RNA interference targeting extracellular matrix metalloproteinase inducer (CD147) inhibits growth and increases chemosensitivity in human cervical cancer cells

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Summary

Overexpression of extracellular matrix metalloproteinase (MMP) inducer (EMMPRIN CD147) has been implicated in the growth and survival of malignant cells. However, its presence and role in cervical cancer cells has not been well-studied. In the present study, small interfering RNA (siRNA) was designed and synthesized to breakdown the expression of CD147. The present data demonstrated that 24 and 48 hours after transfecting CD147 siRNA, both the CD147 mRNA and protein expression were significantly inhibited as determined by quantitative real-time polymerase chain reaction (RT-PCR) and immunocytochemistry. Meanwhile, simultaneous silencing of CD147 resulted in distinctly increasing MMP-9, VEGF, and MDR-1. Further studies demonstrated decreased CD147 expression, resulted in G1/S phase transition with flow cytometry analysis, as well as the resistance of the cells to 5-FU. These findings provide further evidence that CD147 may become a promising therapeutic target for human cervical cancer and a potential chemotherapy-sensitizing agent.

Key words: Endometrial carcinoma; Clear cell carcinoma; Solitary bone metastasis; Treatment.

Introduction

Cervical cancer takes the lives of more than 250,000 women annually worldwide, particularly in under-resourced areas of low-, middle-, and high-income countries [1]. Despite improved understanding of the pathogenesis of this malignancy, patients with advanced cervical cancer still have a poor prognosis despite undergoing conventional therapy with significant side-effects, and the treatment outcome for cervical cancer remains poor. Only modest improvements in survival have been reported and these are attributed mainly to earlier diagnosis [2]. Thus, novel therapies for cervical cancer are greatly needed.

Extracellular matrix metalloproteinase (MMP) inducer (EMMPRIN CD147) is a highly-glycosylated transmembrane protein of 60 kDa with an ectodomain consisting of two regions exhibiting the characteristics of the immunoglobulin superfamily [3]. CD147 is enriched on the surface of tumor cells and stimulates adjacent stromal cells to produce several MMPs [4]. Tumor invasion and metastasis are complicated multi-step processes. Among the requirements is degradation or remodelling of extracellular matrix and basement membrane macromolecules by proteolytic enzymes, among which MMPs are particularly implicated [5]. Elevated CD147 stimulates MMP production in stromal fibroblasts and endothelial cells, leading to extracellular matrix degradation, tumor growth promotion, and metastasis [6]. CD147 is reported to be involved in the progression of malignancies by regulating expression of VEGFs in stromal cells, as well as in tumor cells themselves. CD147 also stimulates the expression of vascular endothelial growth factor (VEGF) and contributes to genesis, growth, and local invasion of malignant cells [7, 8]. The CD147 is overexpressed in multidrug resistant (MDR) cancer cell lines, suggesting that during the development of a multidrug resistance phenotype, the expression of CD147 stimulates MMP activity in MDR cells [9].

The discovery of interfering RNA (iRNA) has generated enthusiasm within the scientific community, not only because it has been used to rapidly identify key molecules involved in many disease processes including cancer, but also because iRNA has the potential to be translated into a technology with major therapeutic applications [10]. These tools have helped to delineate the roles of various cellular factors in oncogenesis and tumor suppression and lay the foundation for new approaches in gene discovery. Furthermore, successful inhibition of tumor cell growth by iRNA aimed at oncogenes in vitro and in vivo supports the enthusiasm for potential therapeutic applications of this technique [11].

Overexpressed biomarkers are of special interest because they may not only be used to predict patient outcome, but may also serve as potential targets in cancer therapy. Extracellular MMP inducer may be one of them [12]. All of this prompted the authors to investigate the putative role of CD147 as a target for anticancer therapy; therefore in this study, three pairs of small interfering RNA (siRNA) were designed directly to down-regulate the expression of CD147 according to its sequence in the Genebank (Accession NM_001728.2) and influences to cell proliferation and chemosensitivity with breakdown of CD147 expression by siRNA as detected in Hela cell line.

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Materials and Methods

Materials and reagent

Bovine serum, methyl thiazolyl tetrazolium(MTT), and dimethyl sulfoxide (DMSO) were purchased. Polymerase chain reaction (PCR) primers were synthesized. Human breast cancer MCF-7 cell originated from Lanzhou University.

Design and synthesis of siRNA

According to human CD147 gene sequence, three small interference sequence BSG1, BSG2, and BSG3 and negative control non-silencing non-BSG were designed and chemically synthesized. All nucleotide sequences of siRNA are shown in Table 1.

Cell culture and siRNA transfection

Human cervical cancer cell lines Hela cells were investigated in this study and cultured in DMEM medium supplemented with 10% bovine serum, 2.05 mM of L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin at 37°C with 5% CO_2. Cells were seeded in a six-well plate at a concentration of 1×10^6 cells/well and allowed overnight growth to reach 80%-90% confluency. Cells were then transfected with different small interference sequences BSG1, BSG2, BSG3, and non-BSG following the protocol set by the manufacturer, and the final concentration of siRNA was 100 nmol/l.

MTT assay

To quantify cell proliferation, cells were seeded in a 96-well culture plates at an optimal density of 5×10^3 cells per well in triplicate wells. Fifty µl per well with five mg/ml MTT solution were added at 48 hour post-transfection. Then, the medium was removed by centrifugation after four hours of incubation at 37°C, 5% CO_2 incubator, and the blue formazan product converted from MTT was dissolved by the addition of 150 µl/well DMSO. The plates were vibrated at room temperature for ten min. The absorbance of each well at 570 nm was read on enzyme-linked immunosorbent assay reader. The cell inhibition ratio calculated as [1-A490 (5-FU/BSG)/A490 (5-FU)] × 100% [13].

Reverse transcription-PCR

Cells were collected and total cellular RNA was extracted using Trizol reagent kit according to manufacturer's instructions. The purity and amount of total RNA were determined using ultraviolet (UV) spectrophotometry. The isolated RNA was converted into cDNA using a reverse transcription kit. The primers corresponding to human CD147, MMP-9, VEGF, MDR-1, and β-actin are shown in Table 2.

In this study, two µl of follow board, one µl of sense, and antisense primers, 2.5 µl of $MgCl_2$ (25 mmol/l), one µl of deoxynucleoside triphosphates, 2.5 µl of 10 × PCR buffer, five units of Taq DNA polymerase, and 14 µl of double-distilled water were used in each PCR reaction in a 20 µl reaction volume. RT-PCR for analysis CD147 was conducted with the following parameters: one four-minute cycle at 94°C, 36 cycles at 94°C for 50 seconds, 65°C for one minute, 72°C for 45 seconds; final extension was at 72°C for ten minutes; moreover, the annealing temperature for analysis VEGF and MDR-1 was regulated respectively at 62°C and 68°C. PCR products were identified with electrophoresis on 1.5% agarose gels and the gray scale ratio was calculated.

Immunocytochemistry

Hela cells were seeded at a density of 1×10^5 cells/well into six-well plates and were transfected with siRNA sequence. Cells were allowed to attach to precoated glass coverslips and fixed the following day in 4% paraformaldehyde and then washed twice with PBS (phosphate-buffered saline), and incubated for another 20 min with 0.5% Triton X-100. Film preparation was washed with DPBS (Dulbecco's phosphate-buffered saline). Following brief proteolytic digestion and blocking of peroxidase, cell slides were incubated overnight with the primary antibody against the respective target protein at a dilution of 1:100 at 4°C. After washing, peroxidase-labelled polymer and substrate-chromogen were then employed to visualize the staining of the interesting proteins targeted.

Table 1. — *Sequences of siRNA.*

Oligonucleoitide	Sequence (5'-3')
BSG1 Sense siRNA strand	CGUCAGAACACAUCAACGATT
BSG1 Antisense siRNA strand	UCGUUGAUGUGUUCUGACGTT
BSG2 Sense siRNA strand	CCUGGUACAAGAUCACUGATT
BSG2 Antisense siRNA strand	UCAGUGAUCUUGUACCAGGTT
BSG3 Sense siRNA strand	UCCAAGUUCUCACCUCUUATT
BSG3 Antisense siRNA strand	UAAGAGGUGAGAACUUGGATT
non-BSG Sense siRNA strand	CCAGGACACAUAUAAGCACTT
non-BSG Antisense siRNA strand	GUGCUUAUAUGUGUCCGGGTT

Table 2. — *Sequences of primer.*

Name	Primer	Sequence	Length (bp)
CD147	Upper	5'-GAGTACTCCTGCGTCTTCC-3'	692
	Lower	5'-CCGGCGCTTCTCGTAGATG-3'	
β-actin	Upper	5'-CTCCATCCTGGCCTCGCTGT-3'	268
	Lower	5'-GCTGTCACCTTCACCGTTCC-3'	
VEGF	Upper	5'-AACCAGCAGAAAGAGGAAAGAGG-3'	192
	Lower	5'-CCAAAAGCAGGTCACTCACTTTG-3'	
MMP-9	Upper	5'-CCC GGA CCA AGG ATA CAG-3'	650
	Lower	5'-GGC TTT CTC TCG GTA CTG-3'	
MDR-1	Upper	5'-AGGCCAACATACATGCCTTC-3'	404
	Lower	5'-GCTCCTTGACTCTGCCATTC-3'	

Flow cytometric

For flow cytometer analysis, cells were seeded in a 24-well plate at a density of 5×10^4 cells/well and incubated at 37°C, 5% CO_2 incubator, then harvested and washed twice with PBS, fixed with ice-cold 70% ethanol at -20°C overnight at 48h post-transfection. Cells were then washed with PBS for three times and stained with propidium iodide (PI) (20 mg/ml) in the dark at room temperature for 20 min. Cell cycle analysis was done with FAC station equipped with Cell Quest, and the cell cycle phase distribution was calculated from the resultant DNA histogram using Multicycle AV software. Each group detected in triplicate experiments and mean were calculated [14].

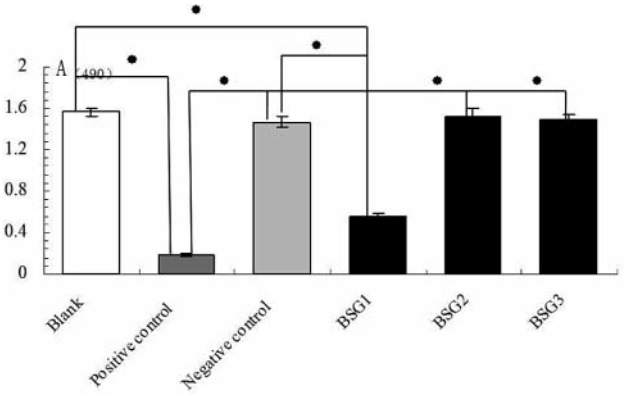
Drug sensitivity

To assess the effect on chemosensitivity to 5-FU of siRNA sequences, siRNA tansfected cells (5×10^3/well) were cultured for six hour in 96-well plates, and the culture solution with was replaced a fresh one. Then 5FU (20 µl/ml) were added and incubated for another 48 hours. Cells were treated with MTT as described earlier. Each group contained five wells. The cell survival inhibition ratio calculated as [1-A490 (5-FU/BSG)/ A490 (5-FU)] × 100% [15].

Statistical analysis

Results are expressed as means ± standard deviation (SD). Statistical analyses were performed using SPSS statistical software (SPSS17.0). Student's t-test was used for comparison between two groups. Significance was defined as $p < 0.05$.

Fig. 1A



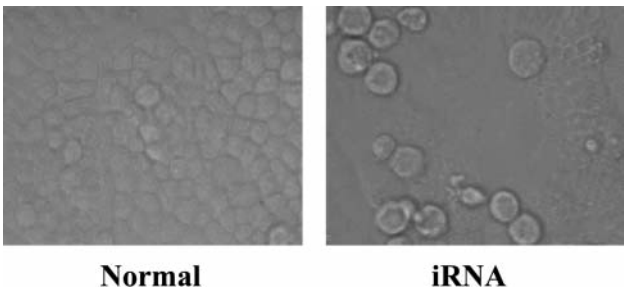


Fig. 1B

Figure 1. — Effects of siRNA-CD147 on cervical cancer Hela cells proliferation. Figure 1A shows the effect of siRNA-CD147 on Hela cell proliferation and was assessed by the MTT assay and each group contained three wells. * means $p < 0.05$. Figure 1B shows the effect of siRNA-CD147 on cell modality, Compared with controls, some distinctions, as shrinkage, megaly, and vacuole on cells surface were distinguished in BSG1 group.

Results

Breakdown of CD147 significant inhibition of cell proliferation

Overexpression of CD147 is known to stimulate cell growth. To assess the effects of CD147 breakdown in cell proliferation, three siRNA BSG1-3 were chemically synthesized to examine proliferation rate of cells and screen better interference sequence. After 48 hours of transfection, compared with the negative group, the CD147-siRNA BSG1 significantly decreased the growth rate of cervical cancer Hela cells ($p < 0.05$). The cell inhibition rate of tumor growth achieved to 56%. In contrast, there was little effect for BSG2, BSG3 (Figure 1A). The effects on cell modality of transfected interference sequence for 48 hours were observed by an inverted microscope. The authors found that cells of control non-BSG groups grew well, while CD147 silenced BSG1 groups exhibited a worse growth state. Compared with controls, some distinctions, as shrinkage, megaly, and vacuole on cells surface, were distinguished in BSG1 group. The distinctions are shown in Figure 1B.

iRNA-mediated inhibition of CD147 mRNA and protein levels in cervical cancer cells

The authors adopted siRNA to break down the expression of CD147 in cervical cancer cells, and transfection of the CD147-siRNA into cervical cancer cells led to a remarkable inhibition of CD147 mRNA expression by quantitative RT-PCR. In terms of brightness of the bands with analysis with BANDSCAN 5.0 software, the blank control and β-actin groups exhibited stronger expression than did BSG1 groups (Figures 2A, B).

The expression of CD147 protein in cervical cancer cells transfected with CD147-siRNA was strongly suppressed. Immunocytochemistry staining analysis demonstrated that buffy masculine marker located on the endochylema and cell membrane and distributed dispersedly after 72 hours transfection. Compared with the blank group cells, chromatosis extent of BSG1 group cells is more identifiable. In contrast, there were little changes for BSG2, BSG3 in the expression of CD147 protein. The said delineation demonstrated that CD147 protein is down-regulated in the transfection, with which BSG transfected (Figure 2C).

Breakdown of CD147 blocked the cervical cancer cell proliferation

To further confirm that the breakdown of CD147 on cell proliferation, cell cycle was also detected with flow cytometry. When the expression of CD147 gene was silenced, there was an obvious change of cell cycle distribute in cervical cancer Hela cells (shown in Figure 3), and the number of cells was increased significantly in G0/G1 phase from 53.73 ± 1.4% to 86.33 ± 2.3%, and those in S and G2/M phases were reduced sharply from 46.27 ± 1.9% to 13.67 ± 1.1%. In the silencing group, the proportion of G1 phase cells increased, however, the proportion of S phase cells reduced significantly, indicating the cell proliferation index was significantly reduced. There was no significant change in the fluorescence control group and in the blank group.

Down-regulation of CD147 reduced mRNA expression of MMP-9, VEGF and MDR-1 in cervical cancer cell

To verify whether the invasion, metastasis-related genes, and multi-drug resistance (MMP-9, VEGF, and MDR-1) was suppressed by siRNA against CD147(CD147-siRNA), the authors determined the mRNA levels of these genes in tumor tissues in cervical cancer cell by quantitative real-time RT-PCR, using specific primers and probes for MMP-9, VEGF, and MDR-1 with β-actin as internal control. The relative quantification results show that tumor cells transfected with CD147-siRNA-BSG1 significantly reduced the mRNA levels of

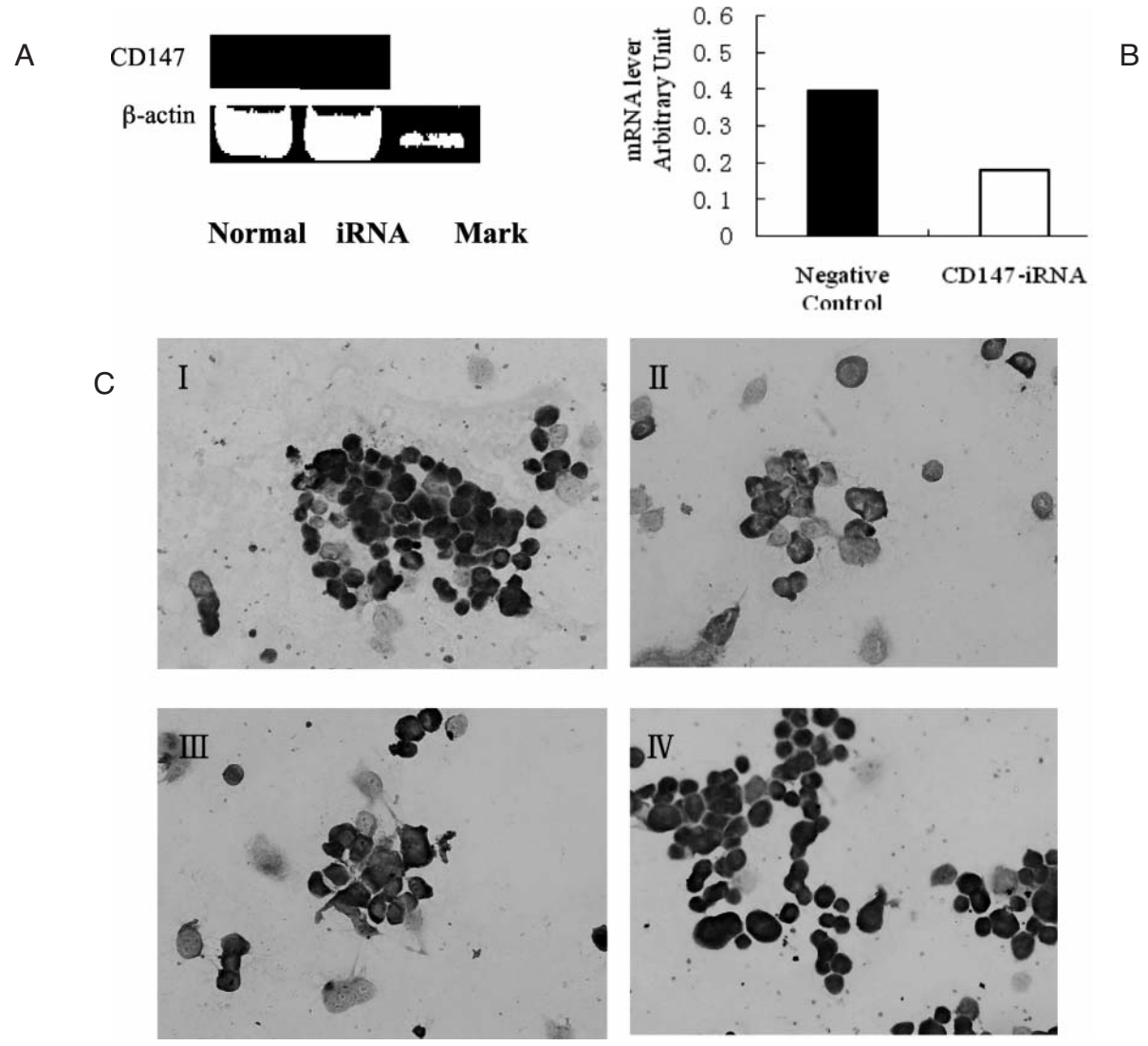


Figure 2. — Suppression of CD147 mRNA and protein levels in cervical cancer cells by siRNA. Figure 2A shows iRNA effects of siRNA-CD147 in human cervical cancer cells by analysis with RT-PCR. Figure 2B shows the signal intensity of all band analysed with bioinformatics software. Figure 2C: iRNA effect on CD147 protein expression evaluated by immunocytochemistry. I: represents Hela/non-BSG; II: represents Hela/BSG1, III: represents Hela/BSG2; IV: represents Hela/BSG3. Experiments were designed with non-silencing siRNA as negative group (100 nmol/l non-BSG) and siRNA-CD147 groups (100 nmol/l BSG1, BSG2 and BSG3).

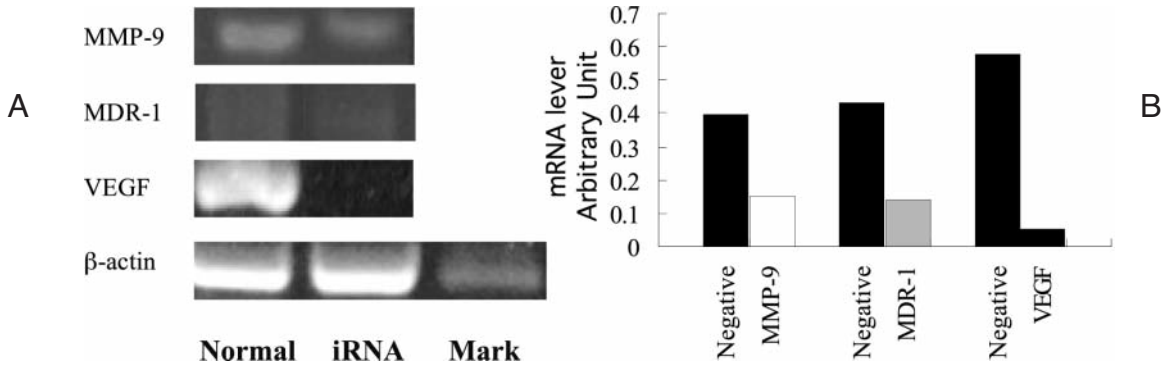


Figure 3. — The effects of down-regulation of CD147 on mRNA expression of MMP-9, VEGF, and MDR-1 in cervical cancer cell. Figure 3A: mRNA expression of MMP-9, VEGF and MDR-1 was analyzed with RT-PCR and the content of the band was evaluated with BANDSCAN 5.0 software. Figure 3B: The signal intensity of b-actin had no discernible change between the BSG1 group and negative control non-BSG group. Compared with non-BSG group, signals intensity of MMP-9, MDR-1, and VEGF were distinctly weakened.

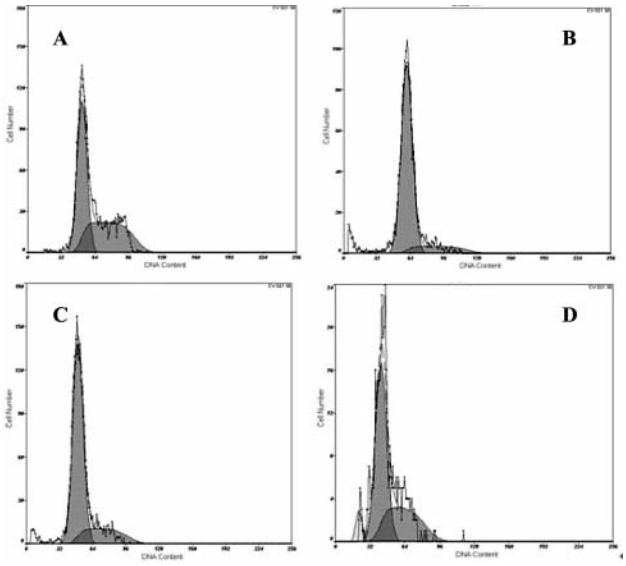


Figure 4. — Flow cytometric analysis of the cell cycle. A: represents Hela/non-BSG; B: represents Hela/BSG1, C: represents Hela/BSG2, D: represents Hela/BSG3.

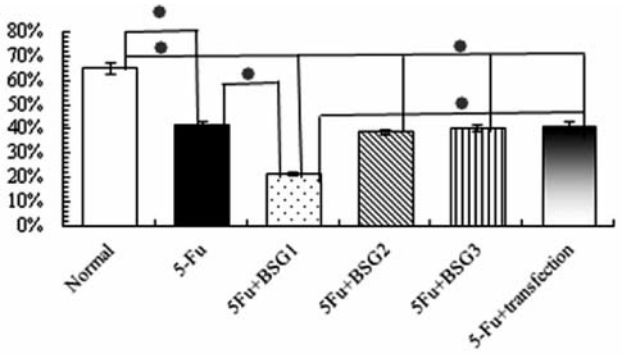


Figure 5. — Effects of siRNA-CD147 on chemosensitivity of cervical cancer cell. Drug sensitivity calculation for the cell survival inhibition ratio, of negative group (5FU and EntransterTM-R) was 36%, while inhibition ratio of siRNA BSG1 groups (5FU and 25 nmol/l BSG1) was achieved in 67%. * $p < 0.05$.

MMP-9, VEGF, and MDR-1, compared to those in cervical cancer cell negative groups (shown in Figures 4A, 4B).

Down-regulation of CD147 enhanced the chemosensitivity of cervical cancer cell

Hela, Hela-negative iRNA, and Hela-CD147-iRNA cells were treated with 5-FU to determine chemosensitivity. As shown in Figure 5, 5-FU reduced the growth of all cell lines. Compared with blank and negative RNAi cells, BSG1-RNAi cells displayed increased sensitivity to 5-FU ($p < 0.05$), especially at 25 nmol/l, which inhibition ratio of siRNA BSG1 groups was achieved in 67%. The iRNA mediated CD147 down-regulation may synergistically enhance the cytotoxicity of 5-FU.

Discussion

As a tumor-derived MMP inducer, CD147 stimulates fibroblast and endothelial cells to facilitate tumor invasion, metastasis, and angiogenesis [16]. The interaction between CD147 and MMPs have been reported [17]. Expression of CD147 was enhanced in a variety of human carcinomas and correlated with tumor progression and invasion by inducing the production of MMPs by stromal cells [18]. Special attention was devoted to MMP-2 and MMP-9 enzymes. Host-derived MMP-9 contributes to tumor incidence and proliferation in a model of skin carcinogenesis [19]. The silencing of CD147 by siRNA resulted in the decreased proliferation and invasion of A375 cells and the expression of VEGF, constitutively elevated in these cells, were down-regulated in vitro [20]. Study of Yang [21] showed that breakdown of CD147 on viability of cells grown as attached monolayer or suspension culture core was different and breakdown of CD147 could inhibit cancer cell survival by regulating intercellular contacts and promote anoikis. The result about RNAi inhibited expression of CD147 in Hela cells for the first time was related here. Expression level of MMP-9 mRNA was down-regulated on account of silencing CD147 via RNA interference. Besides, the proliferation was inhibited remarkably in Hela cell line.

There are some essential features for malignant tumors, such as immortalized cellular proliferation, activated invasiveness into the surrounding stroma, distant metastasis, and angiogenesis via VEGF production [22]. Cancer cells acquire resistance to anti-cancer drugs to draw assistance from the MDR phenotype frequently. Distant metastasis and MDR, the major obstacles to the effective treatment of malignant tumors, remain to be overcome [23].

VEGF, a homodimeric lycoprotein of the platelet-derived growth factor family, plays a pivotal role in tumor angiogenesis and lymphangiogenesis which are crucial for tumor growth, invasion, metastasis [24] and VEGF production and affects the outcome in patients with tumors [25, 26]. Numerous clinical studies have demonstrated that the elevated expression of VEGF is strongly correlated with the density of tumor microvessels, the potential for malignancy, and a negative patient prognosis [27]. In this data, not only was the expression of the CD147 gene and protein suppressed in cervical cancer cells, but also the expression of VEGF. The deduction that expression of CD147 was positively correlated with the expression of VEGF in cervical cancer cells needs to be confirmed.

Chemotherapy is highly-effective in treating a number of gynecologic malignancies; however, its effectiveness often diminishes with repeated exposure due to the emergence of MDR [28]. Overexpression of CD147 promotes

tumor cells metastasis and confers the MDR to P-gp substrate drugs in breast cancer cells MCF-7 [29]. Analogical dependability was reported in multidrug resistant cancers cells MCF-7, AdrR, KBV-1, and A2780Dx5 [30-33]. The fluoropyrimidine drug 5-fluorouracil (5-FU) is widely used in the treatment of gastrointestinal, breast, head, and cervical cancer [34, 35]. In fact, the combination of 5-FU with other anticancer agents, such as cisplatin as a neoadjuvant chemotherapy, has improved the response rate for cervical cancer [36]. However, some patients have a poor response to 5-FU-based chemotherapy. In the present study, down-regulation of CD147, which provoked MDR-1 expression degression, not only influenced proliferation and apoptosis, but also increased chemosensitivity to 5-FU in Hela cells. This suggests that CD147 can protect pancreatic cancer cells from chemotherapy-induced apoptosis and CD147-iRNA break down inhibit the protective effect of increasing chemosensitivity. This result showed that CD147 is an adjuvant chemotherapy target of tumor.

The FCM result shows that the cell proliferation index in CD147 siRNA transfected cervical cancer Hela cells was decreased significantly. However, there was no significant change in the fluorescence control and blank group, which indicated that the cell cycle of Hela cells was inhibited while cells in quiescent stage increased. That is, the proportion of G1 phase cells increased, however, the proportion of S phase cells reduced significantly in the silencing group [37].

In mammals, siRNA is expected to become a powerful tool, not only for large-scale gene silencing essential for functional genomics, but also for therapeutic purposes, including anti-cancer treatments [38, 39]. The successful employment of an iRNA-based gene breakdown technique depends on the proper design or selection of the siRNAs, and the adoption of an effective strategy to deliver the siRNAs to the target cells or tissues [40], since it was discovered that not all siRNAs are equally potent in their ability to silence the gene products [41]. Although design algorithms have been improved over the last few years, there is still a risk that not all siRNAs chosen will result in significant break down within the given experimental setting [42]. Therefore, performance of each siRNA must still be proven experimentally when selecting the most efficient siRNA for loss-of-function studies.

In conclusion, the down-regulation of CD147 using iRNA successfully reduced cervical cancer Hela cells proliferation in vitro. These findings provide further evidences of involvement of CD147 in a variety of cancer key cellular events as a versatile signaling orchestrator, and suggest that CD147 would be a promising gene-targeting therapy for cervical cancer.

Acknowledgments

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