Original Article
CD147 promotes cervical cancer migration and invasion by up-regulating fatty acid synthase expression

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Abstract: CD147 is a transmembrane glycoprotein that when highly expressed contributes to tumor progression. In the present study, we investigate the clinical relevance of CD147 expression in CCSC tissues and evaluate the association between CD147 expression and cervical lymph node metastasis; CD147 was detected using immunohistochemistry. To functionally analyze the role of CD147 in CCSC cell lines in vitro, SiHa cells were employed, whose endogenous CD147 was artificially downregulated, by using lentiviral-based transfection. Moreover, we have confirmed that knockdown of CD147 led to reduced levels of cellular lipid content in shCD147 cells by BODIPY staining. Cell invasion and migration were analyzed using transwell assays and wound healing. Angiogenesis and lymphangiogenesis were assessed by an endothelial cell tube formation assay. Our data showed that highly expressed CD147 up-regulated the major lipogenic genes, FAS and ACC1 to promote de novo lipogenesis, and knockdown of CD147 significantly inhibited the migration and invasion of CSCC cells. The culture supernatants of CD147 knockdown cells significantly inhibited vascular and lymphatic endothelial cell tube formation. Our results suggest that CD147-mediated FAS and ACC1 overexpression are major regulators of cervical cancer growth and metastasis.

Keywords: Cervical cancer, CD147, fatty acid synthase, migration, invasion

Introduction
De novo lipogenesis has been increasingly recognized to be involved in oncogenesis [1]. Reports have shown that continuous de novo lipogenesis provides cancer cells with lipids and lipid precursors for membranes formation and lipid-based post-translational modification of proteins to support rapid growth of tumor cells [2, 3]. Further evidence for the de novo lipogenesis in tumors came from the observation that fatty acid synthase (FAS) as a key enzymes involved in de novo fatty acid synthesis is overexpressed in breast cancer independent of the amount of circulating lipids [4]. Subsequent researches have demonstrated that FAS is overexpressed in many types of cancers, including lung cancer, ovarian, and esophageal cancer [5-7]. However, the underlying mechanisms for the regulation of de novo lipogenesis in cancers are still not completely understood. Therefore, it is necessary to investigate the lipid metabolism in cancer.

CD147, also named as the extracellular matrix metalloproteinase inducer (EMMPRIN), is a transmembrane glycoprotein, is highly expressed in various human cancers, including cervical carcinoma [8, 9]. Multiple independent laboratories demonstrated that the greatly expressed of CD147 expression significantly contributes to tumor growth, metastasis and angiogenesis [10, 11]. Previous studies have also reported correlations between the expression levels of CD147 and de novo lipogenesis in hepatocellular carcinoma [12, 13].

Cervical cancer ranks as the 4th most frequently diagnosed cancer and the 4th leading cause of cancer death in females globally [14]. In China, this disease ranks second in incidence and mortality behind breast cancer, despite the existence of effective screening methods. Although surgical techniques and perioperative treatments have been advanced, the prognosis of CSCC still remains poor due to the local recurrence and distant metastasis
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One would ask whether CD147 would modulate fatty acid metabolism by upregulate lipogenic enzymes (FAS and ACC1) and lead to the development of cervical cancer likewise as a critical regulator in other types of cancer.

In the present study, we examined the association of CD147 and lipogenic enzymes (FAS and ACC1) expression with cervical cancer tissues and two different cervical cancer cell lines, C33a, and SiHa. Furthermore, we were performing experiments using two different CC cell lines to investigate whether alter expression of FAS and ACC1 and increase the lipogenesis with different CD147 expression levels. Moreover, the proliferation, migration and invasion of cervical cancer cells activated by CD147 were also investigated.

Materials and methods

Patients

A total of 85 patients with previously untreated, clinically gynecological diagnosed was performed in all cervical cancer patients for staging in accordance with the International Federation of Gynecology and Obstetrics (FIGO) criteria and pathologically confirmed, who underwent surgery between June 2015 and December 2018 at the Department of Gynecology in the First Affiliated Hospital in Xinjiang Medical University, were retrospectively enrolled in the present study. The patients ranged in age from 36 to 71 years (median, 53.6 years; Table 1).

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Regarding the other histopathological characteristics, 36 cases were poorly differentiated, 10 cases were moderately differentiated and 39 cases were well differentiated. In addition, 32 were lymph node metastasis. Informed consent was obtained from all patients and controls participating in this study. The study was approved by the ethics committee of our hospital.

Immunohistochemistry

To examine the staining pattern of various target proteins in cervical tissues, the fixed preparations were first dewaxed in dewaxing agent (Zhong Shan Goldenbridge Biotechnology Co. Ltd, China) and rehydrated in alcohol, blocked with endogenous peroxidase inhibitor (Zhong Shan Goldenbridge Biotechnology Co. Ltd, China) at room temperature for 30 min and then incubated with CD147, FAS, ACC1 (abcam) overnight at 4°C. IHC staining was following the manufacturer’s protocol. Two experienced pathologists independently evaluated the staining scores. According to the staining intensity and distribution, immunostaining scores were semiquantitatively estimated. IHC scores of 7-8 were classified as high expression, scores of 3-6 were moderate expression and scores of 0-2 were low expression, respectively.

Cell lines and cell culture

Two cervical cancer cell lines SiHa, C33a, and human immortalized cervical squamous epi-
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The human cervical epithelial (H8) cells line, human lymphatic endothelial cell (HLEC), and human umbilical cord vascular endothelial cells (HUVEC) were purchased from Shanghai Cell Collection (Shanghai, China). Cultured in DMEM medium (BI). Cells were cultured in a 37°C incubator filled with 5% CO₂ and routinely passaged at 90% intensity. The culture medium was supplemented with 10% fetal bovine serum and 100 units/mL penicillin and streptomycin (BI).

Lentiviral infection, endothelial cell tube formation assay

Construct lentiviral vectors and lentivirus were produced by GENECHEM (Shanghai, China). ShCD147 target-CCAGAATGACAAAGGCAAGAA-, sh-NON target-TTCT CGAACGTGTCACGT-. Stable cell lines were generated by infection of cell lines and lentivirus, which was carried out in 6-well plate with free serum DMEM medium. SiHa cell was transduced with lenti-shCD147 at the infection MOI =10, at 37°C with 8 μg/ml polybrene and enhance for 72 h, according to manufacturer guidelines. Then, culture medium with 10% FBS was replaced and cells were continuously cultured for 6-8 days followed by selection with flow cytometry. Lymphangiogenesis was detected by a lymphatic endothelial cell tube formation assay according to the manufacturer's protocol.

Quantitative RT-PCR

Total RNA of cervical cancer cell lines cells was extracted by using TRIzol reagent (Invitrogen) and was reverse transcribed into 2 mg cDNA with Revert Aid First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Realtime PCR was performed using SYBR Green Premix PCR Master Mix (Roche, Mannheim, Germany) according to manufacturer protocols. Quantitative RT-PCR was carried out using the FastStart Universal SYBR Green Master (Roche) on an Applied Biosystems ABI 7900 Real time PCR System (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers for human CD147 and glyceraldehyde-3-phosphate dehydrogenate (GAPDH) were as follows: CD147-F5'-TTCATC TACGAGTAGCGCCG-3'; CD147-R5'-CAGGAAGTAGCTTCCGC-3'; GAPDH-F5'-TGGGTCAAGGATTTCCCTG-3'; GAPDH-R5'-GGCATGACTGGTGTCAAG-3'. The gene expression level was normalized using GAPDH as an internal reference gene and the average relative change was calculated using 3-5 determinations by relative quantification, applying the delta-delta cycle threshold method. All reactions were performed in triplicate.

Western blot

The cells were digested with trypsin, then washed with cold PBS, and lysed with RIPA. The protein concentration was determined by BCA protein assay kit (Bio-Rad, Hercules, CA, USA). The protein was quantified to 1.5 μg/ml by adding the loading buffer, protein, and PBS in proportion. After 2 h of 100 V electrophoresis, it was transferred to PVDF membrane. Closed with blocking solution, it was incubated with specific antibodies against CD147, FAS, ACC1 (Abcam, CA, USA), GAPDH (Proteintech, Rosemont, IL, USA) and Cell Signaling. ChemiScope Mini chemiluminescence instrument was used to detect and photograph the gray value of the target protein and calculate the expression of the target protein.

BODIPY 493/503

Place the cells in a confocal culture dish and incubate in 37°C for 20 h. After washing with PBS twice, and add diluted bodipy (1:300), 500 ul. Protected from light was 30 minutes at room temperature. Aspirate bodipy and washed 3 times with PBS for 3 minutes each time. Add DAPI dropwise, avoiding light for 5-10 minutes at room temperature. Aspirate DAPI and wash 2-3 times with PBS for 3 min each time.

Migration assay

Cell migration was analyzed using Wound Healing in a 6 well plates. Briefly, 1 ml of cells (1×10⁵ cells/ml) were transferred into each well and incubated at 37°C and 5% CO₂. After appropriate cell attachment was achieved (24 h), migration was assessed, at various time points (0, 24, 36 and 48 h), and images were captured with Nikon ECLIPSE TS100 epifluorescence microscope using NISElementsAR3.1software.

Invasion assay

Transwell assays were performed as described previously. Routinely, 8.0-μm Matrigel-coated Transwell supports from Becton Dickson Canada (BD) were used to evaluate cell invasion.
5000 cells were suspended in 100 μL of free serum DMEM and seeded in the upper chamber. The bottom chamber was filled with 600 μL of DMEM with 10% FBS. Cells allowed invading for 24 hours followed by fixation in 4% Paraformaldehyde Fix Solution. The under side of the membranes were stained with Giemsa solution for 20 minutes and the cells at the upper side of the membrane were removed using a wet cotton swab. We counted the number of cells that had migrated in a Nikon ECLIPSE TS100 epifluorescence microscope. Results were based on analysis of 10 fields (×10) in random fields.

Statistical analyses

All statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.) and Prism5.0 software (GraphPad). The data are presented as the mean ± SD of at least three independent experiments. Mann-Whitney test was used to test continuous variables for differences in CD147 IHC scores between tumor and normal tissues. The relationships between CD147 expression level and clinicopathological characteristics were tested by the X² test or Fisher exact test. Correlations between CD147, FAS, and ACC1 were analyzed by Spearman rank correlation analysis. Results were considered significant at P<0.05.

Result

CD147 protein overexpression is associated with malignant phenotypes

To estimate whether CD147 is dysregulated in human cervical cancer, we performed IHC on 85 CSCC samples; the antibodies were tested on formalin-fixed, paraffin embedded, normal cervical tissues, and CSCC. Representative staining patterns for the CD147 were shown in Figure 1A. CD147 was expressed in most of the cervical cancer cells, but only in basal cells of the normal cervical epithelia, and all positive staining was localized on the cell membrane. Expression findings of the CD147 were summarized in Table 1. As shown in Table 1, the percentage of CD147 positive cells was 78.8% in the cervical cancer tissues; the high expression of CD147 had a significant difference in lymph node metastasis and invasion depth. To provide further evidence, we measured the expression of these fatty acid synthase FAS and ACC1 in cervical cancer tissues with different CD147 levels using IHC staining (Figure 1B).

To determine whether CD147 expression exhibited similar patterns in cervical cancer cells line, we detected the expression of CD147 in SiHa, C33a, and immortalized cervical (H8) cells. CD147 protein levels in SiHa, C33a, and H8 were (2.207±0.1933), (1.325±0.3382), (0.1028±0.008740) respectively. As expected, the expression level of CD147 was significantly higher in SiHa and C33a cells than in immortalized H8 cells (Figure 1C). Additionally, compared to the C33a cell line, CD147 protein levels were elevated in SiHa cell lines. These results further supported the up-regulation of CD147 expression in cervical cancer cells.

CD147 promotes lipid metabolism by upregulate fatty acid synthase FAS and ACC1 expression in CC

To determine whether CD147 regulates lipid metabolism in CC cells, we first generated stable CD147-knockdown CC cells that originally expressed high levels of CD147 (shCD147). Western blot analysis confirmed the lentiviral infection efficiency of CD147 (Figure 2A) and the successful alteration in CD147 expression. The levels of intracellular TAG and PLs were measured by lipophilic fluorescence dye BODIPY 493/503 staining. Subsequently, our data showed that the levels of lipids were reduced in shCD147 cells, which indicated that CD147 knockdown decreased the intracellular contents of neutral lipids in SiHa cells (Figure 4A). To better explain the mechanisms by which CD147 regulates lipid metabolism, we analyzed levels of fatty acid synthase (FAS and ACC1) in cells expressing different levels of CD147. Knockdown of CD147 decreased FAS and ACC1 expression (Figure 2A). Overall, CD147 exerted significant effects on FAS and ACC1 in cervical cells, and the findings suggest that CD147 can regulate FA synthesis of cervical cells.

CD147 improves the migration and invasion of cervical cancer cells line and lymphangiogenesis in vitro

The above results demonstrate that CD147 great expression is correlated with tumor progression and metastasis. Migrate and invade is crucial for tumor metastasis. Thus, we carried out in vitro experiments to investigate the role
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of CD147 on cell migration and invasion in cervical cancer cells. Wound healing and Transwell invasion assays were conducted and results showed that CD147 knockdown markedly decreased migration capacity in a time-dependent manner (Figure 3A). A significant reduction in cell invasion was also observed (Figure 3B). These results suggest that lipid metabolism may accelerate the metastasis of CC cells. Moreover, we examined the effect of CD147 on the tube formation of lymphatic and vascular endothelial cells that is important for tumor lymph node metastasis. The culture supernatants of CD147 knockdown cells significantly inhibit lymphatic and vascular endothelial cells tube formation compared with those of the corresponding control cells (Figure 4B, 4C).

Discussion

The emergence of metastasis is the leading cause of cancer-related death and invasion represents the initiate step of the migration of malignant cells away from the primary site. As a
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Transmembrane glycoprotein, CD147 regulates a variety of cellular processes, including cell reproduction, embryo implantation, neural network formation, inflammation, glucose metabolism, fatty acid metabolism, and angiogenesis [17-19]. Due to its role in controlling cell growth, angiogenesis, and reprogramming of metabolism, CD147 has been linked to the metastasis of cancer. Therefore, CD147 may be a potential as anti-metastatic drug targets in cancer therapies.

The present results show that CD147 greatly expressed in cervical cancer and express much more CD147 protein notably associated with the lymph node metastasis and poor differentiation with cervical cancer. The result is consistent with the report that highly expressed

Figure 2. Western blot analyses for protein levels of the key fatty acid synthase FAS and ACC1 in the indicated cells with different CD147 expression levels. Data are expressed as means ± SEM. *P<0.05; **P<0.001. All experiments were performed at least three times.

Figure 3. Representative figures of cell migration (left) and cell invasion (right) in Siha cells. Bar graph summarizing the number of invasion per field, a Knockdown of CD147 decreased cell migration and invasion, Data are expressed as means ± SEM. **P<0.001. All experiments were performed at least three times.
CD147 promotes lymphangiogenesis and cervical cancer cells metastasis by lipogenesis. A. The neutral lipids content was detected by double staining with BODIPY 493/503 dye and Hoechst in the indicated cells. B. HUVEC was pretreated with the culture medium supernatants of the cervical cancer cells to investigate the effects of CD147 on the tube formation. C. HLEC was pretreated with the culture medium supernatants of the cervical cancer cells to investigate the effects of CD147 on the tube formation.

CD147 in squamous cell carcinoma of the tongue and neck is at significant risk of cancer progression and mortality [20, 21]. In addition, similar reports have suggested by Rosenthal EL et al., whereby the high expression of CD147 promote the progression of head and neck squamous cell carcinoma. Notably, CD147 expression has been associated with lymph node metastasis in laryngeal squamous cell carcinoma [22]. In hepatocellular carcinoma cells CD147 significantly contributes to the reprogramming of glucose metabolism through a p53-dependent way and it is also regulates the lipid metabolism in hepatocellular carcinoma cells by activation of Akt/mTOR signaling pathways; meanwhile knockdown CD147 significantly inhibited the proliferation, migration, and invasion of HCC cells [13]. The result suggested that the highly expressed regulates invasion and metastasis in cancers by controlling lipid metabolism.

It has been proven that aggravated fatty acid synthesis metabolism in tumor cells is reflected in enhance expression of pivotal enzymes for lipid biosynthesis [3]. In rapidly growing cancer cells, large amount of lipids is needed for synthesis of membranes, signaling molecules and lipoproteins. Indeed, increased de novo fatty acid synthesis in cancer cells is through multiple mechanisms, most of which involve the increased expressions of key lipogenic enzymes FAS and ACC. Accumulating evidence has shown that FAS is highly expressed and strongly related with tumor malignant progression [23, 24]. Clinical data showed a positive correlation between FAS expression and tumor angiogenesis in primary colorectal cancer patients [25]. FAS expression promoted peritoneal metastasis of ovarian cancer in part through the induction of epithelial mesenchymal transition [26]. HYO et al. addressed that elevated levels of Prolyl-isomerase Pin1 strongly correlated with elevation of FAS in human breast cancer and promote breast cancer growth and survival [26]. ACC1 is the rate-limiting enzyme in the fatty acid synthesis Pathway, which carboxylates acetyl-CoA to form malonyl-CoA. As well, the highly expressed of ACC1 was greatly related with multiple aggressive biological characteristics of human cancer, such as vascular invasion and poor differentiation and up-regulation of ACC1 was also significantly correlated with poorer overall survival and disease recurrence in human cancer patients [27]. Taken together, in cancer cells, de novo lipogenesis by FAS and ACC1 are regulated by different pathways, which can be involved in cell signaling.
In the present study, to demonstrate the role of CD147 in the regulation of fatty acid synthesis in cervical cancer, we examined the effects of CD147 on several key fatty acid synthesis enzymes, including FAS and ACC1 in cervical cancer cells. Consequently, significant down-regulation of FAS and ACC1 protein and mRNA levels were observed compared with the levels in controls in SiHa cells after CD147 knockdown. To provide further evidence, we measured the expression of these fatty acid synthase in cervical cancer tissues with different CD147 levels using IHC staining and obtained consistent results and cellular staining with BODIPY 493/503 showed that CD147 knockdown decreased the intracellular contents of neutral lipids in SiHa cells. Because migrate and invade is crucial for tumor lymph node metastasis, we further determined whether CD147 promotes the tube formation of lymphatic and vascular endothelial cells and found that high expression of CD147 significantly promoted lymphatic endothelial cells tube formation. Thus, we conclude that CD147 promotes lymph node metastasis through the fatty acid synthesis in cervical cancer.

In conclusion, CD147 as a promising prognostic biomarker for cervical cancer promotes migration and invasion through the regulating fatty acid synthase expression. It would be interesting to further study the underlying mechanisms of CD147 to reprogramming of lipid metabolism by FAS and ACC1.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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