Original Article

Downregulation of CD147 inhibits skin squamous cell carcinoma cell proliferation, migration, and invasion by inhibiting phosphorylation of ERK

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Abstract: Purpose: The aim of the current study was to investigate the effects of CD147 on skin squamous cell carcinoma cells, examining related mechanisms. Methods: Normal human keratinocyte cell line (HaCaT) and human skin squamous cell carcinoma cell lines (HSC-5, SCL-1, and A431) were cultured. HSC-5 cells were transfected by CD147 siRNA (CD147-siRNA group). The siRNA-Negative Control group and Blank group were set. Normal HSC-5 cells and cells of the CD147-siRNA group were cultured in 1640 medium containing 100 μmol/L U0126. They were named the U0126 group and CD147-siRNA + U0126 group, respectively. Moreover, qRT-PCR was used to detect CD147 mRNA expression levels. Western blotting was performed to detect CD147, ERK, and p-ERK protein expression levels. MTT assays, cell scratch tests, and Transwell experimentation were used to detect cell proliferation, migration, and invasion. Results: CD147 relative expression levels in skin squamous cell carcinoma cells were significantly upregulated (P < 0.01). Compared with the Blank group and siRNA-Negative Control group, p-ERK expression, as well as HSC-5 cell proliferation, migration, and invasion, were declined in the CD147-siRNA group, U0126 group, and CD147-siRNA + U0126 group (P < 0.05). Compared to the CD147-siRNA group and U0126 group, p-ERK expression, as well as HSC-5 cell proliferation, migration, and invasion, were decreased in the CD147-siRNA + U0126 group (P < 0.05). Conclusion: Downregulation of CD147 may inhibit proliferation, migration, and invasion of skin squamous cell carcinoma cells by inhibiting phosphorylation of ERK.

Keywords: CD147, HSC-5, proliferation, migration, invasion, p-ERK

Introduction

Twenty percent of patients with skin malignancies are diagnosed with skin squamous cell carcinoma. It is one of the most common skin malignancies [1]. Morbidity and mortality rates caused by skin squamous cell carcinoma have increased year by year [2]. Complete tumor excision, accompanied by chemotherapy or radiotherapy, is the most common treatment method for most skin squamous cell carcinoma patients. However, some refractory patients are at a risk of high metastasis and mortality [3].

Researchers have found that targeted therapy at the molecular level can achieve better therapeutic effects, to a certain degree [4, 5]. However, the discovery of tumor specific biomarkers is still a challenge, especially for skin squamous cell carcinoma. Relevant molecular biomarker studies, concerning skin squamous cell carcinoma, are rare. CD147, a membrane-bound glycoprotein, has been shown to play a role in promoting the growth and metastasis of several types tumors, including prostate cancer and gastric cancer [6-9]. Chu et al. [10] demonstrated that upregulation of CD147 was positively correlated with invasion, metastasis, and TNM staging in gastric cancer patients. They concluded that CD147 might be an indicator of tumor recurrence and prognosis in gastric cancer patients. Kaira et al. [11] showed that, in patients with pancreatic cancer, CD147 could form a complex with LAT1 and ASCT2. It was identified as a negative prognostic predictor in
pancreatic cancer. Yue et al. [12] suggested that CD147 expression levels were positively correlated with MMP-9, which has been correlated with the development of cancers and relapse. Su et al. [13] revealed that CD147 knockdown could suppress tumor growth of melanomas by downregulating GLUT-1 via PI3K/Akt pathways, providing a new therapeutic regimen for melanoma treatment. It has also been found that CD147 also plays an important role in skin squamous cell carcinoma development. They recommend CD147 as a biomarker and as a potential therapeutic target for skin squamous cell carcinoma [14, 15]. However, the specific mechanisms of CD147 in skin squamous cell carcinoma remain unclear.

In the current study, the mechanisms of CD147 on skin squamous cell carcinoma cells were examined. This study aimed to provide guidance and a theoretical basis for the molecular treatment of skin squamous cell carcinoma, clinically.

**Materials & methods**

**Cell culturing**

Normal human keratinocytes (HaCaT) and human skin squamous cell carcinoma cell lines (HSC-5, SCL-1, and A431), purchased from American Type Culture Collection, were cultured in 24-well plates with 1640 medium at a density of 1*10^4 cells per well, respectively. The 1640 medium consisted of fetal bovine serum by a volume fraction of 10%. These cells were incubated in a carbon dioxide incubator with 5% CO_2 and 95% humidity at 37°C for 48 hours.

**Cell transfection**

HSC-5 cells in the logarithmic growth phase were collected and inoculated into 6-well plates at a concentration of 1*10^5/L. A total of 1,000 μL cell suspension was added into each well. After incubation in the incubator for 24 hours, they were transfected with CD147 siRNA or negative control plasmids using Lipofectamine 2000. The sequence of CD147 siRNA was as follows: 5’-GATCCGTGACAAAGGCAAGACGTCTTCAAGAGAGCATTTGTCATTGGAAATTC-3’, 5’-AGCTTTTACTAAAAATGACAAAGGAAGCCTCCTTGACGTCTTGTGCAAGC-3’. HSC-5 cells that were successfully transfected with CD147 siRNA were collected and designated as the CD147-siRNA group. Furthermore, HSC-5 cells transfected by CD147 negative control plasmids were collected and named the siRNA-Negative control group. HSC-5 cells without any treatment were set as the Blank group. Cells in each group were seeded in 24-well plates at a density of 1*10^4 cells per well. They were incubated in the carbon dioxide incubator with 5% CO_2 and 95% humidity at 37°C for 48 hours.

**U0126 treatment**

Normal HSC-5 cells and HSC-5 cells, successfully transfected with CD147 siRNA, were collected and prepared as a cell suspension with 1640 medium containing 30 μmol/L U0126 (an ERK phosphorylation inhibitor). These cells were seeded in 24-well plates at a density of 1*10^4 cells per well. They were incubated in the carbon dioxide incubator. They were named the U0126 group and CD147-siRNA + U0126 group, respectively.

**RNA extraction and qRT-PCR detection**

After incubation of 48 hours, the cells were collected. Total RNA was extracted using the RNeasy Plus mini kit (Qiagen, Valencia, CA). The extraction procedure was carried out in strict accordance to kit instructions. RNA concentrations and purity levels were detected. Moreover, the cDNA template was synthesized by reverse transcription. PCR amplification was conducted in a 20 μL reaction volume containing 1 μL cDNA template and 2 μL primers. Amplification conditions were as follows: Pre-denaturation at 95°C for 10 minutes, denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 34 seconds. The same reaction process was circulated 40 times. β-actin was used as an internal reference. Primer sequences of CD147 and β-actin are shown in Table 1. Data was analyzed using the 2^ΔΔCt method.

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>CD147-F</td>
<td>5'-CCATGCTGGTCTGCAAGTCAG-3'</td>
</tr>
<tr>
<td>CD147-R</td>
<td>5’-CGTTTATGAGGCCCTTGGTC-3’</td>
</tr>
<tr>
<td>β-actin-F</td>
<td>5’-CTGGAACGTTGAAGTGACA-3'</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>5’-AAGGGACTTCTGTAACACGCA-3’</td>
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Western blot analysis

In the current study, 30 μmol/L U0126, known as an ERK phosphorylation inhibitor, was used to treat normal HSC-5 cells and HSC-5 cells that were successfully transfected with CD147 siRNA. ERK and p-ERK protein relative expression levels in HSC-5 cells of each group were determined by Western blotting. First, the total protein of cells was extracted after incubation for 48 hours. Protein concentrations were determined with the BCA kit. A total of 30 μL protein was electrophoresed on 12% SDS-polyacrylamide gels. The protein was then transferred onto PVDF membranes using the wet method. The membranes were blocked with 5% fat-free dry milk in TBST for 1 hour. The primary antibody (mouse anti-human CD147 monoclonal antibody, mouse anti rabbit E-cadherin, MMP-9, ERK, and p-ERK, 1:1000, Abcam, UK) was added to incubate for 12 hours at 4°C. Next, TBST was used to wash the membranes 3 times. HRP-labeled goat anti-mouse IgG secondary antibody (1:5000, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) was then added for 1 hour of incubation at room temperature. Finally, chemiluminescence was used to visualize CD147 proteins and β-actin was used as a reference.

MTT assays

For cells in each group, a total of 20 μL MTT (5 mg/mL, Sigma) was added to each well after incubation for 24, 48, 72, and 96 hours, respectively. The upper liquid was discarded after another 4 hours of incubation. Afterward, 150 μL DMSO was added to each well and lightly shaken until the crystals dissolved completely. Absorbance values (OD₄₉₅ value) of each well were measured at 495 nm. Cell proliferation ability was determined according to OD₄₉₅ values.

Cell scratch testing

HSC-5 cells of each group were treated with serum-free 1640 medium for 24 hours. They were then collected and seeded in 6-well plates at a density of 5×10⁵/mL cells per well, respectively. After 72 hours of incubation, a scratch was made by pipettes and the floating cells were cleared out with PBS. A total of 2 mL of 1640 medium containing 10% fetal bovine serum was added into each well. These cells were still incubated in a 5% CO₂ incubator for 12 hours. Cell migration ability was evaluated according to the scratch wound healing rate. Scratch wound healing rate was calculated by the following formula: scratch wound healing rate = (initial scratch wound width-scratch wound width after 12 h)/initial scratch wound width*100%.

Transwell experiment

HSC-5 cell invasion ability levels of each group were determined through Transwell experimentation. Cells suspended in serum-free 1640 medium were placed on the top of Matrigel-coated polycarbonate Transwell filters at a density of 5×10⁵/mL cells. Medium supplemented with serum was used in the bottom chamber. After incubation for 48 hours, cells invading to the bottom of the membranes were fixed for 10 minutes with 100% methanol. Air-drying was performed before staining with DAPI. Furthermore, the number of cells penetrating the membranes was counted under a microscope, aiming to assess cell invasion ability.

Statistical analysis

Present data was processed with SPSS 18.0 statistical software. Data are expressed as mean ± SD and were analyzed by one-way analysis of variance, followed by Tukey’s post-hoc testing, comparing the means. P < 0.05 indicates that differences are statistically significant.

Result

Upregulation of CD147 in skin squamous cell carcinoma cells

Compared with HaCaT cells, relative expression levels of CD147 mRNA and proteins in HSC-5, SCL-1, and A431 were all significantly upregulated (P < 0.01). Relative expression levels of CD147 mRNA and proteins in HSC-5 cells were the highest (Figure 1A, 1B). Therefore, HSC-5 cells were selected for subsequent studies.

Downregulation of CD147 in HSC-5 cells by siRNA transfection

After transfection, CD147 mRNA and protein relative expression levels in the CD147-siRNA group were significantly lower than those in the
Blank group and siRNA-Negative Control group ($P < 0.01$) (Figure 2A, 2B), suggesting that siRNA transfection successfully inhibited CD147 expression in HSC-5 cells.

**Inhibition of p-ERK expression by silencing CD147**

There were no statistically significant differences in relative expression of ERK among the Blank group, siRNA-Negative Control group, CD147-siRNA group, U0126 group, and CD147-siRNA + U0126 group ($P > 0.05$). However, p-ERK relative expression levels of the CD147-siRNA group, U0126 group, and CD147-siRNA + U0126 group were significantly declined, compared with the Blank group and siRNA-Negative Control group ($P < 0.01$). In addition, compared to the CD147-siRNA group and U0126 group, relative expression of p-ERK in the CD147-siRNA + U0126 group was significantly lower ($P < 0.05$) (Figure 3). Present results indicate that p-ERK expression in HSC-5 cells was inhibited through silencing CD147.

**Downregulation of CD147 could inhibit HSC-5 cell proliferation through inhibiting expression of p-ERK**

MTT assays showed that, at 48-96 hours, the OD$_{495}$ value of CD147-siRNA group was significantly lower than that of the Blank group and siRNA-Negative Control group ($P < 0.05$). CD147 silencing may inhibit the proliferation of HSC-5 cells. At the same time, significantly lower OD$_{495}$ values in the U0126 group were found, compared with the Blank group and siRNA-Negative Control group at these three time points ($P < 0.05$). Results suggest that the proliferation of HSC-5 cells was also inhibited by inhibition of p-ERK. In addition, at 72 hours and 96 hours, the OD$_{495}$ value of the CD147-siRNA + U0126 group was not only much lower than that of the Blank group and siRNA-Negative Control group.
Control group ($P < 0.05$), but also significantly lower than that of the CD147-siRNA group and U0126 group ($P < 0.05$). Present results suggest that downregulation of CD147 inhibited HSC-5 cells proliferation by inhibiting p-ERK expression (Figure 4).

**Downregulation of CD147 could inhibit HSC-5 cell migration through inhibiting expression of p-ERK**

Cell scratch test results showed that cell wound healing rates were significantly lower in the CD147-siRNA group than the Blank group and siRNA-Negative Control group ($P < 0.05$), indicating that HSC-5 cell migration ability levels were significantly inhibited by silencing of CD147. Also, significantly declined cell wound healing rates were found in the U0126 group, compared with the Blank group and siRNA-Negative Control group ($P < 0.05$), indicating that downregulation of p-ERK had significant inhibitory effects on migration abilities of HSC-5 cells. Moreover, cell wound healing rates of the CD147-siRNA + U0126 group were significantly decreased, compared with the Blank group and siRNA-Negative Control group ($P < 0.05$). Moreover, compared with the CD147-siRNA group and U0126 group, the cell wound healing rate of the CD147-siRNA + U0126 group dramatically declined ($P < 0.05$) (Figure 5A). In addition, expression of E-cadherin, a migration-associated protein, was also detected. Results revealed that the change trend of E-cadherin relative expression was completely opposite to the change trend of cell wound healing rates of each groups (Figure 5B). The above data indicates that downregulation of CD147 could inhibit HSC-5 cell migration ability levels through inhibiting expression of p-ERK.

**Downregulation of CD147 could inhibit HSC-5 cell invasion through inhibiting expression of p-ERK**

Transwell assays were performed to explore the effects of CD147 on the invasive abilities of HSC-5 cells. Compared with the Blank group or siRNA-Negative Control group, significantly decreased invasive cell numbers were found in the CD147-siRNA group ($P < 0.05$), suggesting that downregulation of CD147 could inhibit HSC-5 cell invasion. Results also showed that invasive cell numbers of the U0126 group were obviously lower than those of the Blank group or siRNA-Negative Control group ($P < 0.05$). Downregulation of p-ERK showed significant inhibitory effects on invasion ability levels of HSC-5 cells. Furthermore, invasive cell numbers of the CD147-siRNA + U0126 group were
Figure 5. Downregulation of CD147 inhibited HSC-5 cell migration through inhibiting p-ERK expression. A. Cell scratch testing to detect the migration of cells in each group; B. Western blot to detect E-cadherin expression of cells in each group. *P < 0.05 compared with the Blank group or siRNA-Negative Control group. #P < 0.05 compared with the CD147-siRNA group or U0126 group.

Figure 6. Downregulation of CD147 inhibited HSC-5 cell invasion through inhibiting p-ERK expression. A. Transwell experiment to detect the invasion of cells in each group; B. Western blot to detect MMP-9 expression of cells in each group. *P < 0.05 compared with the Blank group or siRNA-Negative Control group. #P < 0.05 compared with the CD147-siRNA group or U0126 group.

not only lower than those of the Blank group and siRNA-Negative Control group (P < 0.05), but also lower than those of the CD147-siRNA group and U0126 group (P < 0.05) (Figure 6A). The change trend of MMP-9 relative expression was in accord with the change trend of invasive
Discussion

In the current study, the mechanisms of CD147 on skin squamous cell carcinoma cells were studied. Results demonstrated that expression of CD147 in skin squamous cell carcinoma cells was upregulated. Downregulation of CD147 was shown to inhibit the proliferation, migration, and invasion of HSC-5 cells by inhibiting expression of p-ERK. Moreover, downregulation of CD147 may promote E-cadherin expression and inhibit MMP-9 expression by inhibiting p-ERK.

CD147 is an important influencing factor in tumor cell growth, development, differentiation, and progression. It has been considered a novel target for antitumor therapy [16]. Chu et al. found that CD147 overexpression increased the risk of invasion and metastasis in gastric cancer, an independent prognostic factor of gastric cancer overall survival rates, as well as disease-free survival rates [10]. Xu et al. also revealed that, in colorectal cancer tissues, CD147 was upregulated. This aggravated the invasion abilities of tumors. More importantly, their research indicated that the ability of CD147 to promote invasion ability was acted on through MAPK/ERK signaling pathways [17]. Consistent with these studies, present results also suggest that expression of CD147 in skin squamous cell carcinoma cells was upregulated, further confirming the role of CD147 in malignant neoplasms and providing a theoretical basis for treatment of skin squamous cell carcinoma at the molecular level.

In addition, the current study examined the mechanisms of CD147 in skin squamous cell carcinoma cells. Results showed that downregulation of CD147 could exert inhibitory effects on skin squamous cell carcinoma cell proliferation, migration, and invasion by inhibiting expression of p-ERK. Furthermore, downregulation of CD147 may also promote E-cadherin expression and inhibit MMP-9 expression by inhibiting p-ERK. E-cadherin is a migration-related protein. Downregulation of this protein will lead to increased tumor cell migration ability levels [18]. MMP-9 can increase the invasive ability of tumor cells [19]. Upregulation of E-cadherin and downregulation of MMP-9 provide inhibitory effects on tumor cell migration and invasion. In this research, inhibition of CD147 was shown to upregulate E-cadherin and downregulate MMP-9. Relevant mechanisms were through inhibiting p-ERK expression. Moreover, p-ERK, as one of the crucial factors in tumor progression, can be accumulated in the nucleus. Extracellular stimuli can be transferred from the cell surface to the nucleus by p-ERK during the process of intracellular signal transduction [20, 21]. The function of activated ERK pathways has been examined in many cancers, such as renal cell carcinoma, lung cancer, breast cancer, and colorectal carcinoma [22-24]. Zhao et al. researched the impact of p-ERK on non-small cell lung cancer. They found that p-ERK expression was an independent prognostic factor leading to poor prognosis. Its overexpression was closely related to histological type and tumor differentiation. They concluded that p-ERK is a prognostic marker for non-small cell lung cancer [25]. Vicent et al. [26] further confirmed that upregulation of p-ERK could be used as a sign of advanced and invasive non-small cell lung cancer. The current study found that CD147 silencing and the use of U0126 could both obviously decrease relative expression levels of p-ERK. Under the dual impact of CD147 silencing and U0126 using, more significant declines of p-ERK relative expression levels occurred and significant inhibitory effects of HSC-5 cell proliferation, migration, and invasion were found. Results suggest that the impact of CD147 on HSC-5 cells was through acting on the expression of p-ERK. Xu et al. [27] revealed that, in colorectal cancer, high expression of CD147 could activate MAPK/ERK signaling pathways. They explained that CD147 could promote expression of p-ERK in colorectal cancer cells and ERK inhibitor abrogated CD147-induced invasion and migration. Xiao and colleagues demonstrated that, in hepato-cellular carcinoma, ERK signaling pathways might be activated in CD147-induced cell migration [28]. The current study was consistent with previous studies, suggesting that downregulation of CD147 could exert an inhibitory effect on skin squamous cell carcinoma cell proliferation, migration, and invasion by inhibiting expression of p-ERK. This article first explored the mechanisms of CD147 in skin squamous cell carcinoma, providing important guid-
CD147 expression in skin squamous cell carcinoma

The current study had some limitations. First, only HSC-5 cells were selected for subsequent studies. HSC-5 cells had the highest CD147 mRNA and protein relative expression levels. Therefore, using HSC-5 cells for follow-up studies would be more representative and persuasive. In addition, follow-up studies could not be performed on all cell lines due to limitations of laboratory conditions. Thus, only HSC-5 cells were selected for subsequent studies. In future studies, tests concerning the other two cell lines will be conducted. Second, the current study only showed phosphorylation of ERK to be the mechanism of CD147 mediated HSC-5 cell proliferation and invasion. This study could not implement the other parts of MAPK signaling pathways because of limitations of laboratory conditions.

In conclusion, the current study investigated the effects of CD147 on proliferation, migration, and invasion of skin squamous cell carcinoma cells. Results showed that downregulation of CD147 could inhibit proliferation, migration, and invasion of skin squamous cell carcinoma cells through inhibiting expression of p-ERK. The current study may provide important guiding significance, laying a theoretical foundation for clinical targeted therapy of skin squamous cell carcinoma.

Disclosure of conflict of interest

None.

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