5-Fluorouracil upregulates the activity of Wnt signaling pathway in CD133-positive colon cancer stem-like cells

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[Abstract] Background and Objective: CD133-positive colon cancer stem-like cells (CSCs) are resistant to the conventional cytotoxic drug 5-fluorouracil (5-FU). Wnt signaling pathway plays important roles in colon cancer carcinogenesis and metastasis, and regulates the self-renewal capacity of CSCs. In the present study, we explored the impact of 5-FU on Wnt signaling pathway of CD133-positive colon CSCs, and the relation between Wnt signaling pathway and drug resistance of CD133-positive colon CSCs. Methods: Magnetic activation cell separation was used to collect CD133-positive cells from colon cancer cell line DLD1, which was transfected with luciferase reporter for Wnt signaling activity. The activity of Wnt signaling pathway was compared between CD133-positive and CD133-negative cells. After the treatment with 1 µg/mL of 5-FU, the cell proliferation rates of DLD1 cells, CD133-positive cells, and CD133-negative cells were compared. After the treatment with 1 µg/mL and 10 µg/mL of 5-FU for 48 h, Wnt activity was compared between CD133-positive and CD133-negative cells. The expression of CD133 and cell apoptosis of CD133-positive cells was detected after exposure to 50 ng/mL of dkk1 (DKK-1), a Wnt pathway inhibitor. Results: After the treatment with 5-FU, the cell proliferation rate of CD133-positive cells was higher than that of CD133-negative cells and the sensitivity of CD133-positive cells to 5-FU decreased. Wnt activity was higher in CD133-positive cells than in CD133-negative cells [(46.3 ± 0.3)% vs. (33.9 ± 2.7)%,  P = 0.009]. After the treatment with 1 µg/mL and 10 µg/mL of 5-FU, Wnt activity of CD133-positive cells was (90.1 ± 10.0)% (P = 0.012) and (52.9 ± 2.5)% (P = 0.047), respectively, whereas that of CD133-negative cells was (35.5 ± 3.3)% (P = 0.434) and (26.5 ± 0.4)% (P = 0.046), respectively. CD133 expression in CD133-positive cells decreased from (87.2 ± 5.3)% to (60.8 ± 3.1)% (P = 0.022) after treatment with DKK-1, whereas the cell apoptosis rate increased from (11.8 ± 0.2)% to (28.3 ± 0.6)% (P = 0.013). Conclusions: Wnt activity is higher in CD133-positive DLD1 cells than in CD133-negative DLD1 cells. 5-FU can upregulate Wnt activity of CD133-positive colon CSCs. Blocking Wnt activity may reverse drug sensitivity of CD133-positive cells to 5-FU.

Key words: Colon cancer stem-like cell, 5-fluorouracil, Wnt signaling pathway, CD133, drug sensitivity

Colorectal cancer is one of the most common tumors among worldwide. The incidence of colorectal cancer is the third of all malignant tumors. Although the incidence of colorectal cancer has declined in western countries, that is still increasing in China. The treatment of colorectal cancer has made great progress recently, however, about 50% of patients relapse after treatment, indicating that improving the treatment of colorectal cancer is still necessary[1].

Drug resistance has been the bottleneck in increasing the efficacy of chemotherapy. The growing evidences indicate that the tumors originating from epithelial such as colorectal cancer initiate from a small part of cancer stem
cells or cancer initiating cells with the capacity of self-renewal, which were defined as cancer stem like cells (CSCs). CSCs have the capacities of self-renewal, differentiation, and inducing cell proliferation, invasion and metastasis. Chemoresistance of CSCs is one of the reasons for tumor recurrence [3]. Recently, specific markers are applied for the identification of CSCs, such as CD133, CD44, CD166, and ESA in colon CSCs [4,5].

Wnt signaling pathway regulates self-renewal of cells and the formation of multiple organs. The disorder of this pathway induces tumor formation. Classical Wnt signaling pathway is mediated by β-catenin. Wnt ligand inhibits kinase activity of β-catenin degradation complex through direct interaction between Lrp5/6 and axin, causing β-catenin accumulation in the cytoplasm, translocation into nucleus, and interaction with transcriptional factor LEF/TCF. Thus, β-catenin regulates the transcription of Wnt target genes and promotes cell proliferation [5]. More than 80% of sporadic colonic cancers have stable β-catenin mutations, and continuing activation of classical Wnt signaling pathway plays important roles in the initiation and development of colorectal cancer [6]. Activation of endogenous Wnt signaling pathway results in a gross disruption of crypt architecture and a disproportionate expansion of CD133 expression in the crypt base, and then induces the formation of high grade intraepithelial neoplasia and crypt adenoma, indicating the potential relation between CD133-positive colorectal CSCs and Wnt signaling pathway [7].

We have found that 5-flourouracil (5-FU) upregulated stem cell marker CD133 expression in colon cancer cell line DLD1 cells. 5-FU is applied for accumulating CD133 overexpression cells in vitro. Overexpressed CD133 cells have stronger capacity of clone formation and tumorigenesis [8,9]. CD133-positive stem cells resist 5-FU. Therefore, the effect of 5-FU on the activity of Wnt signaling pathway of CD133-positive colorectal CSCs, the relation between the activity of Wnt signaling pathway and drug resistance of stem cells, and the reverse of drug resistance by blocking Wnt signaling pathway all warrant further studies. In this study, we compared cell growth inhibition and Wnt activities of 5-FU treated CD133-positive and CD133-negative cells to discuss the effect of 5-FU on Wnt activities of CD133-positive colorectal stem cells. Additionally, we discussed the drug resistance of CD133-positive colon stem cells through blocking Wnt signaling pathway.

Materials and Methods

Reagents

5-FU was purchased from Sigma, dissolved in double distilled water to a final concentration of 100 g/mL, and stored at −20°C. Wnt inhibitor dickkopf (DKK)-1 was purchased from PeproTech. PBS was from Invitrogen. Penicillin, streptomycin, and fetal bovine serum were purchased from Gibco. Dulbecco’s modified Eagle’s medium (DMEM) was from Invitrogen. Anti-CD133 antibody, magnetic beads, and isomers were purchased from Miltenyi. Actinomycin D (7-AAD) was purchased from BD Biosciences. Trypan blue was from Sigma Aldrich. Magnetic separation MiniMAC™ was purchased from Miltenyi. Flow cytometry, fluorescence activated cell analysis (FACS) Canto II, was from BD Biosciences.

Cell culture

Colon cancer cell line DLD1 (a reporting system of Wnt signaling pathway), a kindly gift from Professor Moon of Stem Cell Centre, University of Washington, was cultured in DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (GIBCO) at 37°C in 5% CO2. Cells were cultured no more than 10 passages.

FACS

Single cell suspension was mixed with anti-CD133/2 (293C3-PE) or mouse IgG2b-PE according to the ratio of 10:1, and stained for 30 min at 4°C. Cells were washed by PBS and treated with 7-AAD to exclude the dead cells. CD133-positive cells were detected by Flow Cytometry. All experiments were repeated three times.

Magnetic activated cell separation (MACS)

Single cell suspension was mixed with anti-CD133/1 magnetic beads at 4°C for 30 min. Cells were eluted through cylinder. CD133-positive cells were adsorbed by magnetic beads and negative cells were washed away. After magnetic separation, cell viability was evaluated by trypan blue. The purity of CD133-positive and CD133-negative cells was detected by flow cytometry. Cells were mixed with anti-CD133/2 or mouse IgG2b-PE and stained for 30 min. Cells were eluted and treated with 7-AAD to exclude the dead cells. CD133 positive cells were detected by flow cytometry. All experiments were repeated three times.

Cell growth curve

A total of 5.8 × 10^6 cells (DLD1, DLD1 with CD133⁺ and DLD1 with CD133⁻, respectively) were seeded onto plates containing 1 μg/mL of 5-FU. Cells were collected after 24 and 48 h and then stained with trypan blue. Cell number was counted and a growth curve was draw.
Luciferase activity detection

Cells with TCF reporting system were cultured onto 96 well plates. $1 \times 10^6$ PLB (BD Bioscience) was added to each well. The plates were agitated for 20 min at room temperature. Cell lysate was scraped from the plate and stored in Ep tube. Luciferase was added into 96 well plates with cell lysis buffer. Firefly and Renilla activity were detected on the fluorescence measuring instrument. Wnt activity was identified by the ratio of Firefly and Renilla.

DKK-1 inhibition

DKK-1 (50 ng/mL) was used to inhibit Wnt signaling pathway of CD133-positive cells. After 48 h, cells were collected and the expression of CD133 was detected by flow cytometry. DKK-1 was removed 48 h after treatment, and 1 $\mu$g/mL of 5-FU was added. Cell apoptosis was tested 48 h after staining with 7-AAD, and the ratio of apoptosis cells was detected by flow cytometry.

Statistical analyses

Statistical data was analyzed by SPSS13.0 software. Data was presented as mean $\pm$ standard deviation (SD). The t test was used to compare the differences of two groups. $P < 0.05$ was considered as statistically significant.

Results

Expression of CD133 in DLD1 cell line and MACS separation

As CD133 expression was $(31.2 \pm 2.5)\%$ in colon cancer cell line DLD1 by flow cytometry, we used CD133 as a marker to distinguish two groups of cells. CD133-positive and -negative cells were separated by MACS. Cell purity was detected by FACS. The cell purity of CD133-positive and -negative cells were $(84.3 \pm 4.7)\%$ and $(87.2 \pm 5.3)\%$, respectively, as shown in Figure 1.

Figure 1  CD133-positive cells separated from colon cancer cell line DLD1
CD133 expression rate in DLD1 cells was $(31.2 \pm 2.5)\%$. After magnetic activated cell separation (MACS), the purity of CD133-positive and -negative cells were $(84.3 \pm 4.7)\%$ and $(87.2 \pm 5.3)\%$, respectively.
Cell growth comparison

The growth curves of DLD1 cells, CD133\(^+\) cells, and CD133\(^-\) cells treated with 5-FU are shown in Figure 2. Under treatment with 5-FU, CD133\(^+\) cells grew faster than did DLD1 cells and CD133\(^-\) cells (\(P = 0.031\) and 0.011), while under normal condition, the growth rate of the three groups did not show any differences, indicating that sensitivity of CD133\(^+\) cells to 5-FU was lower than that of CD133\(^-\) cells (\(P = 0.012\)).

![Figure 2: Growth curves of unfractionated DLD1, CD133-positive, and CD133-negative cells cultured with 1 \(\mu\)g/mL of 5-FU](image)

A total of \(57 \times 10^6\) DLD1 CD133\(^+\), DLD1, and DLD1 CD133\(^-\) cells were cultured in 5-FU media. After 1 day of culture, cell number were (112.0 \pm 12.1) \times 10^6, (86.0 \pm 11.9) \times 10^6, (49.0 \pm 9.8) \times 10^6, respectively. After 2 days of culture, cell number were (140.0 \pm 7.3) \times 10^6, (111.0 \pm 12.7) \times 10^6, (87.5 \pm 13.3) \times 10^6, respectively. DLD1 CD133\(^+\) cells were more resistant to 5-FU treatment compared with either DLD1 or DLD1 CD133\(^-\) cells.

Wnt Activity

As shown by luciferase detection, Wnt activities of non-separated DLD1 cells, CD133\(^+\) cells and CD133\(^-\) cells were \(41.2 \pm 1.4\)%\(\), (46.3 \pm 0.3)%\(\) and \(33.9 \pm 2.7\)%\(\), respectively. Wnt activity was higher in CD133\(^+\) cells than in CD133\(^-\) cells (\(P = 0.009\)), suggesting higher capacity of self-renewal of CD133\(^+\) cells.

Effect of 5-FU on Wnt signaling pathway of CD133\(^+\) cells and CD133\(^-\) cells

CD133\(^+\) cells and CD133\(^-\) cells were treated with 1 \(\mu\)g/mL and 10 \(\mu\)g/mL of 5-FU. Wnt activities were detected. Both low dose of 5-FU (1 \(\mu\)g/mL) and high dose of 5-FU (10 \(\mu\)g/mL) upregulated Wnt activity of DLD133\(^+\) cells, from \(46.3 \pm 0.3\)% to \(90.1 \pm 10.0\)% (\(P = 0.012\)) and \(52.9 \pm 2.5\)% (\(P = 0.047\), respectively. Wnt activity of CD133\(^+\) cells untreated was \(33.9 \pm 2.7\)%\(\). After treatment with 1 \(\mu\)g/mL and 10 \(\mu\)g/mL of 5-FU, the wnt activity were \(35.5 \pm 3.3\)% (\(P = 0.434\)) and \(26.5 \pm 0.4\)% (\(P = 0.046\), respectively.

Effect of DKK-1 on Wnt signaling pathway of CD133 positive cells

After 48 h of treatment with 50 ng/mL of DKK-1, the expression of CD133 in CD133\(^+\) cells decreased from \(87.2 \pm 5.3\)% to \(60.6 \pm 3.1\)% (\(P = 0.022\) (Figure 3A). Apoptosis rate induced by 5-FU increased from \(11.8 \pm 0.2\)% to \(28.3 \pm 0.6\)% (\(P = 0.013\) (Figure 3B).

Discussion

In this study, we used CD133 to separate cancer stem cells from DLD cell line and compared Wnt activities between CD133\(^+\) cells and CD133\(^-\) cells. We found that Wnt activity of CD133\(^+\) cells was higher than that of CD133\(^-\) cells. 5-FU increased Wnt activity of CD133\(^+\) cells. High dose of 5-FU inhibited Wnt activity and proliferation rate of
CD133-negative cells. The sensitivity to 5-FU of CD133-positive colon stem cells treated with DKK-1, a Wnt signaling pathway inhibitor, increased significantly.

Primary or secondary drug resistance is the biggest bottleneck in chemotherapy for cancer. Cancer stem cells, which are hardly removed, are not sensitive to treatment and contribute to tumor recurrence and metastasis. Several studies have found that CSLCs or cancer cells expressed stem cell markers are resistant to cancer therapy. CD133-positive CSLCs separated from glioma cells of surgical resection are resistant to ionizing radiation therapy because of DNA repair activity\(^9\). The sensitivity to oxaliplatin and 5-FU of CD133\(^+\) cells and serum-free-cultured small spherical cells is lower than that of CD133\(^-\) cells\(^9\). Anti-IL-4 receptor antibody reduces the activity of CD133\(^+\) cells and increases the efficacy of chemotherapy by reducing the level of survivin, and then induces apoptosis of small spherical cells. Inhibiting IL-4 in vivo or neutralizing IL-4 antibody can reduce the ectopic tumor implanted subcutaneously, identifying the resistance of the model of cancer stem cells and CSLCs to chemotherapy and revealing the mechanism of resistance to drugs. Woodward et al.\(^{11}\) found that breast cancer stem cells expressed stem cell markers were resistant to radiotherapy, and ectopic expression of Wnt and \(\beta\)-catenin enhanced the radiation-resistant cell populations, suggesting that Wnt signaling pathway was correlated with the capacity of self-renewal and the resistance to therapy. Flahaut et al.\(^{12}\) reported that in gene expression profiling of doxorubicin (DoxR)-resistant and -sensitive parental cell lines, the MDR1 gene was included in the identified upregulated genes, however, the highest overexpressed transcript in both of the two cell lines was the frizzled-1 Wnt receptor (FZD1) gene, an essential component of the Wnt/\(\beta\)-catenin pathway; FZD1 upregulation was shown to mediate sustained activation of the Wnt/\(\beta\)-catenin pathway, and specific micro-adapted short hairpin RNA-mediated FZD1 silencing induced parallel strong decrease in the expression of MDR1 and restoration of drug sensitivity in FZD1-silenced cells. Noda et al.\(^{13}\) found that drug resistance of liver cancer cells to 5-FU was associated with Wnt signaling pathway; Wnt target genes only expressed in drug-resistant liver cancer cells, and activation of Wnt gene induced the drug resistance of liver cancer cells to 5-FU.
However, the impact of drugs on Wnt signaling pathway in cancer stem cells and that of Wnt activity on drug resistance of CSLCs have not been reported. We had established CD133-positive cells as colon stem cells and found that common chemotherapy drug 5-FU increased CD133 expression in colon cancer cells and the stemness of cells [9]. In this study, we used CD133 as a marker to isolated stem cells from DLD1 cell line. Our results showed that Wnt activity of CD133-positive cells was higher than that of CD133-negative cells, which was consistent with the self-renewal capacity of CSLCs. 5-FU enhanced the activity of Wnt signaling pathway significantly, suggesting that 5-FU did not inhibit Wnt activity of CD133+ cells, but enhanced Wnt activity and self-renewal capacity of these cells. Repetitive stimulation by 5-FU may activate Wnt signaling pathway and increase the drug resistance. Growth rate of CD133+ cells treated with 5-FU was higher than that of CD133- cells. The sensitivity of CD133+ cells to 5-FU was lower than CD133- cells, but increased after treatment with DKK-1, suggesting that blocking Wnt signaling pathway can reverse the drug resistance of CD133+ cells. Therefore, Wnt signaling pathway played important roles in drug resistance of colon CSLCs.

In summary, we found that 5-FU activates Wnt signaling pathway of CD133+ colon cancer stem cells. The sensitivity of CD133+ cells to 5-FU may increase through inhibiting Wnt signaling pathway. Wnt signaling pathway involved in regulating the drug resistance of CD133+ cells. 5-FU increased Wnt activity of CD133+ cells, which associated with chemoresistance of cancer stem cells. Blocking Wnt signaling pathway may enhance the sensitivity of CSLCs to chemotherapy drugs. Further studies are needed to explore the sensitive location of Wnt signaling pathway, the effect on drug sensitivity by blocking Wnt signaling pathway, and the inhibitory effect of combination of anti-Wnt activity and chemotherapy drugs.

References