Downregulation of CD147 expression by RNA interference inhibits HT29 cell proliferation, invasion and tumorigenicity in vitro and in vivo

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Abstract. We investigated the effect of CD147 silencing on HT29 cell proliferation and invasion. We constructed a novel short hairpin RNA (shRNA) expression vector pYr-mir30-shRNA. The plasmid was transferred to HT29 cells. The expression of CD147, MCT1 (lactate transporters monocarboxylate transporter 1) and MCT4 (lactate transporters monocarboxylate transporter 4) were monitored by quantitative PCR and western blotting, respectively. The MMP-2 (matrix metalloproteinase-2) and MMP-9 (matrix metalloproteinase-9) activities were determined by gelatin zymography assay, while the intracellular lactate concentration was determined by the lactic acid assay kit. WST-8 assay was used to determine the HT29 cell proliferation and the chemosensitivity. Invasion assay was used to determine the invasion of HT29 cells. In addition, we established a colorectal cancer model, and detected CD147 expression in vivo. The results showed that the expression of CD147 and MCT1 was significantly reduced at both mRNA and protein levels, and also the activity of MMP-2 and MMP-9 was reduced. The proliferation and invasion were decreased, but chemosensitivity to cisplatin was increased. In vivo, the CD147 expression was also significantly decreased, and reduced the tumor growth after CD147 gene silencing. The results demonstrated that silencing of CD147 expression inhibited the proliferation and invasion, suggesting CD147 silencing might be an adjuvant gene therapy strategy to chemotherapy.

Introduction

Colorectal cancer is the third most common cancer in men (10.0% of the total, ~663,000 cases) and the second in women (9.4% of the total, ~570,000 cases) worldwide (1). Approximately 608,000 colorectal cancer deaths were estimated worldwide, accounting for 8% of all cancer deaths, making it the fourth most common cause of death from cancer. The traditional treatment of colorectal cancer is generally drugs, radiotherapy and chemotherapy, but the effect of these methods are not satisfactory, the mortality rate of colorectal cancer still remains high. The study of Center et al (2) indicated that the mortality of colorectal cancer had also been increasing because of tumor relapse and metastasis. However, carcinogenesis is a complicated biological process, and the molecular mechanisms, metastasis phenotype, pathways and regulating genes are not well known (3). Therefore, better understanding of molecular mechanisms underlying proliferation, invasion and survival of colorectal cancer are critical for the development of optimal therapeutic modalities.

CD147 (also called EMMPRIN, basigin, tumor cell derived collagenase stimulatory factor, or human leukocyte activation-associated M6 antigen), is a 43-66-kDa multifunctional glycosylated transmembrane protein, which belongs to the immunoglobulin superfamily (4-7). The protein of CD147 is highly expressed on the cell surface of many tumor cells such as, oral, breast, lung, bladder, kidney, laryngeal, pancreatic, gastric, colorectal cancer, glioma, lymphoma and melanoma (8-13), and was correlated with tumor progression and invasion (14,15) and could also influence lactate transport and glycolysis by its association with lactate transporters.
monocarboxylate transporter (MCT), specifically MCT-1 and MCT-4 (20). MCT-1 and MCT-4 are two members of the proton-linked monocarboxylate (lactate) transporter family, playing a fundamental role in metabolism. CD147 is essential in transporting MCT1 and MCT4 to plasma membrane (21). CD147 was also involved in multidrug resistance of cancer cells via hyaluronan-mediated activation of ErbB2 signaling and survival pathway activity, and multidrug resistance and tumor invasiveness might be linked during the progression of malignant disease (22,23), but the function and mechanism of CD147 remain elusive on proliferation, invasion, metastasis and multidrug resistance of colorectal cancer.

The studies of Zhu et al (12) showed that CD147 expression was high in colorectal cancer and was associated with the colorectal development, and with poor prognosis. The molecular mechanisms involved and the role of CD147 in colorectal cancer, however, remained poorly understood. To determine the role of CD147 in invasiveness, metastasis, growth and survival of colorectal cancer, we used RNA interference (RNAi) technique to knock down the expression of CD147 in HT29 cells, and investigated its roles on proliferation, invasion and the chemosensitivity of colorectal cancer cells.

Materials and methods

Cell culture. HT29 cells, a human colorectal cancer cell line, was provided from the Shanghai Cell Collection, the Chinese Academy of Sciences. Cells were maintained in DMEM (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 g/ml of streptomycin (Gibco BRL) in a 5% CO2 humidified atmosphere at 37˚C.

Design of pYr-mir30-shRNA plasmid construction. The vector pYr-mir30-shRNA was used to generate short hairpin RNA (shRNA) specific for CD147 by selecting the 808-828 fragment as the RNAi target site, and the scrambled control sequence was also synthesized as shown in Table I. These oligonucleotides were annealed and subcloned into the BsaI sites of the vector. These recombinant vectors were designated as pYr-mir30-shRNA-control and pYr-mir30-shRNA, respectively. The vector of pYr-mir30-shRNA included the EGFP gene sequence, so the EGFP protein expression can reflect the CD147 protein expression. All the cloned genes were confirmed by DNA sequencing.

Transient and transfection screening. HT29 cells were plated in 6-well plates at a density of 3x104 cells per well and incubated in 2 ml of growth medium without antibiotics. When the cells reached 80% confluence after 24-h incubation, cells were transfected with pYr-mir30-CD147-shRNA-control and pYr-mir30-CD147-shRNA, respectively, using Lipofectamine 2000 (Invitrogen-Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Forty-eight hours after transfection, HT29 cells were diluted 1:10 for passage and neomycin resistance clones were selected in the medium containing 600 µg/ml G418 (Gibco-BRL) for 2 weeks. The positive clones were picked and expanded to establish cell lines in 300 µg/ml G418. The stable transfection cell clones, designated as HT29/shRNA-control, HT29/shRNA, were verified by quantitative real-time RT-PCR and western blot analysis.

Quantitative real-time PCR assay. Total cellular RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions and reverse transcribed into cDNA using PrimeScript RT reagent kit Perfect Real (Takara). First the cDNA was quantified in a 1:10 dilution on a spectrophotometer. CD147 mRNA expression was evaluated by RT-PCR on an ABI PRISM 7500 real-time PCR apparatus (Applied Biosystems, USA) with SYBR Premix Ex TaqTM II. The primer sequences used for CD147, MCT1, MCT4 and β-actin are listed in Table II. The conditions for real-time PCR were: 95˚C for 30 sec, then 40 cycles at 95˚C for 5 sec, and 60˚C for 34 sec. The mRNA level for CD147 of each sample was normalized to Ct values of the β-actin amplified from the same sample, ΔCt=CtCD147 - Ctβ-actin and the 2-ΔΔ Ct method was used to calculate gene expression change. Samples were measured in triplicates to ensure the reproducibility of the results.

Western blot analysis. Western blot analysis was performed to evaluate CD147, MCT1 and MCT4 protein levels. The cultured tumor cells were washed three times with ice-cold PBS, then the cells were suspended in lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM MgCl2, 100 µg/ml PMSF, 1.0% Triton X-100] on ice for 30 min. Cell lysates were then collected after centrifugation at 12,000 rpm for 5 min at 4˚C. Equal amounts (30 µg) of lysate proteins were separated on 10% SDS-PAGE gels, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% non-fat dry milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 2 h at room temperature, the membrane was probed with mouse anti-CD147 primary antibodies (1:500), rabbit anti-MCT1 primary antibodies (1:500), rabbit anti-MCT4 primary antibodies (1:500) and rabbit anti-human β-actin primary antibodies (1:500) incubated at room temperature for 2 h, followed by incubation in a 1:2,000 dilution of secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Protein bands were detected using ECL detection system (Boster, Wuhan, China). Western blot experiments were performed at least three times.

Gelatin zymography assay. Cells were cultured in serum-free DMEM medium for 24 h, and then harvested in conditioned medium. The gelatinolytic activity of MMP-2 and MMP-9 in the conditioned medium was assayed by electrophoresis on 10% polyacrylamide gels containing 1 mg/ml of gelatin. PAGE gels were run at 100 V in stacking gels, and 100 mA in separating gels, washed in 2.5% Triton X-100 twice every 40 min, and then incubated for 16 h at 37˚C in activation buffer (50 mM Tris-HCl, pH 7.6, 5 mM CaCl2, 0.02% Brij-35). After reaction, the gels were stained with Coomassie Brilliant Blue R-250 for 3 h and destained for 30 min in 20% methanol and 10% acetic acid. White lysis zones indicating gelatin degradations were revealed. This experiment was repeated at least three times.

Intracellular lactate concentration assay. We used a lactic acid assay kit (KeyGen Biotech Co., Ltd., Nanjing, China) to
assess the change of intracellular lactate concentration in HT29 cells after CD147 silencing. Cells (1x10^6) were harvested by centrifugation and were then ruptured by hypotonic salt solution for 1 h at room temperature. The supernatant was retained after centrifuging. The optical density was read at 530 nm. This experiment was repeated at least three times.

**Cell proliferation assay.** Cell proliferation in vitro was analyzed with the formazan substrate, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5 (2, 4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8). Cells were plated in 96-well plates in 100 µl DMEM at a density of 1x10^4 cells per well. After 24, 48, 72, 120 h of culture, respectively, the medium was removed and replaced with fresh medium containing 10% FBS-containing medium and was incubated at 37°C for 24 h. The medium was then removed and added with fresh medium containing cisplatin, paclitaxel, gemcitabine and oxaliplatin (Sigma) with varying concentrations: 0.1, 1 and 10 µM. After 48 h, cells were treated with MST-8 as described earlier. Spectrometric absorbance at 450 nm was measured with a micro-plate reader. Each group was repeated at least three times.

**Invasion assay.** Transwell plates (Corning Costar, Cambridge, MA, USA) were coated with basement membrane Matrigel (20 mg/ml, Becton-Dickinson, Franklin Lakes, NJ, USA) for 4 h at 37°C. Serum-free DMEM containing 1x10^6 cells in 100 µl was added into the upper chamber, the lower chamber received 500 µl of 10% FBS-containing medium and was incubated at 37°C for 24 h. After 18 h, cells that migrated through the permeable membrane were fixed with 100% methanol for 10 min. The membranes with cells were soaked in 0.1% crystal violet for 10 min and then washed with distilled water. The number of cells which attached to the lower surface of the polycarbonate filter was counted at x400 magnification under a light microscope. Results were expressed as mean of triplicate experiments.

**Drug sensitivity assay.** To assess the antitumor drug chemosensitivity, cells were seeded in triplicates on 96-well plates at 1x10^3 cells/well and incubated for 24 h. The medium was then removed and added with fresh medium containing cisplatin, paclitaxel, gemcitabine and oxaliplatin (Sigma) with varying concentrations: 0.1, 1 and 10 µM. After 48 h, cells were treated with MST-8 as described earlier. Spectrometric absorbance at 450 nm was measured with a micro-plate reader. Each group was repeated at least three times.

**In vivo tumor progression assay.** Tumor xenografts were established by subcutaneous injection of 5x10^6 HT29, HT29/shRNA-control, HT29/shRNA cells into the right flank of 4-6-week-old female nude mice, respectively. The experiments were approved by the Experimental Animal Center of University of Yangzhou, Yangzhou, China. All animals were euthanized after 30 days post-inoculation. Harvested tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with H&E. Immunohistochemistry analysis used goat anti-mouse CD147 polyclonal antibody (1:50 dilution, Santa Cruz Biotechnology) to detect CD147 protein expression. Animal experiments were performed in accordance with institutional guidelines for animal care by Nanjing Medical University.

**Statistical analysis.** Statistical analysis was performed by the SPSS software. Each assay was conducted at least three times. All experimental data were expressed as the mean ± SD and assessed by Student’s t-tests and one-way ANOVA at a significance level of p<0.05.
Results

Selection of pYr-mir30-shRNA stable expression transfectants. In our study, we established two vectors, the pYr-mir30-shRNA-control and the pYr-mir30-shRNA. After 48 h of transfect, the transfect cells were treated with 600 μg/ml G418 selection. After two weeks, the stable expression of HT29/shRNA and HT29/shRNA-control cells were obtained. The EGFP expression could be clearly observed as shown in Fig. 1.

The pYr-mir30-shRNA-mediated gene silencing inhibits CD147 expression in HT29 cells. To evaluate the expression of CD147 in HT29 cells, we used the real-time RT-PCR (RT-PCR) and western blotting. The gene of β-actin was selected as the internal gene. A significantly reduced CD147 mRNA level for HT29/shRNA was achieved compared with untreated HT29 cells, respectively (p<0.01) (Fig. 2). In addition, western blot analysis confirmed the downregulation of CD147 protein by the HT29/shRNA (Fig. 2).
The CD147 silencing inhibits MCT1 and MCT4 expression in HT29 cells. Many studies have confirmed the expression of MCT1 and MCT4 are closely associated with CD147 in various cancers. To detect whether the CD147 silencing could reduce the expression of MCT1 and MCT4, we performed real-time RT-PCR and western blotting. The real-time RT-PCR analysis contrasted with the HT29/shRNA-control, the HT29/shRNA mRNA expression of MCT1 was downregulated (p<0.01), but the MCT4 mRNA expression did not significantly change in HT29 cells (p>0.05) (Fig. 2). In addition, western blot analysis demonstrated that the MCT1 protein was downregulation by HT29/shRNA (p<0.01), but the MCT4 protein did not significantly alter in HT29 cells (p>0.05) (Fig. 2).

The CD147 silencing reduces the activity of MMP-2 and MMP-9 in HT29 cells. CD147 contributed to tumor invasion and metastasis by stimulating fibroblasts matrix metalloproteinase production. We used gelatin zymography to investigate the effect of CD147 silencing on HT29 cells reducing the activity of MMP-2 and MMP-9. As shown in Fig. 3, the secretion levels of MMP-2 and MMP-9 in the HT29/shRNA cells show significant difference in the HT29 cells (p<0.01). There was no significant difference between HT29 and HT29/shRNA-control (p>0.05).

The CD147 silencing inhibits the function of lactate transporters in HT29 cells. We examined whether CD147 silencing inhibited the function of lactate transporters used in the lactic acid assay. As shown in Fig. 4, the intracellular lactate concentration of the HT29 cells were increasing after CD147 silencing. There was no significant difference between HT29/shRNA-control and HT29 cells (p>0.05). These results confirmed that the downregulation of MCT1 expression by HT29/shRNA inhibits the function of these transporters in HT29 cells. Thus, the decrease of MCT1 expression is associated with an increase in intracellular lactate concentration.

The CD147 silencing reduces the proliferation of HT29 cells. In order to examine whether the CD147 silencing affects
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Cell proliferation, we used the WST-8 assay to determine the proliferation of HT29, HT29/shRNA-control and HT29/shRNA, respectively. The results (Fig. 5), compared with HT29, show the proliferation of HT29/shRNA was inhibited to 51.03 (p<0.01), 54.26 (p<0.01), 51.92 (p<0.01), 69.94 (p<0.01) and 72.24% (p<0.01) at 24, 48, 72, 96 and 120 h, respectively. There was no significant difference between HT29 and HT29/shRNA-control (p>0.05).

CD147 silencing reduces the invasive ability of HT29 cells in vitro. To examine whether the downregulation of CD147 in HT29 cells affected its invasive ability, we performed a Matrigel Transwell analysis in vitro. The results showed that HT29 and HT29/shRNA-control cells had a similar ability to pass through the Matrigel coated filter (Fig. 6). The number of HT29/shRNA cells passing through the Matrigel was markedly lower than the numbers of HT29 and HT29/shRNA-control cells (p<0.05).

The CD147 silencing increases the sensitivity to chemotherapeutic drugs in HT29 cells. CD147 was found to be overexpressed in multidrug resistance tumor cells and could confer resistance to some antitumor drugs. In order to test whether CD147 silencing affected its sensitivity to chemotherapeutic drugs in HT29 cells, we used WST-8 assay to investigate the sensitivity of HT29 cells to the antitumor drug. As shown in Fig. 7, the chemosensitivity of HT29 cells was significantly increased to the antitumor drug cisplatin (p<0.01), whereas to paclitaxel, gemcitabine or oxaliplatin, there was no significant difference between the HT29/shRNA and HT29/shRNA-control after CD147 silencing (p>0.05).

CD147 silencing inhibited the tumor formation of HT29 cells in vivo. Using RNA interference can effectively reduce the proliferative ability of HT29 colorectal cancer cells in vitro, and we investigated its efficacy in vivo. From the date of vaccination, and every 5 days, we measured the tumor length and width and calculated their volume. As shown in Fig. 8A, the tumor growth was slower in the HT29/shRNA group than that in other groups (p<0.01) and there was no significant difference between HT29 and HT29/shRNA-control (p>0.05).

![Figure 5](image)

Figure 5. Proliferation of HT29 cells stably expressing shRNA. The proliferation of HT29 cells (HT29, HT29/shRNA-control, HT29/shRNA) was analyzed by WST-8 assay. The spectrometric absorbance at 450 nm was measured using a microplate reader. Each group contained three wells and the experiments were repeat three times (see Materials and methods). *p<0.01 compared with HT29.

![Figure 6](image)

Figure 6. Invasive ability of HT29 cells on Matrigel after CD147 silencing. (A) Crystal violet staining results of lower surface filters show the cells invading the Matrigel (x400). (B) The number of cells that invaded through the chamber was evaluated in 3 fields for each experimental group and averaged. The invading cells of each experimental group was counted as the average of the sum of 10 fields of vision under a microscope. *p<0.05 compared with HT29.
difference between HT29 and HT29/shRNA-control group (p>0.05). Hematoxylin and eosin (H&E)-stained examination did not reveal obvious morphological changes among the tumors generated from the three groups, but there were larger areas of necrosis in the tumors formed by injecting with HT29/shRNA cells (Fig. 8B). Immunohistochemistry analysis showed that CD147 protein expression was high in HT29 and HT29/shRNA-control group mice, but was very low in tumors treated with HT29/shRNA (p<0.01) (Fig. 8B).

**Discussion**

CD147 was reported to be more highly expressed on the surface of most human carcinoma cells, and correlated with tumor progression and invasion by stimulating peritumoral fibroblasts to produce elevated levels of several MMPs (25). The present results showed that CD147 silencing resulted in a clear reduction of MMP-2 and MMP-9 expression in colorectal cancer cells, supporting the concept that CD147 was associated with increased expression of MMP-2 and MMP-9 (26). Data have suggested that CD147 stimulated the synthesis of specific MMPs to participate in tumor progression through peritumor fibroblasts (15). MMPs are believed to play important roles in disrupting the balance between growth and anti-growth signals in the tumor microenvironment (27,28). Previous study reported that MMP-2 and MMP-9 were potential prognostic biomarkers of colorectal cancer (29). Development of a new generation of selective inhibitors of MMPs through the pharmacological targeting to colorectal cancer is a promising and challenging area for future research (30). In the present study, we also detected the HT29 cell invasion ability changes using transwell. The results showed that inhibition of CD147 expression reduced the ability of invasion in HT29 cells. The possible mechanism was that CD147 silencing inhibited the secretion of MMPs. In

CD147 is a multifunctional glycoprotein forming homooligomers in a cis-dependent manner in the plasma membrane (24). Based on the high CD147 expression reported in colorectal cancer and the association with colorectal development, we constructed an RNA interference vector, the pYr-mir30-shRNA. We used this vector to decline the CD147 protein expression was high in HT29 and HT29/shRNA-control group mice, but was very low in tumors treated with HT29/shRNA (p<0.01) (Fig. 8B).
order to make the results of the experiment more convincing, we carried out animal experiments. The nude mouse experiments showed that inhibition of CD147 expression reduced colorectal cell tumorigenicity. Immunohistochemical staining suggested that CD147 protein expression was slightly detected in tumor derived from HT29/shRNA cells. These results proved that CD147 silencing could reduce the HT29 cell tumorigenicity, invasion and once again confirmed that the MMPs were associated with cell invasion.

CD147 was able to interact with certain lactate transporters (MCT1 and MCT4) and facilitate their expression on the cell surface. The present results showed that CD147 silencing resulted in a clear reduction of MCT1 expression, supporting the concept that CD147 was an ancillary protein required for the expression of these MCTs (21). Further evidence has demonstrated that the levels of CD147 in the plasma membrane were controlled by silencing or overexpressing of MCT4 (31). Our results showed that the CD147 silencing resulted in a significant reduction of MCT1, but the expression of MCT4 protein did not significantly change in colorectal cancer cells. CD147 inserted H+/lactate symporters, MCTs, in many organizations control the stability and function of plasma membrane, which played a determinant role in metabolic energy (32). In our study, CD147 silencing was able to increase the lactate concentration which might be due to the CD147 silencing leading to the MCT1 protein reduction, and the increase in lactate concentration might reduce the cell growth or other tumor-associated biological activities.

Multidrug resistance (MDR) occurred in tumor cells, tumor stem cells and tumor metastases, which is the main cause of failure in cancer therapy, and upregulated CD147 was observed in many MDR cancer cells (22). In colorectal cancer patients, MDR was also an important cause of treatment failure and mortality. In many different ways, the protein of CD147 could regulate the chemosensitivity of certain chemotherapeutic drugs (33,34). The anticancer drugs cisplatin, paclitaxel, oxaliplatin and gemcitabine are widely used, and potent in head and neck cancer, and also often used in treatment of colorectal cancer.
cancer. In the present study, the results revealed that CD147 silencing increased the chemosensitivity to cisplatin, but not to gemcitabine, oxaliplatin or paclitaxel in human colorectal cancer cell line HT29, suggesting that CD147 was an adjuvant chemotherapy target for colorectal cancer. Cisplatin was shown to be first efficacious compound in the treatment of colorectal cancer, and oxaliplatin consistently exerted antitumor activity in colorectal cancer (35,36), the specific molecular mechanism of resistance to cisplatin are unclear, but several mechanisms of the resistance to cisplatin were proposed, such as reducing drug uptake, increasing drug inactivation, increasing DNA adduct repair and defecting apoptotic response, and we will investigate these mechanisms in the future.

In conclusion, CD147 silencing by RNAi inhibited the proliferation and invasion of cancer cells. The possible mechanism was that CD147 silencing inhibited the MCT1 protein expression, resulting in increased intracellular lactic acid and inhibition of cell proliferation. However, CD147 silencing suppressed the secretion of MMP proteins, thereby inhibited tumor cell invasion and metastasis. CD147 is a key regulator of the multidrug resistance of human colorectal cancer cell line HT29. The results of this study provide new ideas for potential strategies of gene target therapy in colorectal cancer.

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References