 than C1 treatment (Figure 5). C3 treatment resulted in decreased expression of the CSCs-related genes, Sox2 and KLF4, increased expression of the mesenchymal-related genes, Vimentin, and decreased expression of E-cadherin. C4 treatment resulted in decreased Oct-4, Nanog, Sox2 and KLF4 mRNA expression, and reduced expression of Lgr5 mRNA (Figure 5). These findings indicate that the combination of FOLFOX and Mithramycin-A can effectively inhibit the stemness and mesenchymal properties of colorectal cancer stem cells using the Wnt/β-catenin pathway.
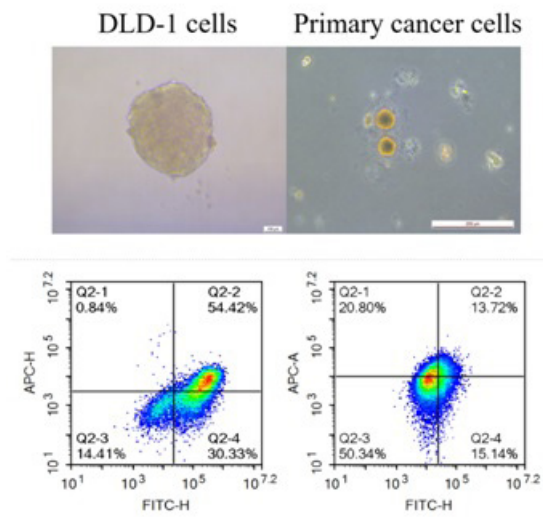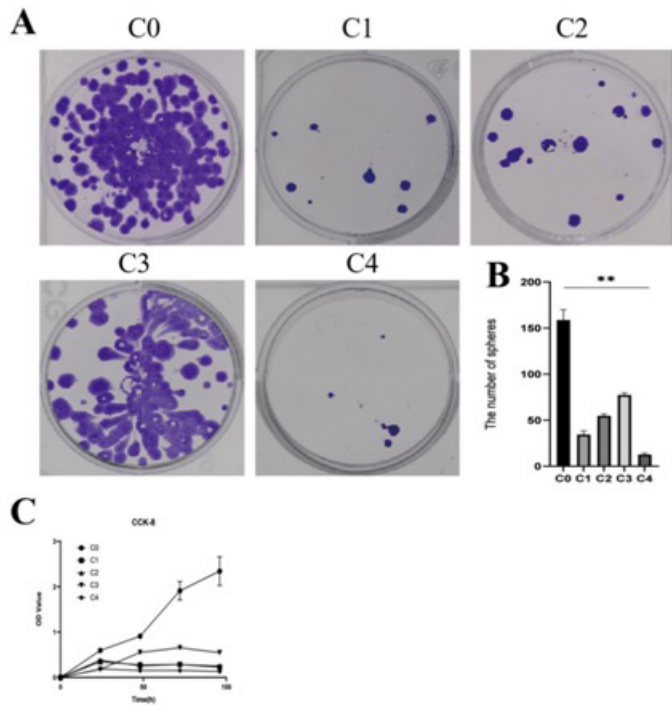
Figure 1: Collection of primary colorectal cancer stem cells and DLD-1 cells by enrichment.
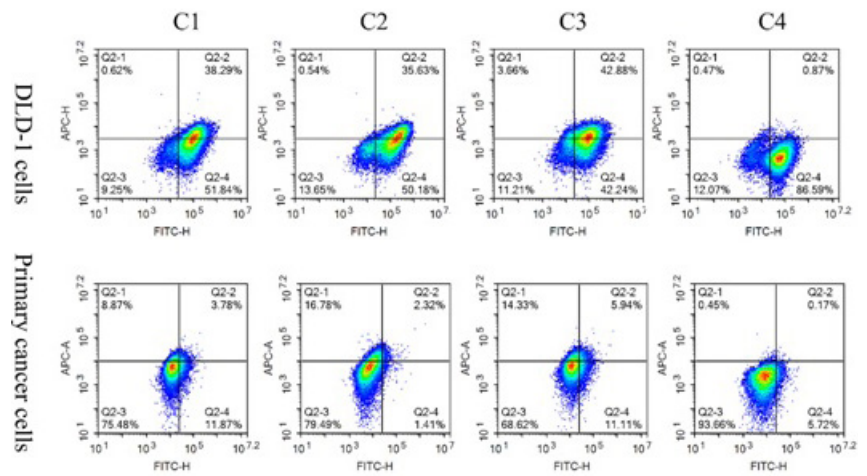
Figure 2: The proportion of CD133+、CD44+、CD133+CD44+ cells in each group of chemotherapy regimens.
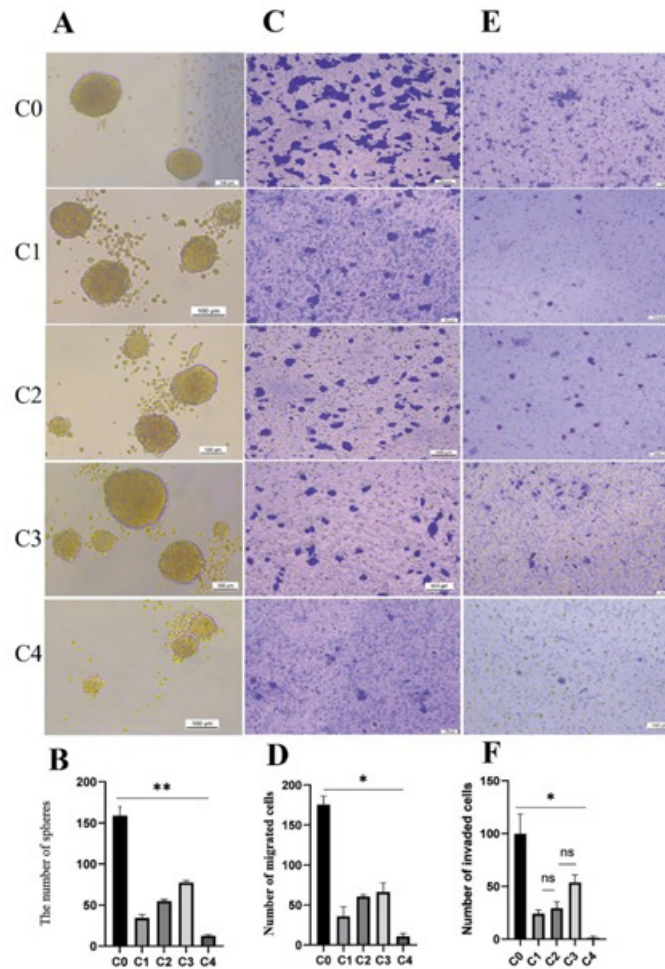
Figure 3: FOLFOX combined with Curcumin, Salinomycin and Mithramycin-A inhibit the stemness and metastasis of CSC.
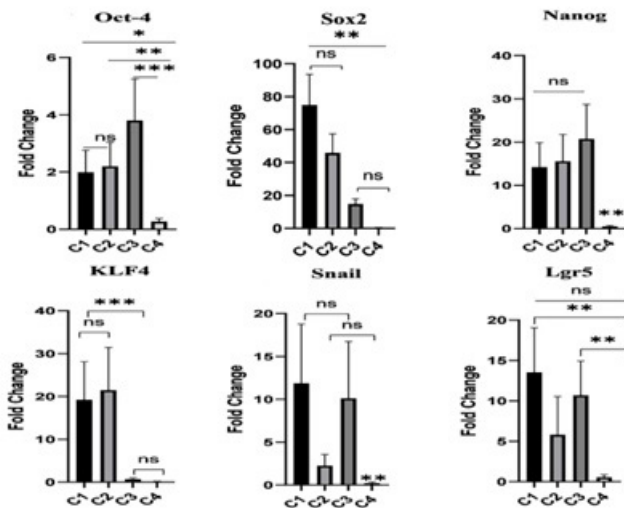
Figure 4: The proliferation of primary colorectal cancer CSCs is weakened after chemotherapy.

Figure 5: The mRNA expression of the core genes of CSCs and the CSCs marker genes of primary colorectal cancer.

Figure 6: The mRNA expression of mesenchymal-related genes and Wnt/β-catenin pathway-related genes.
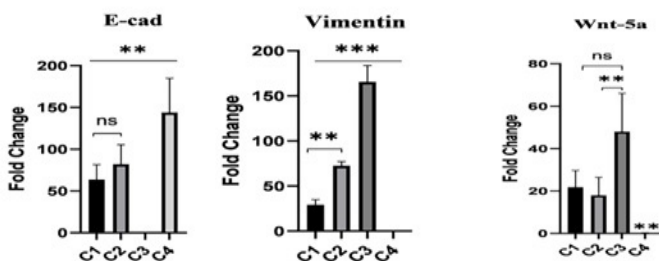
5. Discussion

The treatment of CRC is primarily dependent on surgery and comprehensive treatment that includes radiotherapy and chemotherapy [39,40]. The chemotherapy regimen for CRC has transitioned from single to combined drugs following the development of targeted drugs like Capecitabine, 5-Fu, and Oxaliplatin [41,42]. As FOLFOX treatment continues to be used, a higher proportion of patients are showing resistance [43,44]. This study explored the effectiveness of FOLFOX combined with other drugs on CRC CSCs by obtaining the primary cells of colorectal cancer in northeast Sichuan of China in order to screen out sensitive chemotherapy options for CRC patients. CSC research has led to the identification of more surface markers that can be used to label cancer stem cells, including CD133, CD44, ALDH, Lgr5, and EpCAM [45-49]. CD44+ cells have the characteristics of CSCs, a higher tumor-forming ability than CD44- cells, and lower sensitivity to 5-FU in vitro [50]. CD133 is one of the most well-characterized biomarkers used to isolate CSCs [51]. The current study finds that reduction of CD44+ cells does not affect the proliferation and invasion ability of CSCs, however CD133+ cells are linked to cell proliferation, metastasis, and invasion. Thus, while CD44+ cells may have high tumorigenicity, they are not as likely to be CSCs as CD133+ cells. Mithramycin-A is a polyketide antibiotic that binds to the minor groove of DNA, thus inhibiting binding of transcription factor SP1 to DNA [52]. Research has also shown that Mithramycin-A can inhibit g-MDSCs by blocking PD-L1 receptors resulting in increased T cell infiltration and inhibiting proliferation of CSCs [28]. This study finds that FOLFOX combined with Mithramycin-A can effectively kill CSCs and inhibit the proliferation, migration, and invasion of CRC cells using Wnt/β-catenin and TGF-β1/snail pathways. FOLFOX combined with Mithramycin-A can effectively inhibit the occurrence, metastasis, and recurrence of CRC stem cells, using the Wnt/β-catenin and TGF-β1/snail pathway to inhibit CSCs and mesenchymal characteristics.

6. Funding

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