Original Article
CD13 overexpression promotes rectal cancer cell proliferation by increasing VEGF expression

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Abstract: Objective: To observe the expression level of cluster of differentiation 13 (CD13) in rectal cancer tissues and explore the effect of CD13 overexpression on the biological behavior of rectal cancer cells. Methods: Expression of CD13 in rectal cancer tissues and cancer-adjacent normal tissues was detected via immunohistochemistry and real-time quantitative polymerase chain reaction. The rectal cancer cell line SW1463 was cultured and transfected with CD13 vector at concentrations of 0 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL and 160 μg/mL, respectively. Changes in the proliferation, metastasis, and apoptosis abilities as well as expression of the neovascular marker vascular endothelial growth factor (VEGF) were detected. Results: The protein content of CD13 in rectal cancer tissues was higher than that in cancer-adjacent normal tissues. Expression of CD13 mRNA in rectal cancer tissues was positively correlated with tumor-node-metastasis stage. Transfection of CD13 expression vectors could increase the proliferation and metastasis abilities as well as VEGF expression of SW1463 rectal cancer cells, and reduce the apoptosis rate of SW1463 rectal cancer cells in a dose-dependent manner. Conclusion: CD13 was overexpressed in rectal cancer tissues. Overexpressed CD13 could increase proliferation and metastasis abilities as well as VEGF expression of SW1463 rectal cancer cells and decrease apoptosis of SW1463 rectal cancer cells.

Keywords: Rectal cancer, tumor tissue, cluster of differentiation 13, SW1463 rectal cancer cell

Introduction

According to the World Health Organization statistics on cancer control projects, there are about 200,000 new cases of rectal cancer and about 100,000 deaths each year. Rectal cancer has become the fifth most fatal tumor in China [1, 2]. The high lethality of rectal cancer mainly results from tumor resistance and metastasis. Currently, the main treatment for rectal cancer is radical surgical resection, supplemented by chemotherapy, radiotherapy, and biological therapy, but the five-year survival rate is not satisfactory, and its prognosis is closely related to tumor-node-metastasis (TN-M) clinical stage of patients. The five-year survival rate for stage II patients undergoing radical surgery is 80% compared to 40%-60% for stage III patients [3, 4]. Therefore, to determine the stage, growth, and metastasis of tumor tissues and to explore the molecular mechanisms involved in differentiation, apoptosis, and proliferation of tumor cells are of great significance in guiding the surgical treatment and evaluating the prognosis of patients.

Cluster of differentiation 13 (CD13) is an extracellular enzyme expressed on the cell surface and consists of an N-terminal intracellular domain, a single-pass membrane-penetrating node, an extracellular stem, and a C-terminal extracellular domain [5]. As a tumor marker, CD13 is almost expressed in all tumor tissues, including tumor cells in the skin, lung, ovary, intestine, kidney, bone, prostate, pancreas, thyroid and breast etc. [6]. CD13 expressed on the surface of tumor cells, plays a key role in regulating the metastasis and metabolism of tumor cells since it promotes angiogenesis and tumor growth by shearing and activating angiogenic peptides [7]. Tumor growth and metastasis to adjacent tissues are mainly dependent on neovascularization capacity. Vascular endothelial growth factor (VEGF) is considered as one of the most important markers of neovascularization and promotes expression of CD13 early in the development of cancer [8, 9]. At the same time, activated VEGF in tumor cells can facilitate the formation of neovascularization and metastasis of endothelial cells, which are potent factors inducing and speeding up the

growth of tumor tissues. *In situ* hybridization experiments have confirmed that in the process of rectal cells developing from adenoma to malignant tumors, expression level of VEGF messenger RNA (mRNA) significantly increases [10]. Therefore, the determination of changes in tumor tissue CD13 and neovascular marker VEGF expression can reflect the degree of tumor growth, metastasis, and invasion to some extent.

Current studies have revealed that CD13 may be highly expressed in rectal cancer tissues, and its expression level is closely related to tumor metabolism, proliferation and metastasis. Therefore, this study investigates the expression level of CD13 in rectal cancer tissues and cells, surveys correlations of CD13 expression with proliferation and metastasis of rectal cancer by overexpressing CD13 with the cell line transfection of CD13 expression vectors. It further explores the molecular mechanism of rectal cancer in the processes of invasion and metastasis, thus inserting new ideas and providing experimental basis for the clinical treatment of rectal tumors.

Materials and methods

Inclusion and exclusion criteria

Samples were selected from a total of 60 patients definitely diagnosed with rectal cancer who underwent surgical resection in Shanghai Fourth People's Hospital from January 2015 to December 2016 for the experimental study. All the research operations were approved by the Ethics Committee of Shanghai Fourth People's Hospital.

Inclusion criteria: Patients meeting the diagnostic criteria for rectal cancer included: patients conformed to the indications for surgical resection; patients and their family members obtained the right to be informed and signed the informed consent.

Exclusion criteria: Patients were complicated with other intestinal diseases or acute infection; patients had other lesions with tumor metastasis; failure to obtain the informed consent of patients and their families.

Experimental materials

The rectal cancer cell line SW1463 was purchased from the Cell Bank of the Chinese Academy of Sciences. Plasmid-CMV6 was purchased from OriGene. Polymerase chain reaction (PCR) amplification primers and pCMV6-CD13 expression vector constructed by GenePharma (Shanghai). Transfection reagents were purchased from Takaro and the reverse transcription-PCR experimental kit and apoptosis detection kit were purchased from Roche. CD13 and VEGF antibodies were purchased from Abcam. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Santa Cruz Biotechnology. Immunohistochemical kit was purchased from Google Biotechnology (Wuhan) and the Cell Counting Kit-8 (CCK-8) kit and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit were purchased from Beyotime Biotechnology.

Experimental methods

Sample collection: Tumor tissues and cancer-adjacent normal tissues were collected from rectal cancer patients who met the inclusion criteria. Patients were divided into two groups after their diseases were confirmed by the Department of Pathology. One group was placed and fixed in 10% paraformaldehyde for 48 hours and the other was stored in liquid nitrogen for standby application.

Immunohistochemistry: Paraformaldehyde-fixed tissues were embedded with paraffin, sectioned (4 μm), dewaxed into water and blocked with goat serum for 1 hour. The primary antibody (CD13) was added for incubation at 4°C overnight. Then the secondary antibody was added dropwise for incubation at room temperature for 1 hour, followed by sealing after diaminobenzidine coloration and observation under a microscope.

Real-time quantitative PCR (qPCR): The total RNA was extracted from cancer tissues, complementary DNA (20 μL) reverse-transcribed using the RevertAid RT Reverse Transcription kit, and CD13 (20 μL) was amplified by fluorescence qPCR using the SYBR Green qPCR Master Mix kit. The quantitative analysis of the target gene was conducted with GAPDH as an internal control. CD13 sense primer sequence: 5'-GTAATACGACTCACTATAGGGCCAGGGGC-CTGTACGTTTTTA-3', and antisense primer sequence: 5'-AATTAACCCTCACTAAAGGGCCACCA-GCTCAGTCTTGTCA-3' [11]; GAPDH sense primer sequence: 5'-ACCACAGTCCATGCCATCAC-

3', and antisense primer sequence: 5'-TCCACC-ACCCTGTTGCTGTA-3'.

Cell processing: Rectal cancer cell lines received routine resuscitation, culture, and passage, and were then inoculated into a 6-well plate. After the addition of 10 µL transfection reagent, plasmid and the medium at the corresponding volume, respectively, were mixed with culture medium at the final concentrations of 0 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL and 160 µg/mL. Afterwards, the processed cells were placed in an incubator containing 5% CO_2 at 37°C for 48 h, followed by cell collection to be tested.

Immunoblotting detection: Cell proteins were extracted, and 10% Tris-glycine sodium dodecyl sulfate polyacrylamide gels were prepared for Western blotting. After membranes were transferred by electrophoresis, the hybrid membranes were blocked at room temperature for 90 minutes. After that, primary antibodies for CD13, VEGF and GAPDH were added for incubation at 4°C for 12 hours, and then proteins were placed in secondary antibody for incubation at room temperature for 90 minutes. After full cleaning, the developing solution was added into the developing machine (LAS-4000 chemiluminescence detector, Fujifilm, Japan). The expression levels of CD13 and VEGF in cells transfected with different concentrations of the transfection reagent were detected, and ImageJ software (National Institute of Health) was employed to calculate the gray value, and then semi-quantitative analysis was performed to evaluate the content of the target protein with GAPDH as a reference.

Detection of cell proliferation via CCK-8: After plasmids at different concentrations were transfected for 48 h, the concentration of cell suspensions for digestion and resuspension was adjusted to 1,000 cells/100 µL and 100 µL cell suspensions were added to each well of a 96-well plate. Cells were placed and cultured in an incubator containing 5% CO_2 at 37°C until the cells were adherent to the wall, and the detection was conducted at 6 time points (0 h, 12 h, 24 h, 36 h, 48 h, 72 h) after the adherence, respectively. In addition, a fixed time point was selected for medium change every day, 10 µL CCK-8 solutions were added to each well, and the absorbance value at 450 nm was measured with a microplate reader after culturing for 3 hours.

Cell metastasis assay: Prior to the addition of the transfection reagent, a scratch was made in the bottom center of the cell culture plate, and the scratch area was recorded as S0. After the transfection reagent was added, cells were cultured in an incubator containing 5% CO_2 at 37°C, and the scratched area Sn was recorded at three time points (12 h, 24 h and 48 h) after culture. The cell metastasis rate was calculated by reference to the formula (S0-Sn)/S0.

Detection of cell apoptosis: Cell apoptosis was detected by TUNEL. Cells slid, and were closed and permeable. After the addition of 50 µL of the TUNEL anti-mixture, cells were incubated for 1 hour at 37°C in a dark place, washed thoroughly, and added with 4',6-diamidino-2-phenylindole for 15 minutes of incubation. Then, after washing and mounting, cell count was conducted under a fluorescent microscope. The results were determined using the apoptotic index, namely, apoptotic index = the number of apoptotic cells/total cell number. Finally, any 5 high power fields (>1,000 cells) were counted.
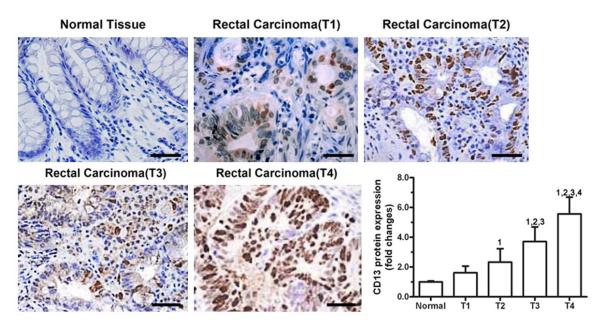
Statistical analysis

SPSS21.0 software was used for statistical analysis. Measurement data are expressed as mean ± standard deviation ($\overline{x}$ ± sd). Correlation analysis was based on Spearman's correlation analysis. One-way analysis of variance was employed for intergroup comparisons. The independent samples t-test was applied for pairwise comparisons. $P<0.05$ represents that the difference is statistically significant.
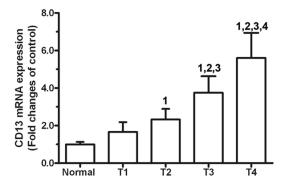
Results

Content of CD13 mRNA and protein in rectal cancer tissues is higher than those in cancer-adjacent normal tissues

Immunohistochemical detection of CD13 showed that CD13 expression in rectal cancer tissues was significantly higher than that in cancer-adjacent tissues, and expression of CD13 in tumor tissues at T1, T2, T3 and T4 was higher than those in the control group. Results also demonstrated that CD13 expression level was positively correlated with TNM stage (**Figure 1**). The expression level of CD13 mRNA in rectal cancer tissues was detected, which evidenced

Figure 1. Detection of the expression of CD13 in rectal cancer tissues and cancer-adjacent normal tissues by immunohistochemistry. T1-T4 represented TNM stage of rectal cancer, respectively, and the scale represented 50 μm. Compared with normal tissues, [1]P<0.05; compared with T1, [2]P<0.05; compared with T2, [3]P<0.05; compared with T3, [4]P<0.05. TNM, tumor-node-metastasis; CD13, cluster of differentiation 13.

Figure 2. Expression of CD13 mRNA in rectal cancer tissues and cancer-adjacent normal tissues. T1-T4 represented the clinical stage of rectal cancer. Spearman's correlation analysis results showed P<0.05, and CD13 expression was positively correlated with TNM stage. Compared with normal tissues, [1]P<0.05; compared with T1, [2]P<0.05; compared with T2, [3]P<0.05; compared with T3, [4]P<0.05. CD13, cluster of differentiation 13; mRNA, messenger RNA; TNM, tumor-node-metastasis.

that the expression level of CD13 was positively associated with TNM stage (**Figure 2**).

CD13 expression level is related to VEGF expression level

Cells were transfected with different concentrations of CD13 overexpression transfection reagent, which showed that the higher the

transfection reagent concentration was, the higher the CD13 expression would be, which was in a dose-dependent manner, and the content of VEGF protein would increase accordingly (**Figure 3**).

Up-regulated CD13 expression facilitates proliferation and metastasis abilities of rectal cancer cells

After transfection with different concentrations of CD13, the culture for rectal cancer cells was terminated at 6 different time points (0 h, 12 h, 24 h, 36 h, 48 h and 72 h), and the proliferation ability of cells was detected by CCK-8. At 0 μg/mL and 10 μg/mL transfection concentrations, there were no significant differences in the absorbance value at each time point compared with 0 hour group (all P>0.05); at 20 μg/mL transfection concentration, the absorbance value significantly increased at 72 h after transfection compared with 0 hour group (P<0.05); at 40 μg/mL and 80 μg/mL transfection concentrations, the absorbance values went up at 48 hours (P>0.05, P<0.05) and 72 hours (P<0.05, P<0.01) after transfection (**Figure 4**). Cell metastasis rates at different time points (12 h, 24 h and 48 h) after transfection with different concentrations of the transfection reagent (0 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL and 160 μg/mL) are shown in **Table 1**.
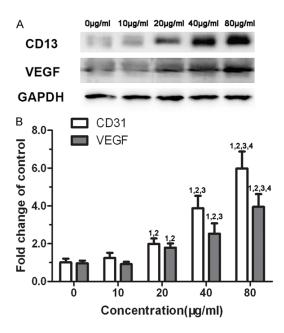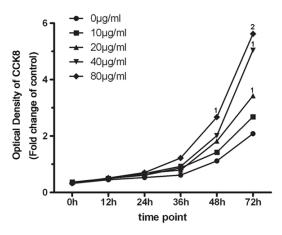
Figure 3. Expression of CD13 and VEGF proteins in SW1463 cells transfected with different concentrations of cells. Compared with 0 µg/mL, [1]P<0.05; compared with 10 µg/mL, [2]P<0.05; compared with 20 µg/mL, [3]P<0.05; compared with 40 µg/mL, [4]P<0.05. CD13, cluster of differentiation 13; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 4. Changes in the proliferation ability of SW1463 cells after CD13 overexpression. The concentration of cell suspensions digested and resuspended at 48 hours after transfection with different plasmids was adjusted to 1,000 cells/100 µL. After the culture in 96-well plates for 0 h, 12 h, 24 h, 36 h, 48 h and 72 h, cells were stained via CCK-8 assay, and the absorbance value at 450 nm was measured using a microplate reader. [1]P<0.05, and [2]P<0.01. CCK-8, Cell Counting Kit-8; CD13, cluster of differentiation 13.

Transfection could significantly boost cell metastasis rate in a dose-dependent manner,

and the higher the transfection concentration was, the higher the cell metastasis rate would be.

Up-regulation of CD13 expression inhibits apoptosis of cancer cell lines

After 24 hour-transfection of rectal cancer cells with different concentrations of CD13 transfection reagent, TUNEL assay was applied to detect the cell apoptosis. The results demonstrated that up-regulation of CD13 expression could inhibit the apoptosis of rectal cancer cells in a dose-dependent manner (**Table 2**).

Discussion

CD13 is one of the relatively mature tumor markers on the cell surface studied in recent years. Studies have found that increasing expression of CD13 on the surface of tumor cells can greatly improve metastasis ability, and that CD13 in the tissue and plasma is correlated with the patient's five-year survival rate, which has been widely applied in the evaluation of the prognosis of patients [12, 13]. A number of studies have confirmed that CD13 expression has a close relationship with the prognosis of malignant tumors such as colon, lung, and pancreatic cancer. The survival rate of CD13 positive patients is significantly lower than that of CD13 negative patients [14-16]. However, little is known about direct correlations of CD-13 expression with metabolism and metastasis of rectal cancer tissues. The study results surfaced that the expression levels of CD13 mRNA and protein in rectal cancer tissues were significantly higher than those in cancer-adjacent normal tissues and the expression level of CD13 in rectal cancer tissues of patients at a higher TNM stage remarkably increased. In addition, cell experimental results revealed that CD13 overexpression increased the proliferation and migration abilities of rectal cancer cells in a dose-dependent manner while inhibiting the apoptosis of rectal cancer cells. All of this is evidence that CD13 expression level can reflect the degrees of cell growth and metastasis to some extent.

At the present stage, the mechanisms of CD13 in enhancing cell proliferation and metastasis abilities as well as in playing its role as a neovascular marker still need further elucidation. It has been reported that CD13 can induce DNA damage by generating excess reactive oxygen

Table 1. Cell metastasis rate after CD13 transfection

Transfection concentration	Cell metastasis rate (%)		
	12 h	24 h	48 h
0 µg/mL	19.87±3.19	29.75±4.72	37.58±4.98
10 µg/mL	29.45±4.01[a]	33.72±5.77[a]	48.86±6.22[a]
20 µg/mL	42.78±5.29[a,b]	50.81±6.98[a,b]	62.53±7.79[a,b]
40 µg/mL	51.42±6.01[a,b,c]	59.16±7.02[a,b,c]	72.25±7.84[a,b,c]
80 µg/mL	56.65±7.05[a,b,c,d]	64.44±7.17[a,b,c,d]	83.46±9.36[a,b,c,d]

Note: CD13, cluster of differentiation 13. Compared with 0 µg/mL, [a]$P<0.05$; compared with 10 µg/mL, [b]$P<0.05$; compared with 20 µg/mL, [c]$P<0.05$; compared with 40 µg/mL, [d]$P<0.05$.

Table 2. Apoptotic index after CD13 transfection

Transfection concentration	Average apoptotic index (%)
0 µg/mL	4.94±0.91
10 µg/mL	4.19±0.91
20 µg/mL	3.76±0.80[a,b]
40 µg/mL	3.29±0.73[a,b,c]
80 µg/mL	0.20±0.63[a,b,c,d]

Note: CD13, cluster of differentiation 13. Compared with 0 µg/mL, [a]$P<0.05$; compared with 10 µg/mL, [b]$P<0.05$; compared with 20 µg/mL, [c]$P<0.05$; compared with 40 µg/mL, [d]$P<0.05$.

species, and the resulted genotoxicity and stress-induced injury can inhibit apoptosis and enhance the proliferation ability of cells, thus inducing the carcinogenesis [17]. This study found that overexpression of CD13 could strengthen the proliferation ability of cells, and CD13 may be indirectly involved in carcinogenesis of rectal cancer tissues. Proteolytic enzyme-mediated degradation of the extracellular matrix plays a crucial role in tumor tissue metastasis. Tumor cells and attached tissue cells can secrete a variety of proteases to participate in the degradation of the basement membrane and extracellular matrix, so as to achieve unlimited invasion and metabolism [18]. It is currently known that the higher the level of CD13 expression in malignant tumor cells, the higher the efficiency of collagen degradation, which promotes the malignant invasion of tumor cells by hydrolyzing the basement membrane and extracellular matrix [19]. In combination with our previous research results, it is speculated that the increased CD13 expression in rectal cancer tissues may be the most direct factor leading to the increased invasiveness of tumor tissues.

It is noteworthy that according to this study, expression of VEGF, an important angiogenic marker in rectal cancer cells, is positively correlated with CD13. It has been reported that the selective knockdown of CD13 expression by small interfering RNAs can inhibit the formation of new blood vessels in human umbilical vein endothelial cells, decrease the expression of VEGF, and inhibit the metastasis of human umbilical vein endothelial cells, with the increased expressions of multiple intracellular adhesion molecules such as type IV collagen, type I collagen and fibronectin. The above results confirm that CD13 is a multifunctional protein that plays an important role in the regulation of the formation of vascular endothelial cells and new blood vessels [15, 20]. Although there is no definitive mechanism confirming that VEGF directly promotes or inhibits the expression of CD13, it is certain that CD13 indeed mediates the formation of VEGF in some way, and VEGF is also involved to some extent in CD13 expression.

In summary, the study results strongly support that CD13 is highly expressed in rectal cancer tissues and cancer cell lines. CD13 was overexpressed using CD13 expression vector, which confirmed its positive correlation with proliferation, invasion, and metastasis of rectal cancer tissues, thus laying a foundation for the clinical treatment of rectal cancer. However, there are still some shortcomings in this study. For example, the sample size was small due to limited conditions, and there might be a certain degree of statistical bias. In the experimental design, CD13 inhibitors were not applied to further confirm the efficacy of targeted therapies with CD13-labeled anti-cancer drugs. Instead, only some theoretical possibilities for treatment were proposed, and both feasibility and effectiveness need to be further confirmed by more specific animal experiments and more in-depth clinical trials, so this will be the research target and direction of our research group in the future.

Disclosure of conflict of interest

None.

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