The effect of omentin-1 on the proliferation and apoptosis of colon cancer stem cells and the potential mechanism

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Summary

Purpose: To investigate the effect of omentin-1 on the proliferation and apoptosis of colon cancer stem cells and the underlying mechanism.

Methods: Colon cancer stem cells were obtained by indirect immune-magnetic beads cultured in serum-free medium, and identified by spheres formation assay, differentiation assay and flow cytometry. Colon cancer stem cells were divided into the control group, the omentin-1 group (1 μg/ml omentin-1), the omentin-2 group (2 μg/ml omentin-1), the omentin-LY group (1 μg/ml omentin-1 and 50 μM LY294002) and the LY group (50 μM LY294002). CCK-8 and flow cytometry were used to detect the proliferation and apoptosis, respectively. Western blot was performed to measure the effect of different concentrations of omentin-1 on phosphorylated Akt.

Results: The colon cancer stem cells were successfully sorted, and the content of CD133+ in colon cancer stem cells reached 80.3%. Omentin-1 inhibited the proliferation and promoted apoptosis of colon cancer stem cells in a dose and time-dependent manner, which could be strengthened by the PI3K/Akt inhibitor.

Conclusions: Omentin-1 could inhibit the proliferation and promote apoptosis of colon cancer stem cells in vitro via the PI3K/Akt pathway.

Key words: adipocytokine, Akt, colorectal cancer, omentin-1, stem cells

Introduction

Colon cancer is a common malignant neoplasm and the third cause of cancer-related death globally. Its incidence is increasing year by year. Epidemiological studies have shown that malignant neoplasms, including colon cancer, are closely related to obesity and metabolic syndrome [1,2]. The risk of gastrointestinal cancer in obese people is about 1.5-2-fold higher than the normal-weight people [3], and the incidence of right colon cancer is over 2-fold than that of the normal population. In addition, obesity is also correlated with breast cancer, endometrial cancer, prostate cancer and liver cancer [4-7]. The International Cancer Research Institute concluded that there is a causal relationship between colon cancer and obesity or overweight.

The mechanism of obesity acting on malignant neoplasms has not been fully understood and the pathophysiological mechanism(s) may be very complicated. Obesity is mainly manifested in an increase in the size and number of fat cells and adipose tissues. Due to influencing the metabolism of other organs and systems, adipose tissue is considered to be an active endocrine organ and can produce hormones/proteins, such as omentin, visfatin, adiponectin, leptin, resistin, serine protease inhibitors, tumor necrosis factor-α (TNF-α) and cytokines including interleukin-6 (IL-6), which are
named as adipokines [8,9]. Among them, omentin, visfatin, adiponectin and visceral adipose tissue-derived serpin (vaspin) have been shown to be four adipokines that can enhance the sensitivity of insulin [10].

It is well known that omentin-1 is an adipokine secreted by adipocytes. Several studies have suggested that omentin-1 is also associated with several malignancies, such as malignant pleural mesothelioma, gastric cancer and kidney cancer [11-14]. Our previous study [15] has found that plasma omentin-1 levels are closely related to colorectal cancer, and omentin-1 may promote cell proliferation and inhibit apoptosis in SW480 cells in vitro. Colon cancer stem cells are a small part of colon cancer cells. The current studies generally suggest that colon cancer stem cells are correlated with the occurrence, development, recurrence, metastasis and chemotherapeutic resistance of colon cancer [16-18]. However, the role of omentin-1 in colon cancer stem cells has not been reported yet. The objective of our study was to investigate the effect of omentin-1 on the proliferation and apoptosis of colon cancer stem cells in vitro, and to explore new ideas for the diagnosis and treatment of colon cancer in the future.

Methods

Materials

The human colon adenocarcinoma cell line SW480 was purchased from the Cell Bank of Shanghai (Shanghai, China). Immunity-magnetic bead sorting and sorting rack, CD133 indirect immune magnetic beads kit, and MS sorting column were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). DMEM high glucose medium and DMEM-F12 medium were purchased from Hyclone (Utah Luogan City, USA). Recombinant human epidermal growth factor (rh-EGF) and recombinant human basic fibroblast growth factor (rh-b-FGF) were purchased from Peprotech (New Jersey, USA). Leukemia inhibitory factor was purchased from Wisent (Nanjing, China). B27 was purchased from Gibco (Los Angeles, USA). LY294002 was purchased from Beyotime (Shanghai, China). Omentin-1 was purchased from USCN (Wuhan, China). CCK-8 Kit and AnnexinV-FITC Kit were purchased from Bestbio (Shanghai, China).

Isolation and culture of colon cancer stem cells

The SW480 cells were incubated in DMEM high glucose medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin. The cells were grown at 37°C, 5% CO2 and saturated humidity. The cells were harvested and 2×10^7 cells were counted. After centrifugation, the supernatant was discarded and re-suspended in a 400 µl buffer solution containing 0.5% albumin from bovine serum (BSA) and 2 mM EDTA to form a single cell suspension. Then, 100 µl fragment crystallizable receptors (FCR) blocker and 50 µl CD133/1 (AC133)-Biotin antibody were added, mixed and placed in a refrigerator at 4°C for 10 min, followed by cleaning with 5 ml buffer solution. After centrifugation at 1530 rpm for 10 min, the supernatant was discarded, re-suspended in a 400 µl buffer solution and then 100 µl Biotin microbeads were added and incubated at 4°C for 15 min. The cells were washed with buffer twice and re-suspended in a 500 µl buffer solution. The labeled CD133 antibody cell suspension was added to the packed column, the cell suspension was spontaneously washed, the column was washed with PBS for three times in which the contents were spontaneously discharged, and finally the separation column was removed from the magnetic field. One ml PBS was used to rapidly rinse the cells in the column, and the cells collected in the culture plate were CD133+ colon cancer stem cells. The ultra-low adhesion 6 well plates were used to reduce cell adherence and to support growth as undifferentiated tumor spheres. The complete serum free medium (SFM) for colon cancer stem cells included DMEM:F12, supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 2%B27, 10 ng/ml Leukemia inhibitory factor (LIF), 20 ng/ml epidermal growth factor (EGF), and 10 ng/ml basic fibroblast growth factor (bFGF). The medium was replaced twice a week, and the cells were passed on when most of them formed a floating ball.

Identification of colon cancer stem cells

The colon cancer stem cells in the logarithmic growth phase were collected, seeded in a 96-well plate and cultured in a DMEM medium containing 10% FBS, to induce stem cell differentiation and adherence. In addition, the stem cells were divided into two groups, including the negative group and the CD133+ group, with 2×10^6 cells in each group. After washing with PBS twice, 500 µl PBS was added to suspend the stem cells. The cells in the CD133+ group were incubated with 2 µl CD133 antibody for 1 hr at 4°C. After washing with PBS, 2 µl phycoerythrin (PE) was added and incubated in the dark at 4°C for 15 min. In contrast, the cells in the negative group were only incubated with PE and CD133 antibody was excluded. Then, flow cytometry was used to analyze the cell surface markers of CD133 in the two groups.

Cell grouping and treatment

There were 5 groups in the present study, including the control group, the omentin-1 group (1 µg/ml omentin-1), the omentin-2 group (2 µg/ml omentin-1), the omentin-LY group (1 µg/ml omentin-1 and 50 µM LY294002), and the LY group (50 µM LY294002).

Cell proliferation assay

The cell proliferation assay was performed with CCK-8. The colon cancer stem cells in the logarithmic growth phase were seeded in a 96-well plate at the density of 15000 cells/ml, with 100 µl fresh serum free medium (SFM) in each well. After incubation for 24 hrs, 10 µl CCK-8 were added into each well, and the absorbance was measured at 450 nm using a microplate reader.
after a 3-h incubation. The experiment was repeated three times. To further understand the temporal effect of omentin-1 on the proliferation of colon cancer stem cells, 1 µg/ml omentin-1 was added into each well, and incubated for 0, 1, 6, 24 and 48 hrs in a humidified atmosphere with 5% CO2 at 37°C, respectively. Then, the same procedure was conducted as described above.

**Flow cytometry assay**

The colon cancer stem cells were re-suspended in SFM at a maximum density of 1.0×10^6 cells/ml, and incubated in a 6-well culture plate with 2 ml SFM in each well. After incubation for 24 hrs, the cells were washed twice with PBS, and a total of 400 µl cell suspension was incubated with 5 µl Annexin V-FITC and 10 µl propidium iodide (PI) at 4°C in the dark for 15 min, immediately followed by flow cytometry on a flow cytometer. The experiment was repeated three times. Flow Jo 7.6 software was used for data analysis.

**Western blot analysis**

Cells were seeded at a density of 2×10^7 cells in 6-well plates and treated with omentin-1 for 24 hrs. Cells were lysed with RIPA lysis buffer containing protease inhibitor cocktail for 30 min on the ice. Then, the cell lysates were centrifuged at 12,000 g for 20 min and the supernatant was collected. Protein loading buffer was added to each sample and boiled for 10 min. BCA protein assay kit (Beyotime, Shanghai, China) was performed to detect the concentration of proteins according to the manufacturer’s instruction. Samples were resolved on 10% SDS-PAGE at 110 mv for 1 hr, and the proteins were transferred onto nitrocellulose membranes at 200 mA for 2 hrs. Then, the membranes were blocked with

![Image of flow cytometry assay and Western blot analysis](https://via.placeholder.com/150)

**Figure 1.** Identification of colon cancer stem cells. (A) Tumor spheres formation observed under inverted microscope (400×). (B) The differentiation of colon cancer stem cells observed under inverted microscope (200×). (C) Identification of colon cancer stem cell surface markers CD133+ by flow cytometry.
5% skim milk for 2 hrs at room temperature. After being washed with PBST (PBS containing 0.1% Tween 20) for three times, the membranes were incubated with rabbit anti Akt IgG, rabbit anti-phosphorylated Akt IgG (Cell Signaling, Boston, USA) and rabbit anti β-actin IgG (Proteintech, Wuhan, China) at 4°C overnight. After that, the membranes were incubated with secondary antibodies for 1 hr at room temperature, and washed with PBST three times. The protein bands were exposed to ECL kits (Thermo, Waltham, USA) and detected by chemiluminescence. Quantity One was used for data analysis.

Statistics

The experimental data were presented as mean±SD. One-way analysis of variance (ANOVA) was used for statistical analyses. P<0.05 was considered statistically significant.

Results

Identification of colon cancer stem cells

We observed that colon cancer stem cells cultured in SFM formed a small size and little tumor spheres on the 1st day. On the 5th day, the quantity of tumor spheres increased significantly. On the 10th day, the tumor spheres became larger, rounder than before, and suspended in SFM (Figure 1A). The differentiation experiment indicated that when colon cancer stem cells were cultured in DMEM medium containing 10% FBS, tumor spheres could still be observed and most of them suspended on the 3rd day, while only a small part of the cells escaped from tumor spheres and adhered into a shuttle type. On the 7th day, most of the cells adhered into a shuttle type. On the 9th day, all tumor spheres were differentiated to SW480 cells and grown as adherent (Figure 1B). Flow cytometry analysis proved that the content of our CD133 positive expression stem cells was 80.3% (Figure 1C).

Effect of omentin-1 on the proliferation of colon cancer stem cells

After incubation for 24 hrs, compared with the control group, the optical density (OD) values of the omentin-1 group, the omentin-2 group, the omentin-LY group and the LY group were all significantly decreased (p<0.05). The OD values of the omentin-2 group and the omentin-LY group were significantly lower than that of the omentin-1 group, and the OD value of the omentin-LY group was obviously lower than the LY group (p<0.05; Figure 2A). 0, 1, 6, 24 and 48 hrs after the treatment of 1 µg/ml omentin-1 on colon cancer stem cells, with the prolongation of the intervention time, OD values of each group showed a statistically significant decreasing trend (p<0.05, Figure 2B).

Cell apoptosis of omentin-1 acting on colon cancer stem cells

After incubated for 24 hrs, the apoptosis of the omentin-1 group, the omentin-2 group, the omentin-LY group and the LY group was significantly increased when compared with the control group (p<0.05). The apoptosis of the omentin-2 group and the omentin-LY group were higher than the omentin-1 group, moreover, the omentin-LY group was obviously higher than the LY group (p<0.05, Figure 3).

The relative protein expression of Akt and pAkt

With the increase of omentin-1 concentration, the protein expression of Akt decreased, pAkt decreased, and the ratio of pAkt to Akt decreased...
Figure 3. Effect of omentin-1 and LY294002 on apoptosis of colon cancer stem cells. (A) The Figure shows the apoptotic effect of omentin-1 and LY294002 on colon cancer stem cells by annexin V-FITC and PI double staining. (B) The Figure shows mean changes of apoptosis rate after intervention of omentin-1 and LY294002 treatment on cultured colon cancer stem cells. Each bar represents mean ± SD (n=3). Colon cancer stem cells in the omentin-1 group and the omentin-2 group were treated with 1 µg/ml and 2 µg/ml omentin-1, respectively. Cells in the omentin-LY group were treated with 1 µg/ml omentin-1 combined with 50 µM LY294002, and cells in the LY group were treated with 50 µM LY294002 alone. Compared with the control group *p<0.05, compared with the omentin-1 group #p<0.05, compared with the LY group Δp<0.05.
Omentin-1 and colorectal cancer

Our previous study has suggested that the level of omentin-1 in CRC patients and healthy persons are all positively correlated with high density lipoprotein cholesterol (HDL-C), while negatively associated with triglyceride (TG), fasting insulin and waist to hip ratio (WHR). We have found that the protein and mRNA expression of omentin-1 in cancer tissues are higher than the adjacent tissues in CRC patients, which can be detected in SW480 cell lysates and supernatants [15]. In patients with CRC, the levels of omentin-1, visfatin and vaspin increase significantly, independent of measures of obesity, and these adipokines may affect CRC through various different mechanisms, in which the direct effects may be more important than indirect effects that are active in the association between obesity and CRC [21]. One prospective cohort study has demonstrated that the concentration of omentin was higher among CRC patients when compared with the controls, moreover, higher circulating omentin concentration is statistically associated with a higher risk of CRC, independent of the known risk factors, adiposity and metabolic biomarkers. Furthermore, omentin has been proven to improve the risk assessment of CRC beyond established risk factors [22]. However, one study has indicated that omentin levels are significantly elevated in stage III colon carcinoma patients treated with surgery and adjuvant oxaliplatin and 5-fluorouracil chemotherapy [23]. TMEM207, linked to the production of omentin-1, is an uncharacterized transmembrane protein. A small-interfering-RNA-mediated knockdown of TMEM207 increased poly-ubiquitination and proteasome degradation of omentin-1 in the cultured CRC cells, and reduced omentin-1 secretion. The downregulation of omentin-1 levels may lead to poor prognosis in patients with advanced CRC [24]. Currently, the views of effect of omentin-1 on CRC are controversial, but no researches have elucidated its real effect on colon cancer stem cells.

Our study isolated colon cancer stem cells marked CD133+ from the human colon cancer cell line SW480 by using the indirect immune-magnetic bead sorting method, and identified these cells by tumor spheres formation assay and cell differentiation experiment. Results indicated that the CD133+ colon cancer stem cells obtained by indirect immune-magnetic bead sorting method and cultured in SFM were able for unlimited proliferation and differentiation as tumor stem cells. Flow cytometry detected the content of CD133+ colon cancer stem cells was 80.3%, which was consistent with the relevant researches [25,26]. Results of different concentrations of omentin-1 on colon cancer stem cells and different time periods all suggested

Figure 4. The phosphorylated Akt (pAkt) and Akt protein were expressed in colon cancer stem cells. (A) The Figure shows pAkt and Akt expression detected by Western blotting analysis after intervention of 1 µg/ml and 2 µg/ml omentin-1 treatment on cultured colon cancer stem cells. (B) The Figure shows mean changes of the relative protein expression of pAkt to Akt after intervention of 1 µg/ml and 2 µg/ml omentin-1 treatment on cultured colon cancer stem cells. Compared with the control group, *p<0.05. Each bar represents mean ± SD (n=3).

Gradually, when compared with the control group. No significant difference of the ratio of pAkt to Akt between the omentin-1 group and the control group was found (p>0.05), however, the ratio of pAkt to Akt in the omentin-2 group was significantly lower than that of the control group (p<0.05; Figure 4).

Discussion

Omentin-1, also known as intelectin-1 (ITLN-1), is a novel adipokine mainly expressed in adipocytes of abdominal adipose tissues [19]. Several authors have suggested that omentin-1 may be closely related to multiple malignant neoplasms, such as prostate cancer, malignant pleural mesothelioma, gastric cancer, kidney cancer, liver cancer and neuroblastoma [11-13,20]. Recent researches have indicated that omentin-1 is also associated with the pathogenesis of colorectal cancer (CRC) [21-24].

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that omentin-1 could promote the proliferation and inhibit the apoptosis of colon cancer stem cells, which were dependent on a time-concentration dependent manner. In addition, our study also demonstrated that the combination of omentin-1 and LY294002 presented synergistic effect on promoting the proliferation and inhibiting the apoptosis of colon cancer stem cells, which were more obvious than using omentin-1 or LY294002 alone.

Akt/PKB is a member of the PI3K signaling pathway, which is a core signaling pathway of stimulating growth factors. PI3K stimulates the intracellular signaling pathways of malignant tumor cells by activating Akt to inhibit tumor cell apoptosis and promote proliferation. In contrast, inhibition of the Akt signaling pathway may induce apoptosis in some malignant tumor cells. The abnormality of the PI3K/Akt signaling pathway can be found in multiple tumors, such as non-small cell lung cancer, gastric cancer, pancreatic cancer, endometrial cancer and cholangiocarcinoma [27-30]. In recent years, the activation of the Akt signaling pathway plays an important role in the proliferation and apoptosis of CRC. LY294002 is an inhibitor of the PI3K/Akt signaling pathway and possesses the ability of decreasing the level of Akt protein phosphorylation to induce CRC apoptosis and promote proliferation, which can also be used in the treatment of CRC [31]. Our study illustrated that omentin-1 could inhibit the activity of pAkt/Akt and the underlying mechanism of inhibiting colon cancer stem cells and promoting apoptosis might be related to the inhibition of Akt activity, which was the same with LY294002, as reported in gastric cancer and neuroblastoma by other researchers [12,20]. Meanwhile, the results showed that omentin-1 could activate the Akt signaling pathway and the AMPK and Akt phosphorylation to inhibit cardiomyocytes apoptosis in the presence or absence of insulin, thus preventing acute ischemic injury in cardiomyocytes [32]. Therefore, it can be speculated the effect of omentin-1 on the Akt signaling pathway was not the same in different cells.

In conclusion, omentin-1 can inhibit the proliferation and inhibit apoptosis of colon cancer stem cells, and is related to a time-concentration dependent manner. In addition, the effect is consistent with LY294002. Furthermore, the mechanism of omentin-1 on Akt may be related to the inhibition of the Akt signaling pathway. By exploring the effect of omentin-1 on colon cancer stem cells, our findings may be helpful to reveal the molecular mechanism of the role of obesity in the development of CRC, and to provide a new approach for the future research and diagnosis of CRC.

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**Conflict of interests**

The authors declare no conflict of interests.

**References**