Effect of the ACY-1 gene on HER2 and TRAIL expression in rectal carcinoma

ZIZHONG XU[1], YATING HU[2] and ZHAOHUI YU[3]

Departments of [1]General Surgery, [2]Endocrinology and [3]Anorectal,
The First People's Hospital Xianyang City, Xianyang, Shanxi 712000, P.R. China

Abstract. The incidence of rectal carcinoma (RC) is increasing and the age at onset of the disease is reducing. Therefore, elucidating the pathogenesis of RC is beneficial for early diagnosis and improving the prognosis. Aminoacylase-1 (ACY-1) is abnormally expressed in various malignant tumor tissues. Furthermore, the human epidermal growth factor receptor-2 (HER2) gene is involved in tumor metastasis and invasion, while tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces tumor cell apoptosis. The aim of the present study was to investigate the effect of the ACY-1 gene on the expression of HER2 and TRAIL in RC. Cancerous and adjacent tissues from RC patients were collected. ACY-1 expression was analyzed by immunohistochemistry. The rectal cancer cell lines HT29 and SW620, and normal colorectal mucosal epithelial fetal human cells were cultured *in vitro*. ACY-1 gene and protein expression levels were tested by reverse transcription-quantitative PCR and western blotting. ACY-1 small interfering RNA (siRNA) was transfected into HT29 and SW620 cells. Cell proliferation was detected by thiazolyl blue MTT assay. Caspase-3 activity was assessed using a commercial kit. HER2 and TRAIL expression levels were determined by western blotting. ACY-1 expression was significantly increased in cancer tissue compared with adjacent tissue (P<0.05). ACY-1 expression was elevated in HT29 and SW620 cells compared with normal colorectal mucosal epithelial cells (P<0.05). ACY-1 siRNA transfected into HT29 cells downregulated its expression, inhibited cell proliferation, enhanced caspase-3 activity, reduced HER2 expression and upregulated TRAIL expression (P<0.05). ACY-1 expression was found to be increased in rectal cancer tissue. Therefore, targeting the ACY-1 gene may regulate HER2 and TRAIL expression levels, and may reduce the occurrence and inhibit the development of rectal cancer.

Correspondence to: Dr Zhaohui Yu, Department of Anorectal, The First People's Hospital Xianyang City, 10 Biyuan Road, Xianyang, Shanxi 712000, P.R. China
E-mail: zhifuyongzhuang@126.com

Introduction

Rectal carcinoma (RC) is a common intestinal malignant tumor belonging to the colorectal cancer class. RC is ranked third and fourth worldwide in terms of global tumor morbidity and mortality, respectively, with an increasing overall prevalence and a trend towards an earlier onset of disease (1,2). With the improvement of living conditions, constant changes in diet structure and lifestyle, and the accelerated pace of work, there has been an increased intake of high-fat, high-protein and low-fiber foods, resulting in dietary disorders (3). RC has become the second most prevalent malignant tumor of the digestive tract behind by gastric cancer (4,5). Diagnosis of RC is limited by the unclear early symptoms; therefore, diagnosis and treatment of RC are usually delayed, and most patients have already reached advanced stages at the time of diagnosis (6). The 5-year survival rate of RC is relatively low (7). The pathogenesis of RC has not been fully elucidated as it involves multiple factors, processes and genes (8,9). Understanding the pathogenesis behind RC is essential to develop early diagnosis procedures and to improve the prognosis, thus a greater understanding of RC pathogenesis is key to improving the survival rate and quality of life of patients.

Aminoacylase-1 (ACY-1) is a zinc-binding enzyme that hydrolyzes N-acetyl amino acids into free amino acids and acetic acid, and is expressed at abnormally high levels in various malignant tumor tissues. It has been shown that ACY-1 is overexpressed in liver cancer and that ACY-1 expression is associated with tumor proliferation, invasion and metastasis (10,11). Human epidermal growth factor receptor-2 (HER2) is a proto-oncogene which is expressed in healthy adults, but its expression increases in more than one-third of tumors (12). HER2 can promote cell proliferation by inhibiting apoptosis, enhancing tumor cell invasiveness, promoting tumor angiogenesis and lymphangiogenesis and promoting tumor occurrence and development (13). In particular, HER2 gene overexpression is closely associated with breast cancer and can be used for clinical monitoring and indicating prognosis (14). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a newly discovered member of the TNF family (15) and can rapidly promote large-scale apoptosis in transformed cells, virus-infected cells and tumor cells (16,17). Therefore, the aim of the present study was to investigate the

effect of the ACY-1 gene on HER2 and TRAIL expression in RC.

Materials and methods

Research objective. A total of 48 RC patients, who had received surgery in the First People's Hospital Xianyang City between May and December 2017, were enrolled for the present study. This included 26 males and 22 females with an average age of 55.8±7.9 (range, 41-75) years. There were 18 cases of mucinous adenocarcinoma, 20 cases of papillary adenocarcinoma, and 10 cases of signet ring cell carcinoma. The inclusion criteria were as follows (6): First time treatment with no adjuvant therapy such as chemotherapy, radiotherapy or biological therapy prior to the surgery. The exclusion criteria were as follows (6): Previous severe heart, lung, liver or kidney failure; cerebrovascular disease; other malignant tumor types; autoimmune diseases; infectious diseases; unable to cooperate with the study; and incomplete clinical data. The study was approved by the Medical Ethics Committee of The First People's Hospital Xianyang City, and the patients or their families had signed informed consent forms. Specimens were collected of intraoperative rectal cancer and adjacent tissue (at least 3 cm from tumor tissue), and stored in liquid nitrogen. Some samples were prepared with 4% neutral formalin solution at 4°C for 15 min for immunohistochemistry.

Main reagents and instruments. The rectal cancer cell lines HT29 and SW620 were purchased from the American Type Culture Collection and the authenticity was verified by Suzhou Genetic Testing Biotechnology Co., Ltd. These cell lines, as well as the normal colorectal mucosal epithelial fetal human cell line (American Type Culture Collection) were cryopreserved by our laboratory. DMEM, EDTA, FBS, penicillin and streptomycin were purchased from HyClone; GE Healthcare Life Sciences. DMSO and MTT powder were purchased from Gibco; Thermo Fisher Scientific, Inc. Trypsin was purchased from Sigma-Aldrich; Merck KGaA. The PVDF membrane was purchased from Pall Life Sciences. All western blotting-related chemical reagents were purchased from Beyotime Institute of Biotechnology. ECL reagents were purchased from GE Healthcare. Rabbit anti-human HER2 monoclonal antibody, rabbit anti-human TRAIL monoclonal antibody, rabbit anti-human ACY-1 monoclonal antibody, and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were purchased from Cell Signaling Technology, Inc. The immunohistochemical streptavidin-perosidase kit (cat. no. SA1098) was purchased from Boster Biological Technology. The RNA extraction kit and the reverse transcription kit were purchased from Axygen; Corning Inc. The caspase-3 activity assay kit (cat. no. 556485) was purchased from BD Biosciences. The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Laboratories, Inc. The ABI 7900 HT Real-time PCR machine was purchased from Applied Biosystems; Thermo Fisher Scientific, Inc. Other commonly used reagents were purchased from Sangon Biotech Co., Ltd.

Immunohistochemistry. Tissue samples were prepared and sectioned to a thickness of 5 μm. Paraffin sections were immunohistochemically stained using the SP kit following the

Table I. Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	ACCAGGTATCTTGG TTG	TAACCATGTCAGC GTGGT
ACY-1	AGTCGACCACTCC ACAGT	GATTCTGTGGTCA CTATATT

ACY-1, aminoacyclase-1.

Table II. ACY-1 expression in RC and adjuvant tissue.

	ACY-1		
Tissue type	(-)	(+ or ++)	Positive rate (%)
Adjacent tissue	42	4	8.33
RC	2	46	95.83[a]

[a]P<0.05 vs. adjuvant tissue group. ACY-1, aminoacyclase-1; RC, rectal carcinoma.

manufacturer's instructions. After blocking with 10% normal FBS at room temperature for 30 min to block endogenous peroxidase, the sections were incubated with ACY-1 monoclonal antibodies (1:1,000) at 37°C for 1 h. Washing was performed with PBS solution three times, and the sections were incubated with biotinylated goat anti-mouse IgG secondary antibodies (cat. no. ab6788; Abcam; 11,000) at room temperature for 20 min. Next, the sections were incubated with streptavidin-biotin complex to amplify the signal at room temperature for 20 min. After 3,3'-diaminobenzidine staining, the sections were counterstained with hematoxylin for 1 min at room temperature, dehydrated with serial gradients of ethanol, and sealed. The sections were then visualized using a light microscope (magnification, x400). Positive expression was determined by the number of cells that contained brown/yellow granules. Samples that contained no brown/yellow stained cells were defined as negative, samples with <50% of cells stained brown/yellow in five random field were defined as positive (+) and when ≥50% of cells were stained brown-yellow, samples were defined as strong positive (++).

Cell culture and grouping. Cells were resuspended in 1 ml fresh DMEM and topped up with 4 ml of fresh DMEM at 37°C and 5% CO_2 prior to seeding. Cells were seeded in 6-well plates at $1x10^5$ cells/cm^2 in high-glucose DMEM (containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C with 5% CO_2. The cells in the 3rd-8th generation and logarithmic growth phase were used for experiments. HT29 cells were randomly divided into three group: A control group; a small interfering RNA (siRNA) (Sigma-Aldrich; Merck KGaA) negative control group (scramble group); and an ACY-1 siRNA (Sigma-Aldrich; Merck KGaA) group.

Liposomal transfection. ACY-1 siRNA was transfected into HT29 or SW620 cells. The ACY-1 siRNA sequence was as
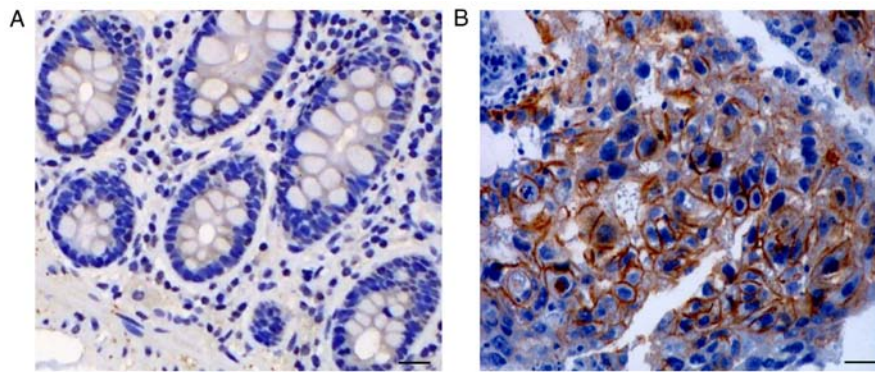
Figure 1. ACY-1 expression in RC tissue and adjacent tissue (magnification, x400). (A) ACY-1 expression in adjacent tissue. (B) ACY-1 expression in RC. Scale bars, 10 μm. ACY-1, aminoacyclase-1; RC, rectal carcinoma.
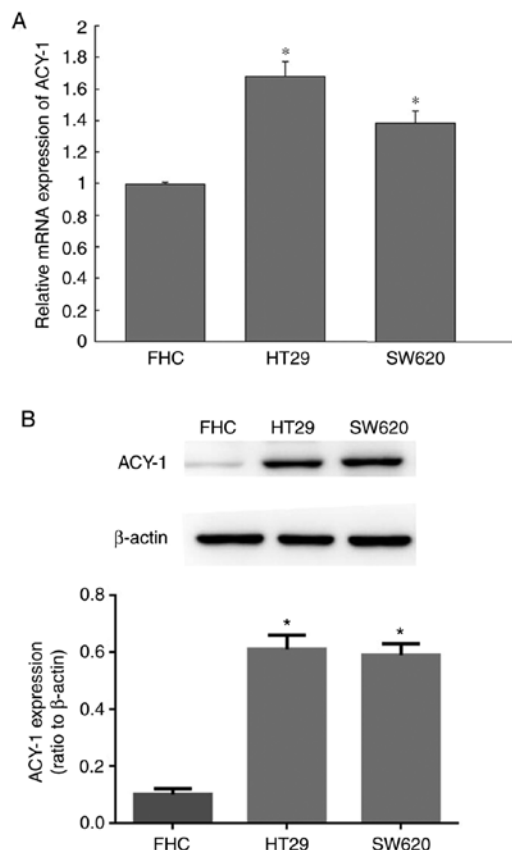

Figure 2. ACY-1 expression in rectal carcinoma cell lines. (A) ACY-1 gene expression in HT29, SW620 and FHC cells. (B) ACY-1 protein expression in HT29, SW620 cells and FHC cells. *P<0.05 vs. FHC. ACY-1, aminoacyclase-1; FHC, fetal human cells.

follows: Forward, 5'-GATAAATGGACTTGGAGAACAG-3' and reverse, 5'-TAGACATGGAGACTTGACAGACT-3'. The siRNA negative control primer sequence was: Forward, 5'-ATT CACCTGCCATGTAT-3' and reverse, 5'-GAACACTAATGT TGACAG-3'. Cells were grown to 70-80% confluence. The ACY-1 siRNA or negative control siRNA was added to 200 μl of serum-free DMEM medium and mixed at room temperature for 15 min. The Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) was mixed with 20 nM ACY-1 siRNA or negative control siRNA and incubated for 30 min at room temperature. The mixture together with 1.6 ml serum-free DMEM, was incubated with the cells at 37˚C with 5% CO_2 for 6 h. The cell culture medium was replaced with DMEM containing serum and cultured for 48 h before experimental research.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tissues or cells using TRIzol® reagent and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions (25˚C for 10 min, 37˚C 120 for min and at 85˚C for 5 min). The primers were designed by Primer Premier 6.0 software (Premier Biosoft) and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). The qPCR reaction was performed using Fast SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) with 35 cycles of 92˚C for 30 sec, 58˚C for 45 sec and 72˚C for 35 sec. GAPDH was used as a reference gene. The relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18).

MTT assay. HT29 and SW620 cells in the logarithmic growth phase were trypsinized and seeded onto a 96-well plate at 3,000 cells/well. The cells were randomly divided into the control group, scramble group and ACY-1 siRNA group, with three replicates per group. After treatment, the cells were given 20 μl of 5 g/l MTT solution for 4 h in the incubator. Next, 150 μl DMSO/well was applied and the solution was shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (A) was measured at 570 nm to calculate cell viability rate. Cell viability rate was calculated with the following formula: Cell viability rate=sample A value/control A value x100%. The experiment was repeated three times (n=3).

Caspase-3 activity detection. The cells were trypsinized and centrifuged at 4˚C at 600 x g for 5 min. Next, the cells were lysed with cell lysis buffer [10 mM Tris-HCl; 10 mM NaH_2PO_4/$NaHPO_4$ (pH 7.5); 130 mM NaCl; 1% Triton X-100; 10 mM sodium pyrophosphate] on ice for 15 min and centri-fuged at 4˚C and 20,000 x g for 5 min. Subsequently, 2 mM Ac-DEVD-AMC (included in the caspase-3 activity kit) was added, the optical density (OD) was measured at 450 nm to calculate the caspase-3 activity.

Western blot analysis. Cells were treated with RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin, 1 mM PMSF, 1.5 mM EDTA, and 1 mM
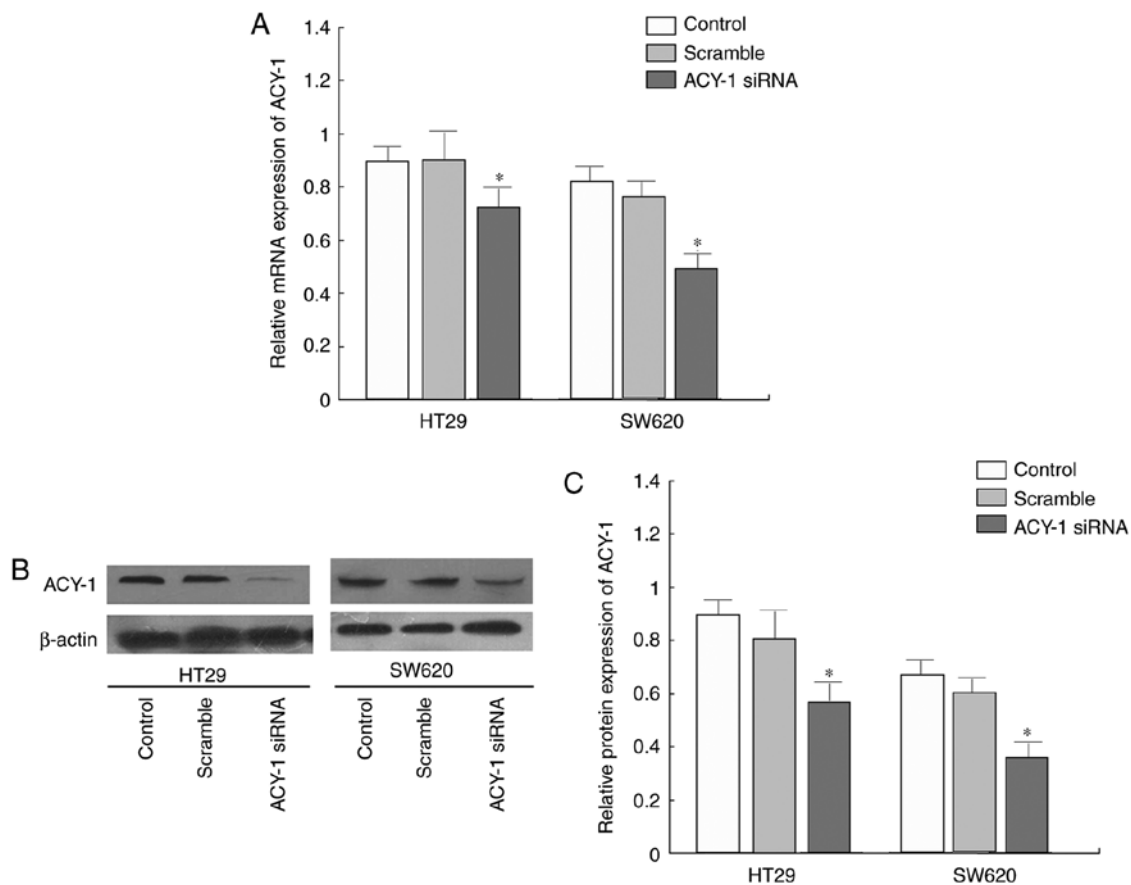
Figure 3. Impact of ACY-1 siRNA on ACY-1 mRNA and protein expression in HT29 and SW620 cells. (A) ACY-1 gene expression in HT29 or SW620 cells. (B) ACY-1 protein expression in TH29 or SW620 cells. (C) ACY-1 protein expression analysis. *P<0.05 vs. retrospective control. ACY-1, aminoacyclase-1; siRNA, small interfering RNA.

sodium vanadate) and quantified by BCA assay. In total, 40 μg of protein was electrophoresed using 10% SDS-PAGE. The gel was transferred to a PVDF membrane by semi-dry transfer at 150 mA for 1.5 h. After blocking with 5% skim milk at room temperature for 1 h, the membrane was incubated with ACY-1, HER2, TRAIL and β-actin primary antibodies (1:1,000, 1:2,000; 1:1,500 and 1:1,000, respectively) at 4˚C overnight. The membranes were then incubated with goat anti-rabbit secondary antibodies (1:2,000) in the dark for 30 min at room temperature. The labelled membrane was imaged using chemiluminescence reagent for 1 min and analyzed by image processing system software and Quantity one software (version 4.52; Bio-Rad Laboratories, Inc.). β-actin was used as a loading control. The experiment was repeated four times (n=4).

Statistical analysis. All data analyses were performed using SPSS 19.0 software (IBM Corp.). The measurement data are presented as the mean ± SD and compared by one-way ANOVAs with post hoc LSD tests. The enumeration data are depicted as percentages and were compared by χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

IHC analysis of ACY-1 expression in RC tissue and adjuvant tissue. Immunohistochemistry was used to detect ACY-1

expression in RC and adjacent tissue. ACY-1 expression was significantly increased in rectal cancer tumor tissue, with positive staining in the cell membrane and cytoplasm. However, the adjacent tissue presented negative for ACY-1 expression (-) (P<0.05) (Fig. 1; Table II).

ACY-1 expression in RC cell lines. ACY-1 gene and protein expression levels were measured between rectal cancer HT29 or SW620 cells and normal colorectal mucosal epithelial cells. It was found that ACY-1 gene and protein expression levels were upregulated in HT29 and SW620 cells compared with control cells (P<0.05; Fig. 2).

Impact of ACY-1 siRNA on ACY-1 mRNA and protein expression in HT29 cells. The effect of siRNA on ACY-1 mRNA and protein expression levels was detected by RT-qPCR. The gene and protein expression levels of ACY-1 were significantly reduced after siRNA treatment (P<0.05; Fig. 3).

Influence of ACY-1 siRNA on HT29 cell proliferation. The effect of ACY-1 siRNA on the proliferation of HT29 or SW620 cells was detected using MTT assays. siRNA transfection significantly inhibited HT29 or SW620 cell proliferation compared with control transfection (P<0.05; Fig. 4).

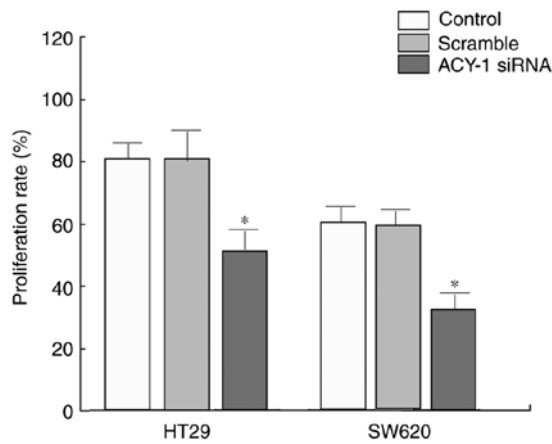*Effect of ACY-1 siRNA on caspase-3 activity in HT29 cells.* The effect of ACY-1 siRNA on the activity of caspase-3 in

Figure 4. Influence of ACY-1 siRNA on HT29 and SW620 cell proliferation. *P<0.05 vs. respective control. ACY-1, aminoacyclase-1; siRNA, small interfering RNA.

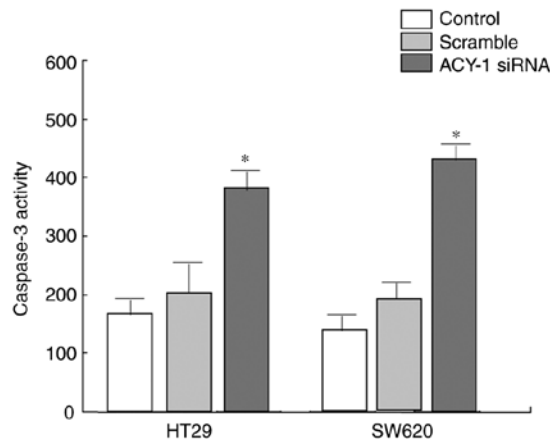
Figure 5. Effect of ACY-1 siRNA on caspase-3 activity in HT29 and SW620 cells. *P<0.05 vs. respective control. ACY-1, aminoacyclase-1; siRNA, small interfering RNA.
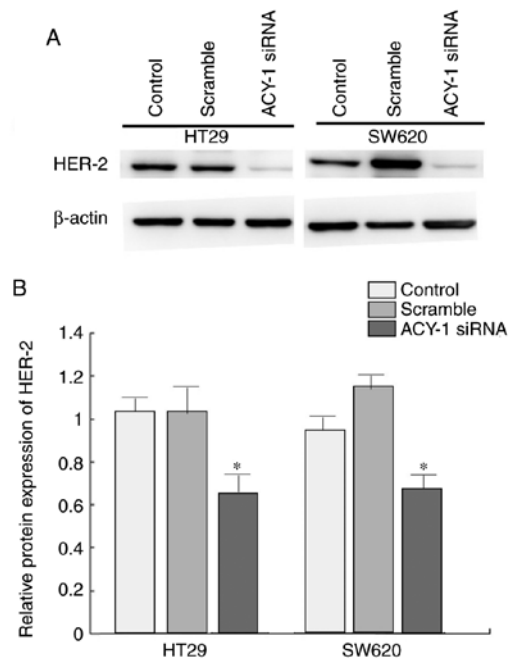

Figure 6. Impact of ACY-1 siRNA on HER2 expression in HT29 or SW620 cells. (A) The impact of ACY-1 siRNA on HER2 protein expression. (B) Semi-quantitative analysis of HER2 protein expression. *P<0.05 vs. respective control. ACY-1, aminoacyclase-1; HER2, human epidermal growth factor receptor-2; siRNA, small interfering RNA.

HT29 or SW620 cells was detected using the caspase-3 activity kit. It was revealed that siRNA significantly enhanced caspase-3 activity in HT29 or SW620 cells compared with control treatment (P<0.05; Fig. 5).

Impact of ACY-1 siRNA on HER2 expression in HT29 cells. Western blotting was performed to analyze the effect of ACY-1 siRNA on HER2 expression in HT29 or SW620 cells. It was demonstrated that siRNA against ACY-1 significantly suppressed HER2 expression compared with the control group (P<0.05; Fig. 6).

Influence of ACY-1 siRNA on TRAIL expression in HT29 cells. Western blotting was performed to analyze the effect of ACY-1 siRNA on TRAIL expression in HT29 or SW620 cells. ACY-1 siRNA significantly increased TRAIL expression compared with the control group (P<0.05; Fig. 7).

Discussion

A previous study has investigated the expression profile of ACY-1 in renal transplantation and as an indicator of

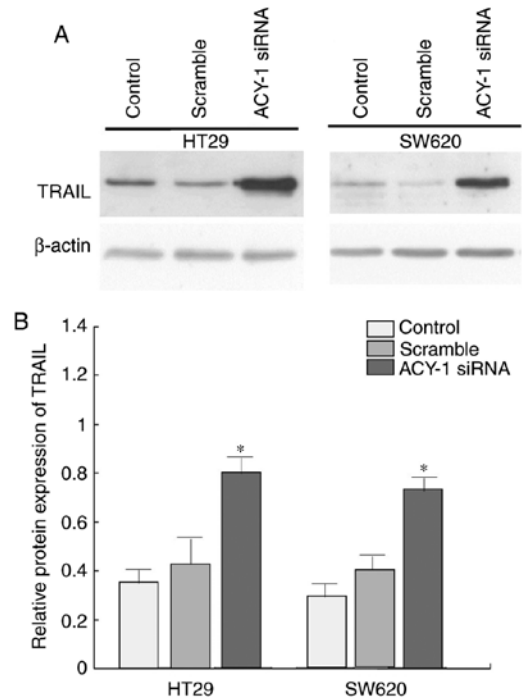

Figure 7. Influence of ACY-1 siRNA on TRAIL expression in HT29 or SW620 cells.(A) the impact of ACY-1 siRNA on TRAIL protein expression. (B) Semi-quantitative analysis of TRAIL protein expression. *P<0.05 vs. respective control. ACY-1, aminoacyclase-1; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

prognosis (19). As one of the acylated amino acid active enzymes, ACY-1 is mainly expressed in the cell membrane and cytoplasm. It can acetylate α-acylated amino acids and enhance protein stability in cells (20). There are relatively few

studies on the role of ACY-1 in tumors. It has been previously found that ACY-1 expression is decreased in liver cancer and renal cell carcinoma (21-23); however, the expression of ACY-1 in rectal cancer has not been clarified. In the present study, ACY-1 expression was shown to be significantly increased in rectal cancer tissues and cells, suggesting that it may be associated with the occurrence and development of rectal cancer.

The proto-oncogene HER2, also known as the neu gene or the c-erbB-2 gene, is involved in several physiological processes, such as cell proliferation and differentiation (24). However, under the influence of external factors, the HER2 gene is abnormally expressed, which may lead to the abnormal activation of its related protein product P185, inducing tumorigenesis and metastasis (25). TRAIL is an apoptosis-inducing ligand; the binding of TRAIL to its receptor promotes the caspase-3 protease cascade and induces apoptosis (26). The present study demonstrated that ACY-1 siRNA decreased the expression of ACY-1 at the gene and protein level, which significantly downregulated the expression levels of HER2, elevated TRAIL expression, suppressed cell proliferation and upregulated caspase-3 activity in HT29 and SW620 cells. This indicates that ACY-1 siRNA in rectal cancer may enhance TRAIL expression by inhibiting HER2 expression, thereby inhibiting tumor cell proliferation and promoting tumor cell apoptosis. In further research, it is necessary to explore the specific target and related mechanisms of ACY-1, and provide relevant evidence for its use in the clinical diagnosis and treatment of RC.

In conclusion ACY-1 expression is increased in rectal cancer tissue. Targeting the ACY-1 gene can regulate the expression of both HER2 and TRAIL which may inhibit the occurrence and development of rectal cancer.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZX and YH performed the experiments and analyzed the data. ZY designed the study and wrote the manuscript.

Ethics approval and consent to participate

The current study was approved by The First People's Hospital Xianyang City and consent was obtained for participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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