Research Article

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EpCAM is critical for tumor proliferation and oxaliplatin chemoresistance in EpCAM\textsuperscript{high}/CD44\textsuperscript{+} colorectal cancer stem cells

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Abstract

Objectives: A small subpopulation of colorectal cancer stem cells (CSCs) possess the ability to self-renew and the capacity to initiate the original tumor. EpCAM\textsuperscript{high}/CD44\textsuperscript{+} cells are regarded as CSCs in colorectal cancer. The present study was undertaken to investigate the significance of EpCAM in the \textit{in vitro} proliferation ability and oxaliplatin chemoresistance of EpCAM\textsuperscript{high}/CD44\textsuperscript{+} colorectal CSCs.

Methods: We applied fluorescence-activated cell sorting (FACS) to separate the EpCAM\textsuperscript{high}/CD44\textsuperscript{+} subset from human colorectal cancer cell line HCT116. We also used siRNA targeting EpCAM to create EpCAM\textsuperscript{−}/CD44\textsuperscript{+} subpopulation. Then we compared EpCAM\textsuperscript{high}/CD44\textsuperscript{+} cells and EpCAM\textsuperscript{−}/CD44\textsuperscript{+} cells for proliferation ability and the chemoresistance to oxaliplatin by CCK8 assay.

Results: The EpCAM\textsuperscript{high}/CD44\textsuperscript{+} subset comprises almost 6.25 \pm 0.09\% in cell line HCT116, and the EpCAM\textsuperscript{−}/CD44\textsuperscript{+} cells displayed a significantly lower proliferation ability and weaker oxaliplatin chemoresistance than the EpCAM\textsuperscript{high}/CD44\textsuperscript{+} cells.

Conclusions: EpCAM is critical for tumor proliferation and oxaliplatin chemoresistance in EpCAM\textsuperscript{high}/CD44\textsuperscript{+} colorectal CSCs.

Keywords: CD44; colorectal cancer; EPCAM; oxaliplatin chemoresistance; tumor proliferation.

Introduction

The prevalence of colorectal cancer increases with age, and it is the 3rd most commonly diagnosed cancer in developed countries [1]. The significant risk factors of colorectal cancer are contributed by the increasingly ageing population, unfavorable modern dietary habits, low physical exercise, obesity, and smoking [2]. Surgery can be applied to remove tumors in almost 2/3 of patients with colorectal cancer that is localized. Radiation therapy is used to cure about 20\% of patients with colorectal cancer, which has spread to other organs, most commonly to the liver [3]. Patients with cancer recurrence can be treated with surgery, and chemotherapy is still the primary therapeutic method for advanced colorectal cancer [4, 5]. There has been substantial progress in the treatment of colorectal cancer recently [6].

Colorectal carcinomas consist of heterogeneous cells [7]. Colorectal cancer stem cells (CSC) have gained great attraction due to their high tumorigenicity and chemoresistance. Many studies have shown that three key pathways play a crucial role in stem cell proliferation and maintenance: the Notch pathway, the WNT pathway, and the bone morphogenetic protein (BMP) pathway [8–10]. Recent studies revealed molecular signatures of colorectal CSCs are associated with patient outcomes. Some CSC-directed therapies are being performed on patients to translate CSC discoveries into the clinics [11].

Several markers are used for colorectal CSC identification, such as epithelial cell adhesion molecule (EpCAM) [12], CD44 [13], CD166 [14], aldehyde dehydrogenase-1 (ALDH-1) [15] and CD26 [16]. EpCAM\textsuperscript{high}/CD44\textsuperscript{+} colorectal CSCs are considered putative colorectal CSCs rich in stem cell genes [17]. However, the significance of EpCAM in the \textit{in vitro} proliferation ability and oxaliplatin chemoresistance of EpCAM\textsuperscript{high}/CD44\textsuperscript{+} colorectal CSCs is elusive. Therefore, this study is to elucidate the great value of
EpCAM in EpCAM<sup>bsh</sup>CD44<sup>+</sup> colorectal CSCs to provide the theoretical foundation for EpCAM-targeted therapies in the future.

Materials and methods

Chemical

A 1 g/L stock solution of Oxaliplatin (Sigma, China) was prepared in a 5% glucose solution, which was diluted by RPMI-1640 medium (Gibco, USA) to make a working solution.

Cell culture

The human colorectal cancer cell lines (HCT116) were obtained from the Institute of Biophysics, Chinese Academy of Sciences. HCT116 cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% streptomycin and penicillin (Sigma, China), which were maintained in an incubator supplied with 5% CO<sub>2</sub> at 37 °C.

Flow cytometry sorting

After washing with Phosphate buffer solutions (PBS), HCT116 cells were stained with FITC-conjugated anti-CD44 and APC-conjugated anti-EpCAM antibodies for 30 min on ice. The BD FACS Aria III cell sorter was applied to analyze and isolate the EPCAM<sup>bsh</sup>/CD44<sup>+</sup> cells.

Gene silencing of EpCAM

The mismatched control and the siRNA targeting human EpCAM were designed and synthesized from Ribobio (Guangzhou, China). Mismatched control siRNA sequences were as follows: sense, UUCUCCGAACGUACGUUdTdT; antisense, ACGUGACACGUUCGGAGAAdTdT. The siRNA sequences for EpCAM were as follows: sense, UUCUCCGAACGUGACGUUdTdT; antisense, ACGUGACACGUUCGGAGAAdTdT. The siRNA sequences for EPCAM were as follows: sense, AACTGAAGTACACTGGCAAGAAdTdT; antisense, AACTGAAGTACACTGGCAAGAAdTdT. The mismatched control siRNA sequences were as follows: sense, CAATGCCAGTGTACTTCAGTT; antisense, AACTGAAGTACACTGGCAAGAAdTdT. The siRNA sequences for EPCAM were as follows: sense, UUCUCCGAACGUGACGUUdTdT; antisense, ACGUGACACGUUCGGAGAAdTdT.

Western blotting

Whole proteins were extracted from HCT116 cells. Thirty mg of protein from each group was separated into each well of a 96-well plate for 24 h, and the old medium was removed. Then 10 μL of fresh medium was added to each well, which was cultured for another 4. A multimode plate reader (EnSpire, USA) was used to compare the cell viability based on the absorbance at 450nm. The detection time was set at 0 day, 1 day, 2 day, 3 day, 4 day, and 5 day.

The assay to test the proliferation ability

A CCK8 kit (Biyuntian Biological Technology Co., LTD, China) was used to evaluate the proliferation ability of EpCAM<sup>bsh</sup>/CD44<sup>+</sup> and EPCAM<sup>bsh</sup>/CD44<sup>+</sup> HCT116 cells. 1 × 10<sup>3</sup> cells were cultured into each well, which was cultured for another 4. A multimode plate reader (EnSpire, USA) was used to calculate the cell viability based on the absorbance at 450nm.

The assay to test the chemoresistance

A CCK8 kit (Biyuntian Biological Technology Co., LTD, China) was used to compare the chemoresistance of EpCAM<sup>bsh</sup>/CD44<sup>+</sup> and EPCAM<sup>bsh</sup>/CD44<sup>+</sup> HCT116 cells. The cells were seeded into the 96 well plates, and the cells were treated with 2, 4, 8, 16, 32, or 64 mg/L Oxaliplatin for 24 h. Then the old medium was removed, and 10 μL CCK8 in 90 μL new medium was added to each well, which was cultured for another 4. A multimode plate reader (EnSpire, USA) was used to calculate the cell viability based on the absorbance at 450nm.

Statistical analysis

Statistical difference was analyzed by GraphPad Prism version 7.0 (GraphPad Software Inc., California, USA). The statistical significance of the data from three independent experiments was

| Gene name | Gene sequence |
|-----------|---------------|
| EpCAM     | Forward 5' GTCGCTCTGCTGCTGCTGCT 3'  |
|           | Reverse 5' CATTCTGCTGCTGCTGCTGCT 3'  |
| β-Actin   | Forward 5' ACTCTTCCTGCTGCTGCTGCT 3'  |
|           | Reverse 5' CATTCTGCTGCTGCTGCTGCTGCT 3'  |
analyzed by Student’s t-test, which is presented as the mean ± SD (standard deviation). p<0.05 or p<0.01 was considered statistically significant.

**Results**

**The proportion of the EpCAM\textsuperscript{high}/CD44\textsuperscript{+} subset in the human colorectal cancer cell line HCT116**

The EpCAM\textsuperscript{high}/CD44\textsuperscript{+} cells are regarded as colorectal CSCs. Anti-CD44 and anti-EPCAM antibodies were used to separate EpCAM\textsuperscript{high}/CD44\textsuperscript{+} subpopulation from the human colorectal cancer cell line HCT116 (Figure 1), which comprises 6.25 ± 0.09% of the whole cell number.

**The EpCAM silencing efficacy in the EpCAM\textsuperscript{high}/CD44\textsuperscript{+} cells**

The EpCAM\textsuperscript{high}/CD44\textsuperscript{+} cells were treated with siRNA targeting EpCAM, RT-PCR was used to detect the mRNA levels of EpCAM, and Western Blotting was applied to evaluate the protein levels of EpCAM. As shown in Figure 2A, B, the mRNA and protein levels of EpCAM decreased significantly after the treatment of EpCAM\textsuperscript{high}/CD44\textsuperscript{+} cells with siRNA targeting EpCAM.

![Figure 1: The proportion of the EpCAM\textsuperscript{high}/CD44\textsuperscript{+} subset in the human colorectal cancer cell line HCT116.](image1)

![Figure 2: The EpCAM silencing efficacy in the EpCAM\textsuperscript{high}/CD44\textsuperscript{+} HCT116 cells (A) Western blot analysis of EPCAM level in the EpCAM\textsuperscript{high}/CD44\textsuperscript{+} HCT116 cells treated with control, mm siRNA or EpCAM siRNA (B) semi-quantitative analysis of EpCAM mRNA levels detected by real-time RT-PCR analysis and (C) semi-quantitative analysis of EpCAM protein levels detected by Western blotting. *p<0.05, **p<0.01 vs. control group.](image2)
The comparison between EpCAM\(^{\text{high}}\)/CD44\(^+\) cells and EpCAM\(^{-}\)/CD44\(^+\) cells in proliferation ability

To evaluate the significance of EpCAM in the proliferation ability of EpCAM\(^{\text{high}}\)/CD44\(^+\) cells, a CCK8 assay was applied to compare the proliferation ability of EpCAM\(^{\text{high}}\)/CD44\(^+\) cells with EpCAM\(^{\text{high}}\)/CD44\(^+\) cells at 0day, 1day, 2day, 3day, 4day and 5day. According to Figure 3, EpCAM\(^{\text{high}}\)/CD44\(^+\) cells showed a significantly lower proliferation ability than EpCAM\(^{\text{high}}\)/CD44\(^+\) cells.

The comparison between EpCAM\(^{\text{high}}\)/CD44\(^+\) cells and EpCAM\(^{-}\)/CD44\(^+\) cells in the chemoresistance

To determine the significance of EpCAM in chemoresistance, CCK8 assay was applied to compare the chemoresistance of EpCAM\(^{-}\)/CD44\(^+\) cells with EpCAM\(^{\text{high}}\)/CD44\(^+\) cells after treatment with different concentrations of oxaliplatin (2, 4, 8, 16 or 64 mg/L) for 24, 48 or 72 h. The results in Figure 4A–C revealed that EpCAM\(^{-}\)/CD44\(^+\) cells showed a significantly lower oxaliplatin chemoresistance than EpCAM\(^{\text{high}}\)/CD44\(^+\) cells control.

Discussion

About 945,000 patients are diagnosed with colorectal cancer, and almost 492,000 patients die from this disease globally each year [18]. The new molecular basis of colorectal cancer has resulted in the emergence of targeted therapeutic methods tested in clinics. Recent research has highlighted that colorectal CSCs play a crucial role in cancer chemoresistance and relapse. Targeting colorectal CSCs through monoclonal antibodies or small molecule inhibitors in vivo studies has demonstrated the effectiveness of these treatments [11]. CD44 is a multifunctional glycoprotein present on the cell surface, and EpCAM is an epithelial cell adhesion molecule [19, 20]. Some investigations suggested that EpCAM\(^{\text{high}}\)/CD44\(^+\) cells are considered as CSCs in colorectal cancer [21].

This study applied fluorescence-activated cell sorting (FACS) to separate the EpCAM\(^{\text{high}}\)/CD44\(^+\) subset from human colorectal cancer cell line HCT116. After quantifying the average proportion of the EpCAM\(^{\text{high}}\)/CD44\(^+\) cells in the cell line HCT116, we used siRNA targeting EpCAM to create EpCAM\(^{-}\)/CD44\(^+\) subpopulation. Subsequently, we compared EpCAM\(^{\text{high}}\)/CD44\(^+\) cells and EpCAM\(^{-}\)/CD44\(^+\) cells for proliferation ability and the chemoresistance to oxaliplatin. Our results suggested that the EpCAM\(^{\text{high}}\)/CD44\(^+\) subset comprises almost 6.25 ± 0.09% cell line HCT116. It also turned out that the EpCAM\(^{-}\)/CD44\(^+\) cells displayed a significantly lower proliferation ability and weaker oxaliplatin chemoresistance than the EpCAM\(^{\text{high}}\)/CD44\(^+\) cells.

The preliminary result of this research will emphasize the significance of EpCAM in the EpCAM\(^{\text{high}}\)/CD44\(^+\) cells to act as a potential target in curing colorectal cancer in the coming years. Antibodies (catumaxomab or edrecolomab) binding to EpCAM combined with oxaliplatin might be a novel method to eliminate EpCAM\(^{\text{high}}\)/CD44\(^+\) cells [22], which will increase the overall survival rates for patients with colorectal cancer. This is sure to reduce the dose of oxaliplatin and improve the patients’ quality of life due to the limited toxicity.

**Figure 3:** Comparison EpCAM\(^{-}\)/CD44\(^+\) HCT116 cells with EpCAM\(^{\text{high}}\)/CD44\(^+\) HCT116 cells for the proliferation ability.

**p<0.01 vs. control group.**
This study has one limitation: we didn’t explore the detailed mechanism at the cellular level. We didn’t compare EPCAM$^{high}$/CD44$^+$ cells and EPCAM$^-$/CD44$^+$ cells for the tumorigenicity and oxaliplatin chemoresistance in tumor xenograft models. Thus, we need to perform further cell experiments to investigate the molecular mechanism and validate its effect on animal models.

In summary, EpCAM is critical for tumor proliferation, and oxaliplatin chemoresistance in EpCAM$^{high}$/CD44$^+$ colorectal CSCs, which indicates targeting EpCAM may be a method to eliminate EPCAM$^{high}$/CD44$^+$ cells to cure patients with colorectal cancer.

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