

Useful detection of CD147 (EMMPRIN) for pathological diagnosis of early hepatocellular carcinoma in needle biopsy samples

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The expression of this protein was significantly elevated in HCC tissue specimens from patients with a low value of serum AST and γ -GTP.

CONCLUSION: CD147 serves potentially as a pathological target for cancer detection of early HCC.

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Key words: CD147; Hepatocellular carcinoma; Needle biopsy

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Abstract

AIM: To make clear whether CD147 (EMMPRIN) expression in pathological tumor samples with a fine-needle aspiration biopsy is useful for pathological diagnosis of early hepatocellular carcinoma (HCC).

METHODS: Twenty-two patients (15 men and 7 women; median age 68 years, range 56-81 years) underwent a liver tissue biopsy in order to make a diagnosis of HCC. Paraffin-embedded liver biopsy tissue samples from 22 patients were stained with anti-CD147 antibody, murine monoclonal antibody 12C3 (Mab12C3) for immunohistochemical analysis. An immunohistochemical analysis of CD147 was performed and the degree of staining compared between tumor and non-tumor tissue. In addition, the degree of staining within tumor tissue was compared according to a number of clinicopathological variables.

RESULTS: The degree of staining of CD147 was significantly higher in tumor tissues than non-tumor tissues, even in tumors less than 15 mm in diameter.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide, involving more than 500 000 new cases yearly, with an age-adjusted incidence of 5.5-14.9 per 100 000 people^[1]. In some areas of Asia and the Middle East, HCC ranks as the most frequent cancer-related cause of death^[2]. The incidence of HCC is also increasing in Europe and the United States^[3]. The early detection of tumors and development of therapies for HCC is likely to improve the prognosis^[4]. Nevertheless, despite improvements in both diagnostic modalities and therapy, in many cases an accurate diagnosis still cannot be confirmed even with diagnostic imaging and the recognition of tumor markers in the serum. Particularly, hypovascular HCC which is often difficult to recognize by computed tomography (CT) requires ultrasound (US) examination for a definitive diagnosis. Tumor biopsy is an important method of evaluation in these cases, particularly in small tumors, less than 15 mm in diameter. Therefore, more sensitive tumor markers for pathological diagnosis are required.

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN) or basigin, is a transmembrane glycoprotein with two immunoglobulin-like

domains. This is part of a family of proteins that includes embigin and neuropilin^[5]. Tumor cell CD147 triggers the production or release of matrix metalloproteinases in the surrounding mesenchymal cells and tumor cells, thereby contributing to tumor invasion^[6-9]. A very high incidence of CD147 expression (> 80% of CD147-positive cases) is detected by immunohistochemical staining in HCC^[10].

A previous paper reported a murine monoclonal antibody (MAB12C3), specific to human ovarian carcinomas was generated by immunizing mice with the human ovarian germinoma cell line (JOHY-2)^[11]. In further research, using phage display libraries, MAB12C3 hybridized with the extracellular region of CD147^[12]. The MAB12C3 reacted with 67.7% (21 of 31 cases) of epithelial ovarian carcinomas, but not with any of benign epithelial ovarian adenomas tested^[11].

Despite extensive studies on small early stage HCCs, the morphological criteria for definite diagnosis of well-differentiated, small HCCs are still questionable^[13]. This study considered whether the use of MAB12C3 against CD147 protein could recognize early stage HCC. MAB12C3 was used for an examination of antigen expression in early HCC tissue specimens and to identify any correlations between the immunohistochemical findings and the clinicopathologic characteristics of the tumors. In this study, small biopsy samples from HCC were examined with immunohistochemical staining. If significant differences are recognized between HCC with non-tumor liver tissues, CD147 may therefore be effective as a diagnostic and therapeutic target in early stage HCC.

MATERIALS AND METHODS

Patients

The study population included 22 patients (15 men and 7 women; median age 68 years, range 56-81 years) who underwent tumor and non-tumor liver tissue biopsy between January 2003 and December 2005, in the Jikei University Daisan Hospital, Tokyo, Japan (Table 1). All patients underwent biopsies to confirm a diagnosis of HCC. These tissue specimens were examined retrospectively. This study was approved by the Jikei University Ethics Committee Institutional Review Board.

Pathologic specimens

Tumor specimens were obtained by a tumor biopsy with a 21 G fine-needle aspiration kit. Non-tumorous liver tissue specimens were obtained by an 18-20 G needle liver biopsy concurrently. Formalin-fixed, paraffin-embedded specimens of liver tumors and non-tumor liver tissues were processed for conventional histologic assessment by hematoxylin and eosin (HE) staining. The tumors were histologically graded (well or moderately differentiated).

Immunohistochemical analysis

For the immunohistochemical analysis, formalin-fixed, paraffin-embedded specimens were dewaxed and used. The specimens were stained using the labeled streptavidin-biotin peroxidase complex method with the Ventana auto-immunostaining system (Ventana Japan, Yokohama,

Table 1 Characteristics of patients undergoing tumor biopsies (*n* = 22)

Features	Median value
Age	68 (56-81)
Sex (Male/Female)	15/7
Plt ($\times 10^3/\mu\text{L}$)	10.0 (5.1-24.5)
AST (IU/L)	68 (21-147)
ALT (IU/L)	61.5 (6-214)
T-Bil (mg/dL)	0.8 (0.4-2.3)
γ -GTP (IU/L)	48 (18-665)
AFP (ng/mL)	21.5 (3-444)
HBs Ag/HCV Ab/Others	3/18/1
Tumor size (mm)	14.5 (8-23)
Cirrhosis (positive/negative)	5/17
Differentiation (well/moderate)	15/7

Data values are expressed as the medians with ranges in parentheses unless indicated otherwise. Normal ranges: Plt (platelet count), $15-35 \times 10^3/\text{L}$; AST (aspartate aminotransferase), 10-33 IU/L; ALT (alanine aminotransferase), 6-35 IU/L; T-Bil (total bilirubin), 0.2-1.2 mg/dL; γ -GTP (γ -glutamyl transferase), 10-50 IU/L; AFP (α -fetoprotein), > 20 ng/mL.

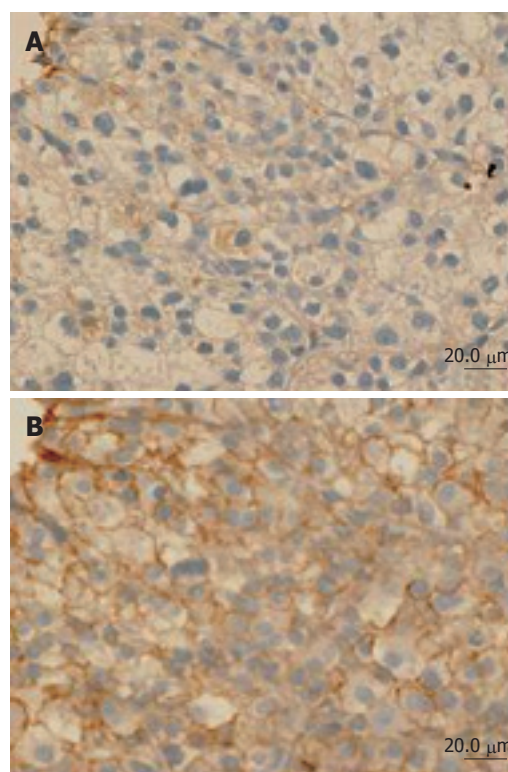


Figure 1 CD147 protein expression of HCC tissues with microwave-stimulated processing. **A:** 10 min of 10 mmol/L citrate buffer (pH 6.0); **B:** 30 min of DAKO antigen retrieval solution.

Japan). A murine monoclonal antibody against CD147 protein, MAB12C3^[12], was used as the primary antibody (manufactured at Department of Biochemistry 1, Jikei University School of Medicine, Japan). The antigen retrieval procedure was performed with a microwave oven in DAKO antigen retrieval solution for 30 min at 95°C to efficiently stain the sample. The immunohistochemical staining was strongest, when performed in a microwave oven in DAKO antigen retrieval solution (Figure 1). The

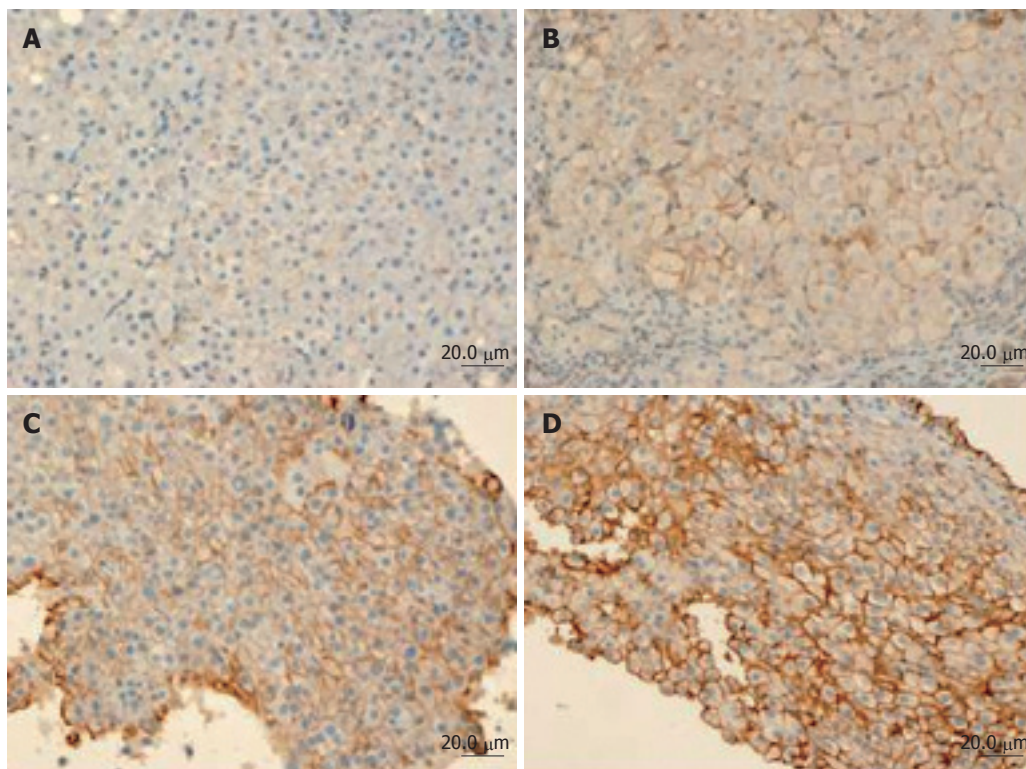


Figure 2 CD147 protein expression of non-tumor liver tissue and HCC tissue specimens. **A:** Non-tumor tissue specimen, very weak expression; **B:** Non-tumor tissue specimen, weak expression; **C:** HCC tissue, moderate expression; **D:** HCC tissue, strong expression.

Table 2 Immunohistochemical scales of tumor and non-tumor biopsy specimens

4-step scales	Tumor tissues <i>n</i> (%)	Non-tumor tissues <i>n</i> (%)	<i>P</i>
Very weak (0)	1 (4.5)	7 (31.8)	< 0.01
Weak (1)	6 (27.3)	6 (27.3)	
Moderate (2)	3 (13.6)	6 (27.3)	
Strong (3)	12 (54.5)	3 (13.6)	
Total	22 (100)	22 (100)	

sections (DAKO Cytomation, Glostrup, Denmark) were developed with 3, 3'-diaminobenzidine with 0.3% H₂O₂ and counterstained with hematoxylin.

For each tissue sample, the fraction of the immunostained cells was recorded, and the staining intensity was estimated using a 4-step scale (0, 1, 2, 3). The tissue specimens were then initially categorized according to arbitrarily predefined criteria into 4 groups, including completely very weakly positive, strongly positive, and 2 intermediate groups. The exact criteria for these groups were as follows: very weak (1+ staining in some cells) (Scale 0); weak (1+ staining in cells) (Scale 1); moderate (2+ staining in cells) (Scale 2); strong (3+ staining in cells) (Scale 3). The examiners were blinded to patients' clinical and histological (HE staining) profile. Two investigators (H.H. and K.N.) evaluated the staining levels independently, after which any discordant evaluations were adjusted by connected microscopes and scored jointly.

Statistical analysis

Statistical analyses were performed by the Wilcoxon signed-rank test and two-sample Wilcoxon rank-sum

(Mann-Whitney) test. *P*-Values < 0.05 were considered statistically significant. All these analyses were performed using STATA 9.1 (STATA Corporation, College Station, Texas, USA).

RESULTS

CD147 expression in HCC and non-tumorous liver tissue

Among all 44 tissues (22 HCC and 22 non tumorous liver tissues), CD147 immunoreactivity was detected on all cell membranes. As shown in Figure 2, CD147 was positively but weakly stained on most non-tumor liver tissues, because the antigenicity was activated by microwave-stimulated processing with 30 min treatment of DAKO retrieval solution (Figure 2B). However, a significant difference was observed in CD147 expression between HCC and non-tumor liver tissues (Table 2, Figure 2). In fact, there was significantly greater expression of CD147 in the carcinoma tissue specimens than in non-tumorous liver tissue specimens, including small tumors measuring less than 15 mm in size (*P* < 0.05).

CD147 expression in tumour aspirates correlates with clinical variables

Twenty-two HCC biopsy specimens were categorized into two groups for each clinical variable, above or below the median value. In these two groups, the CD147 intensity was compared. As illustrated in Figure 3, with regard to tumor size, CD147 was highly expressed in large tumors. In contrast, in the detection of serum AST and γ -GTP level, CD147 was more significant in low value groups. No significant differences were observed by other clinical parameters, such as serum AFP level. In addition, although the CD147 intensity was compared between tumor HCC

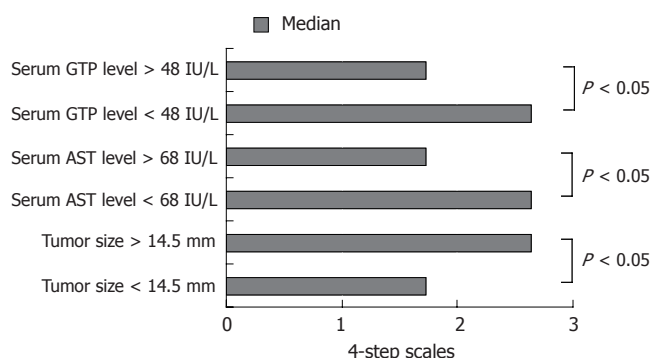


Figure 3 Comparison of CD147 intensity between categories of clinicopathological variables in tumor biopsies.

associated with and without cirrhosis and between well-differentiated and moderately differentiated HCC, no significant difference was observed (Figure 4).

DISCUSSION

CD147 is an adhesion molecule that binds to endothelial cells and fibroblasts, and stimulates the expression of several matrix metalloproteinases associated with the invasiveness of HCC^[14-16]. In a recent study, a significantly positive correlation was identified between the CD147 immunostaining intensity and the histological grading and clinical stage of HCC^[17]. Moreover, this marker may be valuable to differentiate between benign liver nodules and HCC, especially in small lesions with good differentiation^[17]. Most previous reports were based on the analyses of surgically removed tissues. This study observed tumor nodules obtained by the 21G fine-needle aspiration kit before treatment. To enhance the diagnostic efficacy, the most sensitive detection of CD147 in HCC tissues was also investigated. Therefore, the tissue specimens were stained using microwave-stimulated processing with DAKO antigen retrieval solution. The antigenicity of the CD147 protein was activated with microwave-stimulated processing which resulted in the expression of CD147 in some non-tumor tissues. This result showed that the peritumoral tissues, pathologically diagnosed as the non-tumor tissue, may be related to the biological development of the malignant phenotype with CD147. Nevertheless, the CD147 expression in tumor lesions was significantly stronger, even in small tumors. Furthermore, the non-tumorous normal liver tissue and tumor tissue demonstrated significant differences in the expression of the antigen, even in small tumors measuring less than 15 mm in size. This indicates that this marker is therefore useful in the diagnosis of small HCC and also when considering the most appropriate treatment.

Previous studies have demonstrated that CD147 can potentially serve as a target for antitumor therapy. They showed that the CD147 expression could frequently be detected in the vast majority of human malignancies as well as in a subset of benign tumors. Nevertheless, there are significant differences both in the intensity and distribution of CD147 staining among different malignant tumors as well as benign lesions^[10]. In fact, tumors

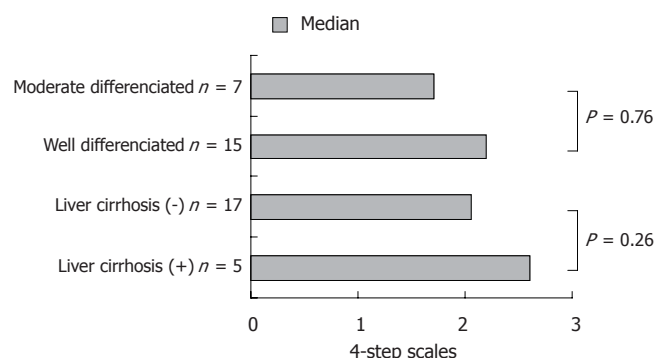


Figure 4 Comparison of the CD147 intensity in tumor biopsy specimens: cirrhosis and tumor differentiation.

expressing high levels of CD147 compared to their normal counterparts include carcinomas of the urinary bladder^[18], breast, lung^[19,20], oral cavity^[21], esophagus^[12], skin^[22], malignant lymphomas^[23,24], and malignant peripheral nerve sheath tumors^[25].

In this study, the staining intensity of CD147 was confined to cell membrane expression of this antigen. CD147 expressed on the tumor cell surface and stimulates nearby fibroblasts and endothelial cells^[26-28]. CD147 has been shown to be an important mediator of tumor-stroma cross-talk, based on the findings that it mediates not only MMP production but also angiogenesis via the stimulation of vascular endothelial growth factor (VEGF)^[29], and anchorage-independent growth and multi-drug resistance in a hyaluronan-dependent fashion^[7,30,31]. Further investigation is necessary to examine the expression of CD147 associated with fibrosis.

In this study, expression of CD147 in HCC biopsies was much stronger than the peritumoral tissue. This result may illustrate the intensity of CD147 expression in a tumor biopsy is rare in peritumoral tissues.

The CD147 expression was higher in the HCC specimens from patients with lower levels of serum AST and γ -GTP. This result may indicate that the CD147 expression of HCC can thus be determined even when the liver function is weak.

In this study, the HCC tissue biopsy specimens were small in size, therefore we could not use an automated method to objectively evaluate the expression of CD147. We therefore require further examinations be used to evaluate the automated method.

In conclusion, HCC tissue biopsy specimens, even from small tumors, expressed CD147 protein at significantly higher levels than non-tumorous liver tissue. The immunohistochemical analysis of the murine monoclonal antibody, MAb12C3, is very useful for the detection of HCC in even needle biopsy specimens. Therefore, CD147 can potentially serve as a useful target for cancer detection in HCC.

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