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Original Article

Involvement of soluble CD147 in the regulation of HCC invasion by active Akt pathway

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Abstract: Purpose: CD147 can induce the expression of matrix metalloproteinases (MMPs) from tumor cells and tumor stromal cells. The aim of this study was to investigate the pathway involved in the regulation of the invasive ability of hepatocellular carcinoma (HCC) cells mediated by soluble CD147. Furthermore, this study sought to validate these finding in HCC patients. Methods: We explored the role of soluble CD147 on the secretion of MMPs via Western blot. Invasion assays were performed with HCC cells to investigate the effects of soluble CD147 on the regulation of invasiveness. Serum CD147 levels were detected by an enzyme-linked immunosorbent assay. Tissues were assayed by immunohistochemistry to detect CD147, MMP-9 and p-Akt expression. Results: Soluble CD147 regulates the invasive ability of HCC cells by association with surface CD147 and induces the activation of Akt-related pathways. The concentration of soluble CD147 in the serum of patients with HCC was significantly elevated when compared to healthy controls ($P < 0.01$). Soluble CD147 levels in serum were positively related with the expression of surface CD147, MMP-9 and p-Akt in tumor tissues ($P < 0.01$). Conclusions: Soluble CD147 regulates the invasive ability of HCC cells by affecting Akt-related pathways and then inducing the expression of MMP-9. Soluble CD147 in serum is a potential diagnostic and prognostic biomarker for HCC.

Keywords: AKT/PKB kinases, hepatocellular carcinoma, soluble CD147

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most frequent cause of cancer death in worldwide. Because of its highly progressive characteristics (i.e. rapid progression, poor response to many therapies and high risk of relapse), HCC has become an increasing threat to human health [1].

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN) or basigin (BSG), is a kind of type I transmembrane glycoprotein belonging to the immunoglobulin superfamily (IgSF) which is wildly expressed in many kinds of cells [2]. CD147 can be broadly divided into 4 isoforms: BSG 1, BSG 2, BSG 3 and BSG 4. The most common type of CD147 is BSG 2, which is composed of 3 domains: an N-extracellular section, a C-intracellular period and a single transmembrane segment [3]. Recent research has indicated that CD147 is closely associated with the initiation and progression of HCC. For example, CD147 can promote the hepatitis B virus (HBV) and hepatitis C virus (HCV)-related carcinogenic process of HCC. Through combination with Cyclophilin A (CyPA), CD147 can interact with the small surface protein of HBV, which promotes the inflammation of the liver and leads to the increase of aspartate transaminase (AST) and alanine transaminase (ALT) in serum [4]. Moreover, through induced expression of CD147, the HCV core protein can promote normal liver cell migration and invasion [5]. All of these effects increase the risk of HCC initiation. CD147 is also associated with the progression of HCC as it induces the expression of extracellular matrix metalloproteinases (MMPs), a zinc ion dependent peptide enzyme family, which plays a crucial role in the process of tumor invasion and metastasis.

Recent studies have suggested that CD147 can potentially increase the invasion and metastatic ability of HCC cells by inducing the secretion of MMP-2 and MMP-9 from fibroblasts or HCC tumor cells [6]. Several pathways have been pointed out as perhaps being involved in the regulation of HCC invasion and metastatic ability which is mediated by CD147, e.g. the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway and the phospholipase A and 5 lipoxygenase catalyzed pathway. However, the clear mechanism involved in is still unknown [7, 8].

Recently, two forms of soluble CD147 have been detected in the condition medium of cancer cells: full-length CD147 and the extracellular domain of CD147, both of which have been demonstrated to play a role in the regulation of cancer progression. In one recent study, serum soluble CD147 was found to be positively correlated with the membrane-bound CD147 level and MMP-2 level in HCC tissues, which indicated that soluble CD147 most likely mainly originates from the shedding of membrane-bound CD147 and demonstrated its involvement in the regulation of invasion ability of HCC cells [9]. As a result, some researchers believe that serum soluble CD147 is a potential prognostic biomarker for HCC.

In this study, we investigated the relationship between soluble CD147 and the Akt pathway in the regulation of MMP-9 expression and cell invasion in HCC cells. Furthermore, we also evaluated the relationship between serum soluble CD147, the activation of the Akt pathway and the expression of MMP-9 in HCC patients.

Materials and methods

Patients

From 70 HCC patients and 30 healthy controls, blood samples (8 ml from each patient collected in non-heparinized tubes) were obtained prior to operation or treatment, centrifuged and stored at -80°C until assayed. In the HCC group, 14 patients were classified as having Barcelona clinic liver cancer (BCLC) stage 0 disease, 33 patients with stage A disease, 16 patients with stage B disease and 7 patients with stage C disease. Primary tumor tissues were obtained either during operation or by needle biopsy.

Cell Culture, treatment and transfection

SMMC-7721 cells were obtained from American Type Culture Collection (ATCC) and were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin, and 2% L-glutamine.

For soluble CD147 treatment, SMMC-7721 cells were induced with 20 μg/ml Human CD147/EMMPRIN/Basigin Protein (Sino Biological Inc.) for 5 days. Predesigned siRNA targeting CD147 was purchased from Life Technologies, Ambion Ltd. Transfections were performed using Lipofectamine-2000 (Invitrogen) according to the manufacturer's protocol. Briefly, cells were grown to 50% confluency for gene silencing. For every 10 cm plate, 20 μM of scrambled or targeting siRNA was mixed with 500 μl Optimem medium (Life Technologies). In a separate Eppendorf tube, 12 μl Lipofectamine solution was mixed with 500 μl Optimem medium and following a 5 minute incubation at room temperature, the mixture was added to the DNA-Optimem mix. After 25 minutes, the transfection mix was added to the cells and the medium was changed 4-6 hours after transfection.

Western immunoblotting

Cells were harvested and sonicated. Total proteins extracted were quantified using the BCA Protein Assay Kit (Beyotime). 10-50 μg protein was mixed with 5 × loading buffer and boiled at 95°C for 5 minutes then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto nitrocellulose membranes (Thermo Scientific). After blocking in 5% non-fat milk/TBST buffer for 1 hour, membranes were incubated in primary antibodies overnight at 4°C. The following primary antibodies were used in the dilution as suggested by the supplier: EMMPRIN (CD147, 1:500, Santa Cruz), MMP-9 (1:500, Santa Cruz), GAPDH (1:1000, Santa Cruz), Akt (1:1000, Cell Signalling), p-Akt (Ser-473) (1:2000, Cell Signalling). After primary antibody incubation, blots were incubated in secondary antibodies for 1 hour. The following secondary antibodies were used in the dilution as suggested by the supplier: Polyclonal Goat Anti-Mouse Immunoglobulins (1:10000, Dako); Polyclonal Goat Anti-Rabbit Immunoglobulins

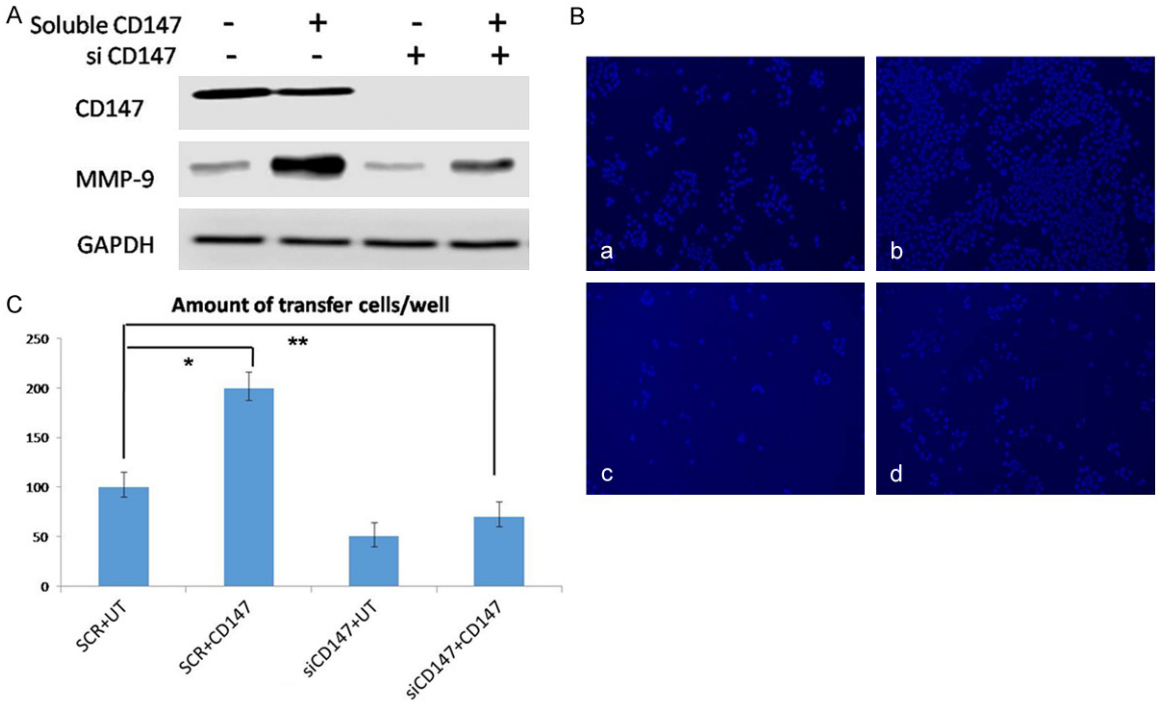

Figure 1. Soluble CD147 regulates the invasion of SMMC-7721 cells via interaction with membrane CD147. A. SMMC-7721 cells were transfected either with a siRNA targeting CD147 or a negative siRNA control. Cells were treated with and without soluble CD147 for 5 days after transfection and cell lists were probed for CD147 and MMP-9. GAPDH was used as a loading control. B. SMMC-7721 cells were transfected with a siRNA against CD147 or a negative siRNA control. Cells were treated with and without soluble CD147 for 5 days after transfection. On day 6, 1 × 10^6 cells/well were seeded into the transwell chamber and the amount of cells transferred was analyzed after 2 days. C. The amount of cells transferred after 2 days in the invasion experiment (*P < 0.01; **P=0.137).

(1:2000, Dako). Immunoreactive proteins were detected using the enhanced chemiluminescence system West Dura Extended Duration Substrate (Thermo Scientific).

Immunohistochemistry (IHC)

All patient sample sections were obtained with the ethical approval from the General Hospital of The Second Artillery Corps of Chinese PLA. Samples were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions. Antigenic activity was measured by first incubating at 97°C for 30 min in Tris-EDTA buffer (Abcam). After washing with TBS, sections were incubated in peroxidase and alkaline phosphatase blocking reagent (DAKO) for 10 minutes to block endogenous peroxidase activity. After 10 minutes of blocking with blocking solution (DAKO), sections were incubated with the primary antibody (p-Akt (Ser473), 1:500; MMP-9, 1:200; CD147, 1:200) for 30 minutes. The slides were rinsed gently with PBS and then incubated with labeled polymer (HRP. Rabbit, DAKO) for 30 minutes. The slides were washed efficiently and enough staining solution (Liquid DAB+ Substrate Chromogen System, DAKO) was applied to each sample for 5 to 10 minutes. After washing, sections were stained with hematoxylin, dehydrated and mounted. Subcellular localization, staining intensity and the proportion of positive cells were recorded. For patient sample slides, the percentage of the positive cells were graded on a scale from 0 to 4 (0: negative, 1: 0-25%, 2: 26-50%, 3: 51-75%, 4: 76-100%). Areas which demonstrated positivity were further quantified from 0 to 3 by staining intensity (1: weak, 2: moderate, 3: intensive). The final score was obtained by combining these two scores together.

Enzyme-linked immunosorbent assay (EILSA)

All patient serum samples were obtained with the ethical approval from the General Hospital of The Second Artillery Corps of Chinese PLA. CD147 EILSA analyses were conducted by using a specific human EMMPRIN/CD147 Qu-

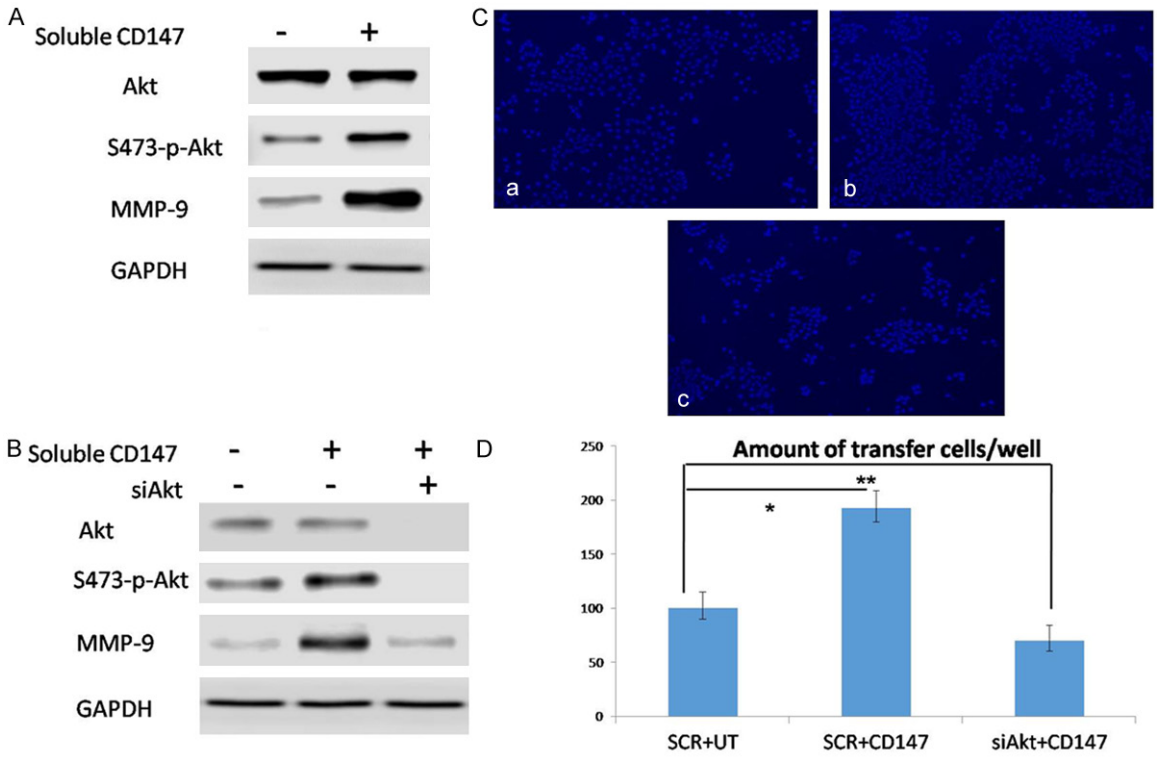

Figure 2. Soluble CD147 regulates the invasion ability of SMMC-7721 cells through interaction with membrane CD147. A. SMMC-7721 cells were treated with or without soluble CD147 for 5 days and cell lysates were probed for Akt, S473-p-Akt and MMP-9. GAPDH was used as an equal loading control. B. SMMC-7721 cells were transfected with siRNA against Akt or a negative siRNA control. Cells were treated with or without soluble CD147 for 5 days after transfection and cell lysates were probed for Akt, S473-p-Akt and MMP-9. GAPDH was used as an equal loading control. C. SMMC-7721 cells were transfected with siRNA against Akt or a negative siRNA control. Cells were treated with or without soluble CD147 for 5 days after transfection and on day 6, 1 × 10^6 cells/well were seeded into the transwell chamber. The amount of cells transferred was analyzed after 2 days. D. The amount of cells transferred after 2 days in the invasive experiment (*P < 0.01; **P=0.159).

antikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the protocols suggested by the manufacturer.

Standard dilutions were prepared as suggested and samples were diluted 1:100. The OD450 was read and the concentration of CD147 was calculated according to the standard curve.

Invasion assays

Invasiveness of SMMC-7721 cells was assayed by using Transwell chambers (Chemicon (Millipore) QCM™, ECM550). 100 μl RPMI 1640 medium with 10% FBS containing 1.0 × 10^5 SMMC-7721 cells was seeded in the upper compartment and incubated for 2 hours, allowing the cells time to attach to the bottom. The lower chamber contained RPMI medium supplemented with 10% FBS. After incubation, the normal RPMI 1640 medium in the upper compartment was aspirated and replaced with serum-free RPMI 1640 medium. After invasion for 48 hours, the cells on both sides of the chamber were fixed with 50/50% acetone/methanol and the top compartment was stained with 1% aqueous eosin. Subsequently, the non-invasive cells on the top side were removed with a cotton swab and invasive cells on the lower surface were stained with DAPI. An image was taken of each well using a UV filter to image nuclei of cells with the 10 × objective. After image acquisition, images were analyzed using the Image-J software. The number of cells on the lower surface was determined.

Statistics

Statistical analyses were carried out using the SPSS software and statistical significance was evaluated with the one-way ANOVA test and t-test. A *P*-value of ≤ 0.05 was considered to be

Table 1. Clinical and demographic characteristics of patients with HCC and healthy controls

Characteristics		*P* Value
Age, years		P > 0.05
Mean ± SD	56.6±8.4	
Median	57.5	
Range	44-79	
Control		
Mean ± SD	58.5±8.6	
Median		
Range		
Sex, n (%)		P > 0.05
HCC		
Male	55 (78.6%)	
Female	15 (21.4%)	
Control		
Male	22 (73.3%)	
Female	8 (26.7%)	
Hepatitis history, n (%)		P < 0.05
HCC	66 (94.3%)	
Control	18 (60%)	

significant. Spearman's correlation test was used to evaluate the relationship between the levels of soluble CD147 in serum with Ser473-phosphorylated Akt or MMP-9 levels in HCC tissues.

Results

Soluble CD147 regulates the invasive ability of SMMC-7721 cells through interaction with the membrane CD147

In order to determine whether soluble CD147 can affect the invasiveness of SMMC-7721 cells, we induced SMMC-7721 cells with the human CD147 protein for 5 days. On day 6, a certain amount of cells were seeded into the transwell chamber and the amount of cells transferred was analyzed after 2 days. As shown in **Figure 1B** and **1C**, when compared with untreated cells, the transferred cell number of soluble treated cells was increased two-fold. To provide further evidence, we also checked the MMP-9 level which is closely related with the invasion ability of HCC cells in both untreated and soluble CD147-treated cells by western blotting. As shown in **Figure 1A**, induction by soluble CD147 significantly increased the expression of MMP-9 in SMMC-7721 cells. These results indicated that soluble CD147 can induce the expression of MMP-9 and can ultimately increase the HCC cell invasion.

Recently, one study suggested that by associating with membrane CD147, soluble CD147 can activate the downstream pathway of membrane CD147 [10]. To determine whether this pathway is also valid in the regulation of invasion in HCC cells, we silenced the expression of membrane CD147 in SMMC-7721 cells and treated these cells with soluble CD147. Unlike normal SMMC-7721 cells, SMMC-7721 cells silenced by membrane CD147 and treated with soluble CD147 cannot induce a significant increase of MMP-9 expression (**Figure 1A**). Moreover, there was no significant increase in cell invasion in these cells which indicated that in order to regulate the invasion of HCC cells, soluble CD147 needs to associate with membrane CD147 (**Figure 1B** and **1C**).

Soluble CD147 increases the invasion ability of SMMC-7721 cells by activating the Akt pathway

Akt-related pathways have been found to be involved in the regulation of HCC cell invasion by up-regulating the expression of MMP-9 [11]. Furthermore, one study suggested that soluble CD147 is potentially involved in Akt activation [9]. To determine whether the Akt-related pathway is also involved in the regulation of HCC cell invasion mediated by soluble CD147, we checked the expression level and activation level of Akt in soluble CD147-treated and untreated cells. As shown in **Figure 2A**, SMMC-7721 cells treated with soluble CD147 can induce phosphorylation and activation of Akt without affecting its expression. To provide further evidence, we silenced the expression of Akt in SMMC-7721 cells and treated them with or without soluble CD147. As shown in **Figure 2B**, cells treated with soluble CD147 induced the phosphorylation and activation of Akt which led to the increased expression of MMP-9. However, silencing of Akt inhibited the increased expression of MMP-9 induction by soluble CD147. Furthermore, we also checked the invasion ability in these cells. SMMC-7721 cells induced with soluble CD147 increased the amount of transfer cells two-fold. However, this effect can be inhibited by silencing the expression of Akt. All these results indicated that soluble CD147 can increase the invasion of HCC cells by affecting the phosphorylation and activation of Akt.

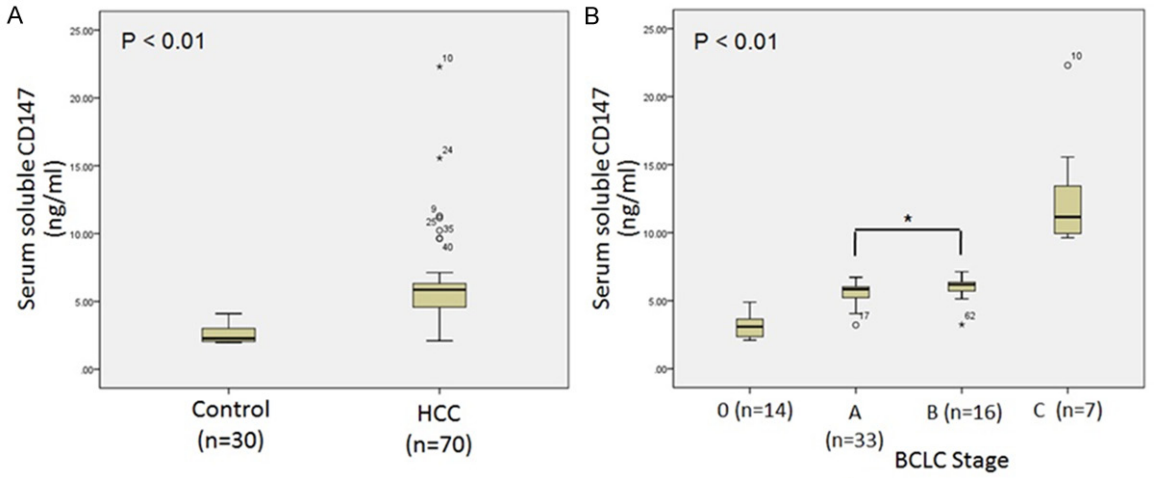

Figure 3. Soluble CD147 is highly present in HCC patients' serum and in more advanced disease stages of HCC. A. Comparison of serum soluble CD147 levels between HCC patients and healthy controls. B. Relationship between serum soluble CD147 levels and BCLC disease stage (*P=0.146).

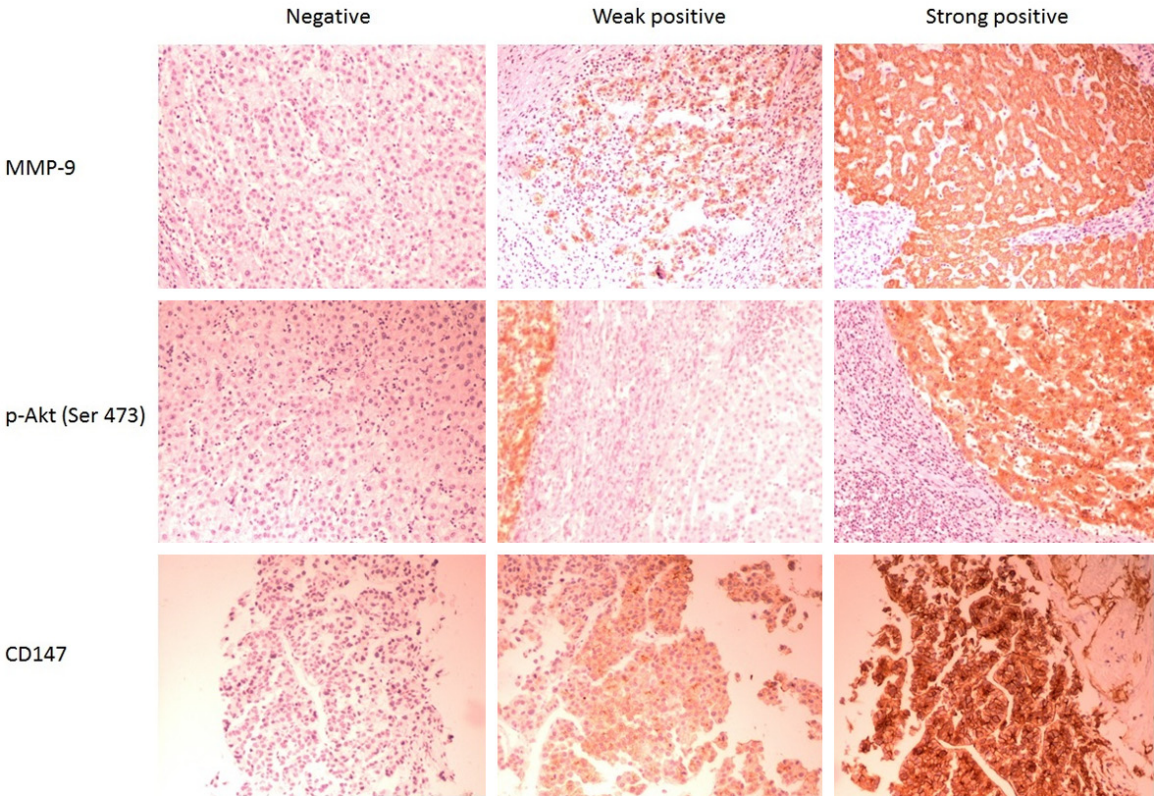

Figure 4. Expression of CD147, p-Akt (Ser 473) and MMP-9 in human HCC tissues assessed by immunohistochemistry (× 200). Representative weak positive, strong positive and negative samples were shown.

Soluble CD147 is increased in serum of HCC patients and is associated with metastasis and invasion of HCC

To evaluate the variation of soluble CD147 concentrations in blood, we tested the serum soluble CD147 of 70 HCC patients and 30 healthy controls by ELISA. The characteristics of HCC patients and the healthy controls are summarized in **Table 1**. There was no significant difference in age and sex. However, hepatitis is more frequent in the HCC group, which is mainly

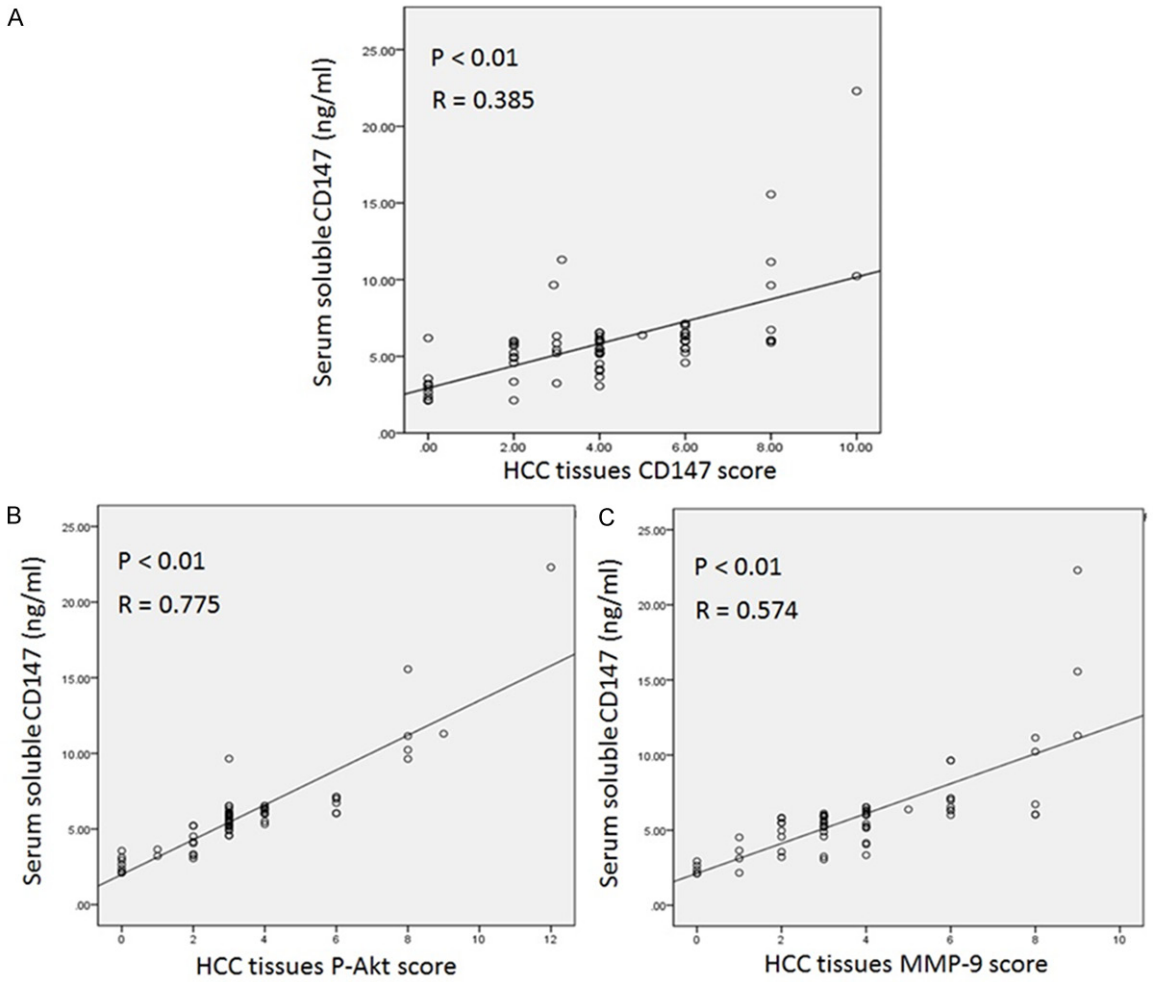

Figure 5. Correlation between serum soluble CD147 and tissue CD147, MMP-9 and p-Akt (Ser 473) expression scores. A. Relationship between soluble CD147 and HCC tissue surface CD147 expression (r=0.385; P < 0.01). B. Relationship between soluble CD147 and HCC tissue p-Akt (Ser 473) expression (r=0.775; P < 0.01). C. Relationship between soluble CD147 and HCC tissue MMP-9 expression (r=0.574; P < 0.01).

because most of the HCC cases present with a background of hepatitis, which is considered to be a risk factor of HCC.

As shown in **Figure 3A**, when compared with the control arm (median: 2.28 ng/ml; range: 1.98-4.10 ng/ml), HCC patients have significantly higher serum soluble CD147 levels (median: 5.87 ng/ml; range: 2.10-22.30 ng/ml; P < 0.01). When correlated with disease stage, a significant difference of serum CD147 was discovered between BCLC stages 0 and A, 0 and B, 0 and C, A and C, B and C. Serum CD147 was increased with increasing disease stage (median: 0: 3.09 ng/ml A: 5.84 ng/ml B: 6.19 ng/ml C: 11.15 ng/ml) However, there was no significant difference between stage A and stage B disease (P=0.146). It is well known that BCLC stage is closely related to the invasion and metastasis of HCC and when combined with the results demonstrating that serum soluble CD147 increases with increasing disease stage, it potentially shows that soluble CD147 is also associated with the metastasis and invasion of liver cancer. More specifically, the IHC of MMP-9, which has been shown to be positively related to metastasis and invasion of liver cancer, was applied to the HCC tissues (**Figure 4**). In total, 53 of 70 HCC cases (75.7%) indicated MMP-9 overexpression and the IHC score of MMP-9 expression in tumors was positively related to the concentration of soluble CD147 in serum (r=0.574, P < 0.01) (**Figure 5C**).

As a result, soluble CD147 is highly present in the serum of liver cancer patients and is associated with the metastasis and invasion of HCC.

Serum soluble CD147 is positively associated with CD147 expression in cancerous tissue and is closely related to activation of Akt in HCC tissue

Some recent studies have shown that soluble CD147 originates mainly from the shedding of membrane-bound CD147. Moreover, it was demonstrated that soluble CD147 can induce the expression of surface CD147 which, in turn, increases the level of soluble CD147. As a result, we think that soluble CD147 stimulated expression of surface CD147, displaying an autocrine CD147 feedback loop in HCC cells. To validate this conclusion, we analyzed the CD147 expression in HCC tissues and correlated it with serum soluble CD147 levels in patients and found that there indeed was a clear positive correlation between them ($r=0.385$, $P < 0.01$) (**Figure 5A**).

In a preclinical model, our group has clarified that soluble CD147 increases the invasive characteristics of SMMC-7721 cells via an activated Akt-related pathway. To validate this result in HCC patients, the immunohistochemistry of p-Akt (Ser473) was applied to 70 HCC tissues and the scores of p-Akt (Ser473) were correlated with the concentration of serum CD147 corresponding to the same patient. In total, 52 of 70 HCC cases (74.2%) demonstrated overexpression of p-Akt (Ser473) and the IHC score of p-Akt (Ser473) expression in tumors was positively related to the concentration of soluble CD147 in serum ($r=0.775$, $P < 0.01$) (**Figure 5B**). These results clearly indicate that Akt is over-activated in HCCs and the activation is positively correlated with the serum CD147 level.

Discussion

As of date, soluble CD147 has been found to exist in the plasma, serum and urine of various cancer patients and has been recognized as a potential diagnostic biomarker for many types of cancers [9, 13]. In one recent study, Wu and colleagues found that full-length soluble CD147 was involved in the regulation of MMP-2 expression in HCC [9]. In this present study, we examined the role of soluble CD147 in the regulation of MMP-9 expression and cell invasion in SMMC-7721 cells. Our results suggested that SMMC-7721 cells treated with soluble CD147 significantly increased the expression of MMP-9 in tumor cells and, as a result, doubled the invasive ability of these cells. Moreover, we also clarified that surface CD147 plays a key role in the regulation of HCC cell invasion mediated by soluble CD147. It is well understood that both tumor cells and cancer-associated fibroblasts are able to regulate HCC invasion through MMP (mainly MMP-2 and MMP-9) production. However, in our study, we did not validate the effects of soluble CD147 in tumor-stroma cells and evidence from other studies has suggested that membrane CD147 also exists in cancer-associated fibroblasts [14]. As we demonstrated that soluble CD147 regulates cancer-related cellular functions by homotypic interactions with surface CD147, this effect might also exist in tumor-stroma cells in which soluble CD147 induces the expression of MMPs. Nonetheless, further studies need to be performed in order to clarify this matter.

Increased PI3K/Akt activation has been shown to significantly increase the proliferation of HCC cells and prevent HCC cell apoptosis [15]. Moreover, activation of Akt is also associated with the migratory activity and invasiveness of HCC cells [16] and is considered to be an independent prognostic biomarker for HCC patients [17]. Akt-related pathways have be found to be involved in the regulation of HCC invasion capability by up-regulating the expression of MMP-9 [11]. Our data suggests that the Akt-related pathway is involved in the regulation of invasion of HCC cells mediated by soluble CD147. Induction by soluble CD147 dramatically increased the phosphorylation of Akt (Ser473) and subsequently increased the expression of MMP-9. Down-regulation of Akt inhibited the soluble CD147-mediated up-regulation of MMP-9 in HCC cells. Similar results can also be found in the invasion assays demonstrating that silencing of Akt inhibits the increase of cell invasion ability induced by soluble CD147. As a result, we speculate that soluble CD147 may activate Akt-related pathways to regulate the expression of MMP-9 and, ultimately, may mediate the invasion ability of HCC cells.

Expression of CD147 in tumor tissues is found to be closely related to disease-free survival and overall survival in patients with various

types of cancers. Overexpression of CD147 contributes to the progression, recurrence and therapy resistance in many cancers [18-20]. As a result, tumor CD147 expression may be considered as a diagnostic and prognostic biomarker for cancers. In our study, by analyzing 70 HCC patients and 30 healthy controls, we confirmed that soluble CD147 is present at high levels in the serum of HCC patients and that the concentration of serum CD147 positively correlates with the expression of CD147 in the cancer tissue. Moreover, we also found that soluble CD147 levels are positively related with tumor MMP-9 expression, Akt phosphorylation and disease stage of the patient (no significance between stage A and stage B disease). Combining all these findings together, we believe that the result we validated in this preclinical model is also suitable for the clinic: soluble CD147 can induce the activation of Akt-related pathways and, in doing so, affects the expression of MMP-9. As a result, we speculate that serum soluble CD147 also plays a role in HCC progression and that it can be used as a diagnostic and prognostic biomarker for HCC patients.

In the present study, we found that soluble CD147 induces the expression of MMP-9 from HCC cells by cooperating with membrane CD147 and an activated Akt-related signaling pathway. Ultimately, soluble CD147 can significantly increase the invasive ability of HCC cells.

In HCC patients, we found that serum soluble CD147 was elevated when compared to healthy controls and that it was positively associated with tumor surface CD147, tumor p-Akt (Ser473) and tumor MMP-9 expression. All of these results validate that soluble CD147 regulates the invasion ability of HCC by affecting the phosphorylation of Akt and subsequently inducing the expression of MMP-9. Our results also showed that serum soluble CD147 levels were higher in later disease stages when compared to earlier stages, which supports the use of serum soluble CD147 as a diagnostic and prognostic biomarker in HCC patients.

In conclusion, this study provides new insight into the role of soluble CD147 in HCC progression.

Disclosure of conflict of interest

None.

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