Identification of CD206 as a potential biomarker of cancer stem-like cells and therapeutic agent in liver cancer

WEIMIN FAN1,2, XUE YANG2, FANG HUANG3, XIANGMIN TONG2, LIFEN ZHU2 and SHIBING WANG2

1The Second Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310000; 2Clinical Research Institute, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014; 3Department of Pathology, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China

Received December 4, 2018; Accepted June 13, 2019

DOI: 10.3892/ol.2019.10673

Abstract. The mannose receptor (CD206) functions in endocytosis and phagocytosis, and plays an important role in immune homeostasis. Tumor-associated macrophages express high level of CD206 and are thought to contribute to cancer progression through tumor immunosuppression, metastasis and angiogenesis. However, the significance of CD206 in the pathology of liver cancer has not been investigated. The present study evaluated the clinical significance of CD206 in the progression and prognosis of liver cancer in pathological tissues from 327 patients. Increased CD206 expression was observed in liver cancer samples compared with healthy adjacent liver tissue (42.8 vs. 62.4%; P<0.05). CD206 expression was significantly associated with tumor size (P=0.009) and metastasis (P=0.041). The recurrence free survival rate of patients with CD206-positive liver cancer was significantly decreased compared with patients with CD206-negative liver cancer (P=0.003). A Cox regression model revealed that liver cancer survival was independently associated with tumor size, metastasis and α-fetoprotein value. The results further revealed that CD206 expression in cancer stem cell (CSC)-like cells was comparable to other internationally recognized biomarkers. Additionally, when CD206 expression was silenced in the liver cancer cell lines HepG2 and PLC/PRF/5 using a short hairpin RNA approach, migration and invasion of the cells significantly decreased compared with controls (P<0.01). CD206 expression in liver cancer significantly influences distant metastasis and spread, resulting in poor patient prognosis. Furthermore, CD206 may be a potential biomarker in CSC-like cells to predict the occurrence of liver cancer.

Introduction

Liver cancer had the seventh highest age-adjusted incidence rate of all types of cancer in the United States of America in 2014, often with high mortality (1-3). Liver cancer requires the detection of small tumors that are often present in asymptomatic individuals (4). Liver cancer is diagnosed by imaging modalities, including computerized tomography and magnetic resonance imaging scans, followed by confirmation via liver biopsy, an invasive procedure (5-7). Although the diagnosis of liver cancer has improved rapidly in recent years, <5% of patients with liver cancer survive >5 years following diagnosis (8,9). Previous studies suggest that high invasiveness and metastasis are the main causes of poor prognosis (10,11). Early liver cancer diagnosis is of paramount importance to therapy and more effective biomarkers to predict the clinical outcome of liver cancer are required.

The mannose receptor (CD206), also known as C-type lectin, is expressed on the surface of macrophages and some subsets of immature dendritic cells (12). CD206 participates in antigen presentation, macrophage endocytosis and is considered a hallmark of tumor-associated macrophages (13). CD206 increased the growth and migration of microglia by promoting their activation (14). Additionally, CD206 expressed on lymphatic endothelium participates in the attachment of various cancer cells to lymphatic endothelium to promote lymphatic metastasis (15-17). Serum CD206 is elevated in patients with multiple myeloma and is a prognostic marker for overall survival (18,19). Furthermore, CD206 has been reported as a novel biomarker for the diagnosis of patients with colorectal and gastric cancer (20,21). Therefore, the investigation of the potential role of CD206 in liver cancer may be beneficial to patients.

The present study evaluated the clinical significance of CD206 in the progression and prognosis of liver cancer. A suspension culture was used to enrich liver cancer stem cell (CSC)-like cells, which acquire the properties of liver CSCs in term of self-renewal, differentiation, quiescence, chemo-resistance and tumorigenicity (22,23). The results obtained
indicated that CD206 may act as a biomarker in CSC-like cells to predict liver cancer occurrence. CD206 promoted the motility and invasiveness of liver cancer cell lines in vitro. Furthermore, it was revealed that the upregulation of CD206 in liver cancer contributes to poor patient prognosis. Thus, novel therapeutic agents targeting CD206 may be beneficial for patients with liver cancer.

Materials and methods

Liver cancer samples and cell lines. The tissue microarrays (TMAs) used in the current study were purchased from Shanghai Biochip Co. Ltd. Written informed consent was obtained from all the patients and the protocol was approved by the Medical Ethics Review Committee of the Zhejiang Provincial People's Hospital. The experiment began in August 2013 and ended in August 2018. The inclusion criteria were as follows: i) diagnosis of hepatocellular carcinoma, ii) patients who signed informed consent and iii) patients with adequate hepatocellular carcinoma tissue to make tissue microarrays (cylindrical liver cancer tissue at least 1 mm in diameter and 1 mm in length). In the tissue microarrays, all the patients had complete clinical data. A total of 327 patients with liver cancer were divided into the following groups according to their respective characteristics: i) age (<55 or ≥55 years) (24), ii) gender (male or female), iii) tumor size (diameter <5 or ≥5 cm) (25), iv) tumor number (single or multiple) (26), v) Edmondson Grade (I+II or III) (27), vi) metastasis (M0 or M1) (28), vii) micro-vascular invasion (absent or present) (29,30), viii) Hepatitis B virus antigen (negative or positive) (31), ix) cirrhosis (negative or positive) (32), x) α-fetoprotein (AFP; <20 or ≥20 µg/l) (33).

Survival time was calculated from the date of surgery to the deadline of 60 months. The liver cancer cell lines HepG2 and PLC/PRF/5 (Chinese Academy of Sciences) were used to study the motility and invasiveness of liver cancer cells. HepG2 and PLC/PRF/5 cells were seeded on 6-well plates (Corning Inc.). Fresh DMEM/F12 with 20 ng/ml human recombinant basic fibroblast growth factor (Merck KGaA), 20 ng/ml human recombinant epidermal growth factor (Merck KGaA), 2% B27 supplement without vitamin A (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/mL penicillin and 100 µg/ml of streptomycin in ultra-low attachment 6-well plates (Corning Inc.). Fresh DMEM/F12 with 20 ng/ml human recombinant basic fibroblast growth factor, 20 ng/ml human recombinant epidermal growth factor, 2% B27 was added to the ultra-low attachment plates every 2 days. Four days later, the tumor spheres were collected by gentle centrifugation with 100 x g for 4 min at 37°C and digested by Accutase (Sigma-Aldrich; Merck KGaA) for 5 min at 37°C to form a single cell suspension for subsequent experiments.

Staining patterns and evaluation. The degree of CD206 immunostaining was evaluated blindly by three pathologists. CD206 expression evaluation was based on the intensity of stained tumor cells. A total of five fields of view were randomly selected in each microarray. Scores were used to represent the intensity of the staining in the cytoplasm or membrane in the microarrays. '0' was non-stained, '1' was weakly stained as light yellow, '2' was moderately stained as brown and '3' was heavily stained as dark brown. In total, five fields of view were observed and scored according to the aforementioned rules. CD206 scores were calculated with scores of 0-5 and 6-12 representing the low and high expression groups, respectively.

Sphere culture and sphere passage. PLC/PRF/5 cells were resuspended to a cell density of 5000 cells/ml, washed to remove serum and suspended in serum-free DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (Merck KGaA), 20 ng/ml human recombinant epidermal growth factor (Merck KGaA), 2% B27 supplement without vitamin A (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/mL penicillin and 100 µg/ml of streptomycin in ultra-low attachment 6-well plates (Corning Inc.). Fresh DMEM/F12 with 20 ng/ml human recombinant basic fibroblast growth factor, 20 ng/ml human recombinant epidermal growth factor, 2% B27 was added to the ultra-low attachment plates every two days. Four days later, the tumor spheres were collected by gentle centrifugation with 100 x g for 4 min at 37°C and digested by Accutase (Sigma-Aldrich; Merck KGaA) for 5 min at 37°C to form a single cell suspension for subsequent experiments. Tumor spheres were centrifuged with 100 x g for 3 min at 37°C to remove the enzyme and resuspended with 20 ng/ml human recombinant basic fibroblast growth factor, 20 ng/ml human recombinant epidermal growth factor and 2% B27 in ultra-low attachment 6-well plates (Corning Inc.) and allowed to reform spheres. The spheres were passaged every 4 days.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). HepG2 and PLC/PRF/5 cells transfected with shCD206 and shNC were washed three times with PBS, and the total RNA was extracted using Trizol (Invitrogen; Thermo Fisher Scientific, Inc.). The extracted RNA was reverse-transcribed into cDNA using PrimeScript™ 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.). qPCR was performed using a KAPA SYBR Green qPCR kit (Roche Diagnostics). The following primer pairs were used: CD206 forward, 5'-GCAGAAGGAGTAAACCACC-3' and reverse,
5'-TGGCACAATGAGCGGTTCGTTTG-3'; Nanog homeobox (Nanog) forward, 5'-AAGGCCCTACACCTGCTCA-3' and reverse, 5'-ACATTAAGGCCCTTCCCACGC-3'; POU class 5 homeobox 1 (Oct4) forward, 5'-GCCGAAAGAGAGAACG CGA-3' and reverse, 5'-ACCCACACATCGGACCACCT CG-3'; SRY-box 2 (Sox2) forward, 5'-TTTTGTCGAAGAC GGAGAACGC-3' and reverse, 5'-TAACTGTCCCATGCGCTGG TT-3'; MYC-binding protein (c-Myc) forward, 5'-GCCAT ACCTGTGGTCTCC-3' and reverse, 5'-CGTCTGGTCTGCAGGCA AACAAGTC-3'; CD44 molecule (CD44) forward, 5'-AGC AACAGCAGACAGCAACCA-3' and reverse, 5'-CTGACACCAG CATTTGTGTTGT-3' and GAPDH forward, 5'-GCTCCCCCTT TTCTTGGACA-3' and reverse, 5'-GGTTGCTGAGGTAGAT CTTTGCC-3'. The following thermostyling conditions were used: Pre-denaturation at 95˚C for 5 min, 35 cycles of 95˚C for 10 sec, 60˚C for 30 sec and, 72˚C for 30 sec and extension at 72˚C for 10 min. mRNA levels were quantified using the 2-ΔΔCt method (20) and normalized to GAPDH. RT-qPCR was performed in triplicate.

Transfection. The short hairpin (sh) CD206 (5'-CGACAA GGAGCTAACCACCC-3') and sh negative control (NC) (5'-GATCCGACTCTTATAAGGCTTC-3') were purchased from GeneCopoeia, Inc. The QIAGEN Plasmid Mini kit (Qiagen GmbH), EndoFectin-Lenti™ (GeneCopoeia, Inc.) and TiterBoost™ reagents (GeneCopoeia, Inc.) were used to generate plasmids delivering shNC and shCD206. The plasmids were co-transfected into 293Ta cells (Chinese Academy of Sciences) with Lenti-Pac™ HIV packaging mix (cat. no. HA1006; 1:5,000; HuaBio) for 1 h at room temperature.

Western blot analysis. HepG2 and PLC/PRF/5 cells stably expressing shCD206 or shNC were lysed using lysis buffer (Beyotime Institute of Biotechnology), 1% complete mini-protease inhibitor cocktail (Roche Diagnostics) and 5 mM sodium fluoride. The total protein was quantified using a bicinchoninic acid assay (Thermo Fisher Scientific, Inc.) and heated for 10 min at 100˚C. A total of 30 µg protein/lane was separated via SDS-PAGE on a 12% gel and transferred to a nitrocellulose membrane (Merck KGaA). The membrane was blocked for 1 h at 37˚C and immunoblotted with primary antibodies against CD206 (cat. no. ab8918; 1:1,000; Abcam), Nanog (cat. no. ab109250; 1:1,000; Abcam), Oct4 (cat. no. ab18976; 1:1,000; Abcam), Sox2 (cat. no. ab79351; 1:1,000; Abcam), c-Myc (cat. no. ab39688; 1:1,000; Abcam), CD44 (cat. no. ab189524; 1:1,000; Abcam) and GAPDH (cat. no. ab9485; 1:5,000; Abcam) overnight at 4˚C. Membranes were subsequently washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. HA1001; 1:50,000; HuaBio) or anti-mouse antibodies (cat. no. HA1006; 1:5,000; HuaBio) for 1 h at room temperature. Protein bands were detected using a ChemiDoc™ MP Imaging system (Bio-Rad Laboratories, Inc.) with a super enhanced chemiluminescence detection kit (Applygen Technologies, Inc.).

Wound healing assay. HepG2 and PLC/PRF/5 cells transfected with lenti-shNC and lenti-shCD206 were seeded on separate 6-well plates at a concentration of 1x10^5 cells/ml. When the liver cancer cells reached ~90% confluency, a marker pen was used to draw a horizontal line on the bottom of the 6-well plates. A cross was drawn every 0.5 cm and 5 lines were drawn across each well. A 20 µl pipette tip was used to scratch the cell layer perpendicular to the horizontal lines. PBS was used to wash the cells three times and serum-free DMEM was added into the plates. The cells were cultured at 37˚C with 5% CO2 for 24 h. Images at 5 fields of view were taken and migration was calculated as follows: (Width of the scratch at 0 h-width of the scratch at 24 h)/width of the scratch at 0 h x100%.

Cell invasion assay. HepG2 and PLC/PRF/5 cells transfected with shCD206 and shNC were serum starved for 24 h prior to the invasion assay. Transwell inserts were placed into 24-well plates, 24-well transwell plate (8.0 µm pore size; Corning Inc.) coated with 20 µg Matrigel and incubated for 120 min at 37˚C to promote uniform gel formation. A total of 100 µl cell suspension at a concentration of 3x10^4 cells/ml was added to the upper chamber, and 500 µl 10% FBS-DMEM medium containing cells transfected with shCD206 or shNC were added to the lower chamber. In the control group, 500 µl 10% FCS-DMEM medium was added to lower chamber. Following incubation for 48 h at 37˚C with 5% CO2, the cells were fixed with 4% formaldehyde at 37˚C for 15 min. Cells were stained with 0.5% crystal violet dye at 37˚C for 10 min. Cells on the Matrigel and microporous membrane layer were removed using a cotton swab and cells which invaded the lower microporous membrane were retained. The number of cells in five randomly selected fields of view was counted using an inverted light microscope (magnification, x100).

Statistical analysis. The Statistical Package for the Social Sciences (version 13.0; SPSS Inc.) was used for statistical analysis. Data are presented as the mean ± standard deviation. For comparison between 2 groups, significant differences were determined using the Student's t-test or Wilcoxon rank test. The χ² or Fisher exact tests were used to assess CD206 expression in patients with HCC. The Kaplan-Meier method and the log-rank test were used to analyze survival curves. The multivariate Cox proportional hazards regression model was used to analyze the univariate factors with prognostic significance in HCC. P<0.05 was considered to indicate a statistically significant difference.

Results

CD206 expression in liver cancer samples and healthy tissue. Immunohistochemical methods were used to assess the expression of CD206 in liver cancer tissue. The staining for CD206 was observed predominantly in the membrane and cytoplasm of liver cancer cells. Three pathologists independently evaluated the liver cancer tissue microarrays under x40 (Fig. 1A, C, E and G) and x200 magnification.
FAN et al: HIGH EXPRESSION OF CD206 IN LIVER CANCER

High levels of CD206 expression were detected in 204/327 (62.4%) of the patients with liver cancer. Positive expression was observed in adjacent healthy liver tissue (5 mm from the liver tumor and histopathologically confirmed) in 140/327 (42.8%) patients. Upon dividing samples into high expression (>6 points) and low expression groups (0-5 points), the expression value of CD206 in liver cancer tissues was 7.69±3.11 points, and the expression value of CD206 in adjacent healthy liver tissue was 3.60±2.17 points (P<0.05). Taken together, these data demonstrated elevated CD206 expression in liver cancer tumors.

Patient characteristics and the association between CD206 expression, liver cancer clinicopathological features and prognosis. The present study investigated whether CD206 expression is associated with the progression of liver cancer. CD206 immunopositivity was not associated with gender, age, tumor number, Edmondson grade, microvascular invasion, hepatitis B virus antigen, cirrhosis and AFP (Table I). The survival time for patients with CD206-negative liver cancer was 51.517±1.781 months and was significantly increased compared with patients with CD206-positive liver cancer (46.067±2.183 months). The Kaplan-Meier survival curves indicated that CD206 expression was significantly associated with overall survival in patients with liver cancer (P=0.003; Fig. II). Additionally, prognosis factors in liver cancer were analyzed by Cox-regression analysis. CD206 positivity was significantly associated with tumor size (P=0.039), metastasis (P=0.022) and AFP value (P=0.002). There was no statistically significant association between CD206 expression and gender (P>0.05), age (P>0.05), cirrhosis (P>0.05), metastasis (P>0.05), AFP (P>0.05) and tumor number (P>0.05) as demonstrated by multivariate analysis (Table II). However, CD206 was significantly associated with the Edmondson grade (P=0.009).

CD206 as a biomarker in cancer stem cells may be used to predict liver cancer. The suspension culture in growth factor-defined serum-free medium may be used to enrich cells associated with the traits of CSCs (34,35). To validate whether liver cancer cell lines acquire these traits when passed through the suspension culture, the liver cancer cell line PLC/PRF/5 was subjected to specific serum-free medium. In ultra-low attachment plates, PLC/PRF/5 gradually formed non-adherent spheroid bodies, termed sphere cells, following culture for 4-6 days (Fig. 2A). RT-qPCR was used to assess the expression of recognized biomarker genes (Nanog, Oct4, Sox2, c-Myc and CD44) and CD206 in the CSC spheres and the parent cells from which they were derived (36-38). The results revealed that, although the mRNA levels of CD206 did not increase to the same levels as Nanog, CD44 and Sox2, CD206 expression was comparable to Oct4, and was significantly increased compared with c-Myc (Fig. 2B). These results were validated at the protein level through western blot analysis, where

(Fig. 1B, D, F and H). CD206 showed positive expression in the cell membrane and cytoplasm of liver cancer cells. High levels of CD206 expression were detected in 204/327 (62.4%) of the patients with liver cancer. Positive expression was observed in adjacent healthy liver tissue (5 mm from the liver tumor and histopathologically confirmed) in 140/327 (42.8%) patients. Upon dividing samples into high expression (>6 points) and low expression groups (0-5 points), the expression value of CD206 in liver cancer tissues was 7.69±3.11 points, and the expression value of CD206 in adjacent healthy liver tissue was 3.60±2.17 points (P<0.05). Taken together, these data demonstrated elevated CD206 expression in liver cancer tumors.

Patient characteristics and the association between CD206 expression, liver cancer clinicopathological features and prognosis. The present study investigated whether CD206 expression is associated with the progression of liver cancer. CD206 immunopositivity was not associated with gender, age, tumor number, Edmondson grade, microvascular invasion, hepatitis B virus antigen, cirrhosis and AFP (Table I). The survival time for patients with CD206-negative liver cancer was 51.517±1.781 months and was significantly increased compared with patients with CD206-positive liver cancer (46.067±2.183 months). The Kaplan-Meier survival curves indicated that CD206 expression was significantly associated with overall survival in patients with liver cancer (P=0.003; Fig. II). Additionally, prognosis factors in liver cancer were analyzed by Cox-regression analysis. CD206 positivity was significantly associated with tumor size (P=0.039), metastasis (P=0.022) and AFP value (P=0.002). There was no statistically significant association between CD206 expression and gender (P>0.05), age (P>0.05), cirrhosis (P>0.05), metastasis (P>0.05), AFP (P>0.05) and tumor number (P>0.05) as demonstrated by multivariate analysis (Table II). However, CD206 was significantly associated with the Edmondson grade (P=0.009).

CD206 as a biomarker in cancer stem cells may be used to predict liver cancer. The suspension culture in growth factor-defined serum-free medium may be used to enrich cells associated with the traits of CSCs (34,35). To validate whether liver cancer cell lines acquire these traits when passed through the suspension culture, the liver cancer cell line PLC/PRF/5 was subjected to specific serum-free medium. In ultra-low attachment plates, PLC/PRF/5 gradually formed non-adherent spheroid bodies, termed sphere cells, following culture for 4-6 days (Fig. 2A). RT-qPCR was used to assess the expression of recognized biomarker genes (Nanog, Oct4, Sox2, c-Myc and CD44) and CD206 in the CSC spheres and the parent cells from which they were derived (36-38). The results revealed that, although the mRNA levels of CD206 did not increase to the same levels as Nanog, CD44 and Sox2, CD206 expression was comparable to Oct4, and was significantly increased compared with c-Myc (Fig. 2B). These results were validated at the protein level through western blot analysis, where
increased expression of CD206 was observed in the sphere cells compared with the parent cells (Fig. 2C). Taken together, these data suggest that CD206, similar to the biomarkers Nanog, CD44, Sox2, Oct4 and c-Myc, has the potential to predict cancer occurrence and progression in CSC-like cells.

CD206 silencing decreases liver cancer cell motility and invasion. To assess the role of CD206 in liver cancer cell migration and invasion, HepG2 and PLC/PRF/5 cells were infected with lentiviruses expressing CD206 shRNA (shCD206) or a scrambled sequence (shNC) to obtain stable expression cell lines. CD206 silencing following shCD206 lentiviral transfection was confirmed by Western Blot analysis (Fig. 3A). Migration (Fig. 3B and C) and invasion assays (Fig. 3D and E) revealed that CD206 silencing significantly decreased HepG2 and PLC/PRF/5 cell migration and invasion compared with the shNC group (P<0.05). These observations indicated that CD206 promotes the motility and invasiveness of liver cancer cell lines.

**Discussion**

Liver cancer is a multigene disease characterized by a high degree of malignancy, rapid development, low survival rates and late detection (9,39). Surgical interventions are often ineffective due to late diagnosis (40). Novel liver cancer diagnostic and therapeutic targets are therefore required.

CD206 is a pattern recognition receptor that identifies the extracellular domains of specific carbohydrate molecules and is highly expressed on the surface of macrophages and immature dendritic cells (41). In the present study, CD206 was expressed in the cytoplasm and on the plasma membrane of liver cancer

---

Table I. Expression of CD206 in liver cancer tissue.

| Clinical parameter                  | Number | Low | High | χ²  | P-value |
|-------------------------------------|--------|-----|------|-----|---------|
| Age (years)                         |        |     |      |     |         |
| <55                                 | 126    | 42  | 84   | 1.601 | 0.206   |
| ≥55                                 | 201    | 81  | 120  | 0.745 | 0.388   |
| Gender                              |        |     |      |     |         |
| Male                                | 266    | 103 | 163  |     |         |
| Female                              | 61     | 20  | 41   |     |         |
| Size                                |        |     |      |     |         |
| <5                                  | 191    | 83  | 108  | 6.913 | 0.009   |
| ≥5                                  | 128    | 37  | 81   | 0.003 | 0.956   |
| Tumour number                       |        |     |      |     |         |
| Single                              | 269    | 101 | 168  | 0.003 | 0.956   |
| Multiple                            | 58     | 22  | 36   |     |         |
| Edmondson grade                     |        |     |      |     |         |
| I+II                                | 202    | 68  | 134  | 3.771 | 0.052   |
| III                                 | 119    | 53  | 66   |     |         |
| Metastasis                          |        |     |      |     |         |
| M0                                  | 294    | 105 | 189  | 4.159 | 0.041   |
| M1                                  | 27     | 15  | 12   |     |         |
| Microvascular invasion              |        |     |      |     |         |
| Absence                             | 122    | 49  | 73   | 0.118 | 0.732   |
| Presence                            | 121    | 46  | 75   |     |         |
| Hepatitis B virus antigen            |        |     |      |     |         |
| Negative                            | 62     | 29  | 3    | 3.101 | 0.078   |
| Positive                            | 259    | 90  | 169  |     |         |
| Cirrhosis                           |        |     |      |     |         |
| Negative                            | 110    | 41  | 69   | 0.008 | 0.928   |
| Positive                            | 217    | 82  | 135  |     |         |
| α-fetoprotein                       |        |     |      |     |         |
| <20                                 | 143    | 49  | 94   | 0.090 | 0.764   |
| ≥20                                 | 123    | 40  | 83   |     |         |

*Total number was <327 due to incomplete pathological data.
FAN et al: HIGH EXPRESSION OF CD206 IN LIVER CANCER

3223

cells. Additionally, CD206 upregulation was observed in liver cancer tissue compared with healthy adjacent tissue obtained from patients with liver cancer. CD206 expression was not significantly associated with gender, age, tumor number, Edmondson grade, microvascular invasion, hepatitis B virus antigen and cirrhosis, but had a positive association with tumor size, metastasis and the AFP value. The present study therefore demonstrated a preliminary association between CD206 and the occurrence and development of liver cancer. Previous studies investigating liver cancer-associated proteins have demonstrated an association between marker expression and cancer staging. The present study revealed that CD206 expression was associated with tumor size, metastasis and the AFP value, indicating that its expression is associated with poor liver cancer prognosis. Furthermore, the association between CD206 expression and survival time revealed that the levels of CD206 were positively associated with poor prognosis. Taken together, these data suggested that high CD206 expression promotes the rapid growth and metastasis of liver cancer.

The present study investigated the direct effects of CD206 knockdown on liver cancer cells lines. The migration and invasion abilities of liver cancer cells decreased when CD206 was silenced, suggesting that high CD206 expression levels promote tumor metastasis and poor prognosis in patients.

Hepatitis C virus (HCV) infection is a main cause of liver cancer (49,50). However, the present study did not investigate

cells. Additionally, CD206 upregulation was observed in liver cancer tissue compared with healthy adjacent tissue obtained from patients with liver cancer. CD206 expression was not significantly associated with gender, age, tumor number, Edmondson grade, microvascular invasion, hepatitis B virus antigen and cirrhosis, but had a positive association with tumor size, metastasis and the AFP value. The present study therefore demonstrated a preliminary association between CD206 and the occurrence and development of liver cancer. Previous studies investigating liver cancer-associated proteins have demonstrated an association between marker expression and cancer staging. The present study revealed that CD206 expression was associated with tumor size, metastasis and the AFP value, indicating that its expression is associated with poor liver cancer prognosis. Furthermore, the association between CD206 expression and survival time revealed that the levels of CD206 were positively associated with poor prognosis. Taken together, these data suggested that high CD206 expression promotes the rapid growth and metastasis of liver cancer.

Uncontrolled self-renewal directly contributes to the progression of liver cancer and other types of carcinomas. The same molecular pathways that regulate self-renewal in normal stem cells also control CSCs (22,38,42). The present study revealed that CD206 is an important biomarker for liver cancer progression in CSCs, similar to other known markers including Nanog, Oct4, Sox2, c-Myc and CD44 (15,43-46). The expression of these markers in CSCs are recognized as evidence of stem cell carcinogenesis (37,47,48). The present study detected the expression levels of CD206 in CSCs and demonstrated high expression at both the mRNA and protein levels, which were comparable with the levels of Oct4 and c-Myc. The results obtained in the current study suggested that CD206 may be used as a biomarker in CSCs to predict liver cancer, highlighting its diagnostic value.

Figure 2. CD206 is a biomarker in cancer stem cells to predict the progression of liver cancer. (A) The liver cancer cell line PLC/PRF/5 formed sphere bodies in suspension culture/ (B) mRNA levels of Nanog, Oct4, Sox2, c-Myc, CD44 and CD206. *P<0.05, **P<0.01 vs. PLC/PRF/5 parents. (C) The expression level of CD206 increased in a similar manner to the expression of the internationally recognized biomarker proteins Nanog, Oct4, Sox2, c-Myc and CD44. CD206, mannose receptor; Nanog, Nanog homeobox; Oct4, POU class 5 homeobox 1; Sox2, SRY-box 2; c-Myc, MYC binding protein; CD44, CD44 molecule.

Table II. Univariate and multivariate Cox regression analysis of the clinicopathological parameters in patients with liver cancer.

| Parameter                                | Univariate analysis | Multivariate analysis |
|------------------------------------------|---------------------|-----------------------|
|                                          | Number              | Regression coefficient| HR  | 95% CI  | P-value | Regression coefficient| HR  | 95% CI  | P-value |
| Sex (male/female)                        | 266/61              | -0.124                | 0.883| 0.556-1.403| 0.598   | -0.186                | 0.830| 0.371-1.857| 0.650   |
| Age (<55≥55 years)                       | 126/201             | -0.027                | 0.973| 0.571-1.658| 0.920   | -0.493                | 0.611| 0.289-1.293| 0.198   |
| Tumor size (<50≥50 mm)                   | 191/128             | 0.489                 | 1.630| 1.025-2.592| 0.039   | 0.254                | 1.289| 0.557-2.982| 0.553   |
| Tumor number (single/multiple)           | 268/58              | 0.059                 | 1.060| 0.582-1.933| 0.848   | 1.008                | 2.740| 1.051-7.143| 0.039   |
| Edmondson grade (I+II/III)               | 202/119             | 0.455                 | 1.577| 0.989-2.515| 0.056   | 1.021                | 2.775| 1.291-5.965| 0.009   |
| Metastasis (M0/M1)                       | 294/27              | 0.160                 | 1.173| 0.629-2.188| 0.022   | 1.293                | 3.644| 1.314-10.104| 0.013   |
| Microvascular invasion (-/+ )            | 122/121             | 0.606                 | 1.834| 1.089-3.087| 0.615   | 0.310                | 1.364| 0.578-3.219| 0.479   |
| Hepatitis B virus (-/+ )                 | 62/259              | 0.002                 | 1.002| 0.558-1.800| 0.994   | -0.526               | 0.591| 0.180-1.937| 0.385   |
| Cirrhosis (-/+ )                         | 110/217             | -0.283                | 0.754| 0.456-1.247| 0.271   | 0.901                | 2.463| 0.912-6.655| 0.076   |
| α-fetoprotein (<20≥20 µg/l)              | 143/123             | 0.920                 | 2.510| 1.395-4.517| 0.002   | 0.647                | 1.910| 0.855-4.264| 0.114   |

aTotal number was <327 due to incomplete pathological data. HR, hazard ratio; CI, confidence interval.
the important role of HCV infection in liver cancer and this is a limitation of the study. Future studies investigating the association between HCV infection and CD206 expression are required.

In summary, the present study revealed that CD206 may be used as a diagnostic tool to screen for liver cancer, and its upregulation in liver tumors represents a therapeutic target to reduce liver cancer cell metastasis. Future investigation of the efficacy of CD206 inhibitors on liver cancer cells as well as an evaluation of the potential toxic effects on normal hepatocytes is required. Such studies may lay the theoretical foundations for future clinical liver cancer diagnosis and treatment.
Acknowledgements

Not applicable.

Funding

The present study was supported by the National Science Foundation of China (grant nos. 81602706 and 81570198), Zhejiang Medical Technology Plan Project (grant no. WKJ-ZJ-1709) and the State Administration of Traditional Chinese Medicine of Zhejiang (grant nos. 2016ZZ007 and 2017ZB006).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SW, LZ and XT conceived and designed the study. WF, XY and FH performed the experiments. SW wrote the paper. SW, LZ and XM reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethical approval and consent to participate

The study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ghouri YA, Mian I and Rowe JH: Review of hepatocellular carcinoma: Epidemiology, etiology, and carcinogenesis. J Carcinog 16: 1, 2017.
2. Altekruse SF, Henley SJ, Cinccolini JE and McGlynn KA: Changing hepatocellular carcinoma incidence and liver cancer mortality rates in the United States. Am J Gastroenterol 109: 542-553, 2014.
3. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer Incidence and Mortality Worldwide: Sources, Methods, and Major Patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
4. Tsai WC, Kung PT, Wang YH, Kuo WY and Li YH: Influence of the time interval from diagnosis to treatment on survival for early-stage liver cancer. PLoS One 13: e0199532, 2018.
5. Ronot M, Clift AK, Vilgrain V and Frilling A: Functional imaging in liver tumours. J Hepatol 65: 1017-1030, 2016.
6. Marrao JA, Aih J and Rajender Reddy K: Americal College of Gastroenterology: ACG clinical guideline: The diagnosis and management of focal liver lesions. Am J Gastroenterol 109: 1328-1348, 2014.
7. Elsayes KM, Hooker JC, Agrons MM, Kielar AZ, Tang A, Fowler KJ, Chernyak V, Bashir MR, Kono Y, Do RK, et al: 2017 version of LI-RADS for CT and MR Imaging: An update. Radiographics 37: 1994-2017, 2017.
8. Ryerson AB, Ehemann CR, Altekruse SF, Ward JW, Jemal A, Sherman RL, Henley SJ, Hoffmann D, Lake A, Noone AM, et al: Annual report to the nation on the status of cancer. 1975-2012, featuring the increasing incidence of liver cancer. Cancer 122: 1312-1337, 2016.
9. Sia D, Villanueva A, Friedman SL and Llovet JM: Liver cancer cell of origin, molecular class, and effects on patient prognosis. Gastroenterology 152: 745-761, 2017.
10. Ghouri S and Mazzaferrro V: Current challenges in liver transplantation for hepatocellular carcinoma. Best Pract Res Clin Gastroenterol 28: 867-879, 2014.
29. Gan W, Zhang MX, Wang JX, Fu YP, Huang JL, Yi Y, Jing CY, Fan J, Zhou J and Qiu SJ: Prognostic impact of lactic dehydrogenase to albumin ratio in hepatocellular carcinoma patients with Child-Pugh I who underwent curative resection: A prognostic nomogram study. Cancer Manag Res 10: 5383-5394, 2018.

30. Couri T and Pillai A: Goals and targets for personalized therapy for HCC. Hepatol Int 13: 125-137, 2019.

31. Zhu R, Huang H, Zhang H, Wang Z, Hu X, Zhai W, Lin Y, Wang J and Zhu H: Prognostic analysis in chronic hepatitis B patients: A retrospective study of 216 cases about Scheuer scores, in situ expression of viral antigens and tissue hepatitis B virus DNA levels. Liver Int 26: 82-89, 2006.

32. Zhu AX, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, Assenat E, Brandi G, Pracht M, Lim HY, et al: Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased alpha-fetoprotein concentrations (REACH-2): A randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 20: 282-296, 2019.

33. Shim JH, Yoon DL, Han S, Lee YJ, Lee SG, Kim KM, Lim YS, Lee HC, Chung YH and Lee YS: Is serum alpha-fetoprotein useful for predicting recurrence and mortality specific to hepatocellular carcinoma after hepatectomy? A test based on propensity scores and competing risks analysis. Ann Surg Oncol 19: 3678-3696, 2012.

34. Zhong M, Zhong C, Cui W, Wang G, Zheng G, Li L, Zhang J, Ren R, Gao H, Wang T, et al: Induction of tolerogenic dendritic cells by activated TGF-beta/Akt/Smad2 signaling in RIG-I-deficient stemness-high human liver cancer cells. BMC Cancer 19: 439, 2019.

35. Wei X, You X, Zhang J and Zhou C: MicroRNA-1305 inhibits the stemness of LCSCs and Tumorigenesis by repressing the UBE2T-dependent Akt-signaling pathway. Mol Ther Nucleic Acids 16: 721-732, 2019.

36. Bamodu OA, Kuo KT, Yuan LP, Cheng WH, Lee WH, Ho YS, Chao TY and Yeh CT: HDAC inhibitor suppresses proliferation and tumorigenicity of drug-resistant chronic myeloid leukemia stem cells through regulation of hsa-miR-196a targeting BCR/ABL1. Exp Cell Res 370: 159-170, 2018.

37. Choi HS, Kim JH, Kim SL, Deng HY, Lee D, Kim CS, Yun BS and Lee DS: Catechol derived from aronia juice through lactic acid bacteria fermentation inhibits breast cancer stem cell formation via modulation Stat3/IL-6 signaling pathway. Mol Carcinog 57: 1467-1479, 2018.

38. Mani SK, Zhang H, Diab A, Pascuzzi PE, Lefrancos L, Fares N, Bancel B, Merle P and Andrisani O: EpCAM-regulated intramembrane proteolysis induces a cancer stem cell-like gene signature in hepatitis B virus-infected hepatocytes. J Hepatol 65: 888-898, 2016.

39. Liu M, Jiang L and Guan XY: The genetic and epigenetic alterations in human hepatocellular carcinoma: A recent update. Protein Cell 5: 673-691, 2014.

40. Orcutt ST and Anaya DA: Liver resection and surgical strategies for management of primary liver cancer. Cancer Control 25: 107327481774621, 2018.

41. Scodeller P, Simon-Gracia L, Kopanchuk S, Tobi A, Kilk K, Saalik P, Kurm K, Squadrito ML, Kopanraju VR, Rinken A, et al: Precision targeting of tumor macrophages with a CD206 binding peptide. Sci Rep 7: 14655, 2017.

42. Maehara O, Ohsashi S, Asano A, Suda G, Natsuizaka M, Nakagawa K, Kobayashi M, Sakamoto N and Takeda H: Metformin regulates the expression of CD133 through the AMPK-CEBPβ pathway in hepatocellular carcinoma cell lines. Neoplasia 21: 545-556, 2019.

43. Palla AR, Piazzolla D, Abad M, Li H, Dominguez O, Schonthaler HB, Wagner EF and Serrano M: Reprogramming activity of NANOGP8, a NANOG family member widely expressed in cancer. Oncogene 33: 2513-2519, 2014.

44. Jerabek S, Merino F, Scholer HR and Cojocaru V: OCT4: Dynamic DNA binding pioneers stem cell pluripotency. Biochim Biophys Acta 1839: 138-154, 2014.

45. Guvench O: Revealing the mechanisms of protein disorder and N-glycosylation in CD44-hyaluronan binding using molecular simulation. Front Immunol 6: 305, 2015.

46. Yoshida GF: Emerging roles of Myc in stem cell biology and novel tumor therapies. J Exp Clin Cancer Res 37: 173, 2018.

47. Suzuki Y, Haraguchi N, Takahashi H, Uemura M, Nishimura J, Hata T, Takemasa I, Assenat E, Brandi G, Pracht M, Lim HY, et al: J Exp Clin Cancer Res 20: 282-296, 2019.

48. Botchkina GI, Zuniga ES, Das M, Wang Y, Wang H, Zhu S, Savitt AG, Roweih RA, Leyman Y, Ju J, et al: New-generation taxoid SB-T-1214 inhibits stem cell-related gene expression in 3D cancer spheroids induced by purified colon tumor-initiating cells. Mol Cancer 9: 192, 2010.

49. Kanda T, Goto T, Hirotsu Y, Moriyama M and Omata M: Molecular mechanisms driving progression of liver cirrhosis towards hepatocellular carcinoma in chronic Hepatitis B and C infections: A review. Int J Mol Sci 20: pii: E1358, 2019.

50. Zahra M, Azzazy H and Moustafa A: Transcriptional regulatory networks in Hepatitis C virus-induced hepatocellular carcinoma. Sci Rep 8: 14234, 2018.