CLEC4M overexpression inhibits progression and is associated with a favorable prognosis in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) remains the most common malignant cancer worldwide. Numerous studies have indicated that C-type lectin domain family 4 member M (CLEC4M) is associated with tumor progression; however, the biological functions of CLEC4M in HCC have not been investigated. In the present study, CLEC4M overexpression was observed to be associated with a favorable patient overall, relapse-free, progression-free and disease-specific survival by using the KMplot™ database. The present study then concentrated specifically on the functions of CLEC4M by performing cell counting kit-8 proliferation, 5-Ethynyl-2'-deoxyuridine and flow cytometric assays. CLEC4M overexpression inhibited proliferation and enhanced apoptosis in Huh7 and PLC/PRF/5 cells. Furthermore, the results demonstrated by using western blotting that CLEC4M overexpression inhibited the Janus kinase 1/signal transducer and activator of transcription 3 pathway, which is involved in various types of tumors including HCC. In conclusion, the present study reported that CLEC4M may be considered as a novel indicator of HCC and may provide a theoretical basis for improving the survival of patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and the third leading cause of cancer mortality worldwide (1). HCC has a high degree of malignancy and develops rapidly, with patients usually having already reached the middle or late stage of the disease at diagnosis (2,3). If patients with HCC are not actively treated, their natural disease course is short and their 5-year survival rate is low (4). Therefore, HCC poses a serious threat to the health and lives of people (5). Actively searching for early-warning markers for HCC prognosis and further studying the molecular mechanism of HCC may provide important theoretical guidance for early intervention for patients with HCC.

C-type lectin domain family 4 member M (CLEC4M), also known as DC-SIGNR, is a Ca^{2+}-dependent C-type lectin that has been reported to be associated with tumor progression (6). For example, a previous study demonstrated that the level of serum CLEC4M in patients with colon cancer was higher compared with healthy controls (7). In addition, studies have also revealed that CLEC4M may promote the occurrence of liver metastases in colon cancer (8) and gastric cancer (9). However, the opposite results were observed in lung cancer and serum CLEC4M levels were lower in patients compared with healthy controls (10). These results indicated a controversial role for CLEC4M in tumor progression. Additionally, while it has been verified that Janus kinase 1/signal transducer and activator of transcription 3 (JAK1/STAT3) pathway serves crucial roles in HCC (11,12), whether there is a link between CLEC4M and this process is still unknown.

Based on the abovementioned problems, the present study investigated the novel role and mechanism of CLEC4M in HCC progression; thus, the present study may provide a theoretical basis for improving the survival of patients with HCC.

Materials and methods

Bioinformatic analysis. As described previously (13), published data in the Oncomine™ database (oncomine.org/resource/main.html) was investigated to analyze the CLEC4M mRNA levels in unpaired non-tumor liver tissue samples and HCC tissue samples to determine the clinical importance of CLEC4M in HCC. The threshold settings were set to P<0.0001, fold change ≥2 and Gene rank=top 10%. The following four datasets were selected for analysis: GSE14520_GPL571 (non-tumor=21, tumor=22) (14), GSE14520_GPL3921 (non-tumor=220, tumor=225) (14), GSE14323_GPL571 (non-tumor=19, tumor=38) (15) and GSE6764 (non-tumor-10, tumor=35) (16).

Additionally, the KMplot™ database (kmplot.com/analysis) was used to analyze the 5-year prognostic value of CLEC4M. The 'Auto select best cutoff' setting was used to divide patients

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with HCC into two groups: The high and low CLEC4M expression groups. Follow-up time was set to 60 months and patients who were still alive at 60 months were censored. Finally, the association between CLEC4M expression and the 5-year overall survival (OS; low expression=90, high expression=274), relapse-free survival (RFS; low expression=88, high expression=228), progression-free survival (PFS; low expression=103, high expression=267) and disease-specific survival (DSS; low expression=89, high expression=273) was assessed.

Cell lines and cell culture. Huh7 and PLC/PRF/5 cells were acquired from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Huh7 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (P/S; Gibco; Thermo Fisher Scientific, Inc.). PLC/PRF/5 cells were cultured in MEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% P/S. All cells were incubated at 37˚C and 5% CO_2.

Lentivirus-mediated CLEC4M overexpression. The lentiviruses were purchased from Shanghai GeneChem, Inc. and the lentivirus-mediated CLEC4M overexpression in Huh7 and PLC/PRF/5 cells was performed according to the manufacturer's protocol. Briefly, a total of 4 μl of lentivirus titer ($1x10^8$ TU/ml) with CLEC4M was added to $1x10^6$ HCC cells at 37˚C for 12 h (CLEC4M overexpression group). Lentivirus without CLEC4M served as the negative control (vector group). The supernatants were then replaced with normal culture medium (DMEM/MEM supplemented with 10% FBS and 1% P/S) and these HCC cells were cultured at 37˚C for 72 h for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. RT-qPCR analysis was performed as previously described (17). Briefly, total RNA was extracted from Huh7 and PLC/PRF/5 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Reverse transcription was performed using a PrimeScript™ RT kit (Takara Bio, Inc.), according to the manufacturer's protocol. SYBR Premix EX Taq™ (Takara Bio, Inc.) was used for qPCR on an ABI 7900 Prism HT (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The PCR primers were as follows: CLEC4M forward, 5'-TGTCCAAGGTCCCCAGCTCCC-3' and reverse, 5'-GAACTCACCAAATGCAGTCTTCAAATC-3'; and GAPDH forward, 5'-AACAGCCTCAAGATCATCAGCA-3' and reverse, 5'-CATGAGTCCTTCCACGATACCA-3'. Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18).

Western blotting. Western blotting was performed as previously described (19). Briefly, protein lysates (25 μg) were separated by using SDS-PAGE and target proteins were detected by western blotting with appropriate primary antibodies. A rabbit anti-CLEC4M antibody was purchased from Abcam (1:1,000; cat. no. ab232709). Anti-GAPDH (1:1,000; cat. no. 2118), anti-phospho-JAK1(p-JAK1; 1:1,000; cat. no. 3331), anti-JAK1 (1:1,000; cat. no. 3332), anti-p-STAT3(p-STAT3; 1:2,000; cat. no. 9145) anti-STAT3 (1:2,000; cat. no. 4904) and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10,000; cat. no. 7074) were purchased from Cell Signaling Technology, Inc.

Cell counting kit-8 (CCK-8) proliferation assay. Huh7 and PLC/PRF/5 cells stably infected with lentivirus ($2x10^3$/well) were seeded in 96-well plates and incubated at 37˚C overnight. These HCC cells were then incubated at 37˚C for 24, 48, 72 or 96 h. CCK-8 (Dojindo Molecular Technologies, Inc.) was used to determine the viability of proliferating cells, according to the manufacturer's protocol. Briefly, HCC cells were incubated at 37˚C for 2 h after adding 10 μl/well CCK8 reagent, and the absorbance was measured at 450 nm using an Infinite M200 Pro Multifunctional microplate reader (Tecan Group Ltd).

5-Ethynyl-2'-deoxyuridine (EdU) assay. EdU (Guangzhou RiboBio Co., Ltd.) was used to determine the proliferative ability of Huh7 and PLC/PRF/5 cells, according to the manufacturer's protocol. Briefly, HCC cells stably infected with lentivirus were seeded in 96-well plates and treated with EdU at 37˚C for 2 h. Subsequently, Hoechst 33342 (Sigma-Aldrich; Merck KGaA) was added to each well and incubated at room temperature for 30 min in the dark. Fluorescence microscopy (Olympus IX50; Olympus Corporation) was used to determine the proportion of EdU-positive cells.

Flow cytometric assay. An Annexin V-FITC/propidium iodide (PI) Cell Apoptosis kit (Nanjing KeyGen Biotech, Co., Ltd.) was used to identify apoptosis of Huh7 and PLC/PRF/5 cells of the CLEC4M or Vector group, according to the manufacturer's protocol. Briefly, 100 μl suspension containing $5x10^5$ HCC cells were incubated with 5 μl Annexin V and 1 μl PI in the dark at room temperature for 15 min. The apoptotic rate (early + late apoptotic) was determined using a BD FACSCalibur flow cytometre (BD Biosciences) and the data was analyzed using FlowJo software (version 7.6.1; FlowJo LLC).

Statistical analysis. All data analyses were conducted using GraphPad Prism software (version 7.0; GraphPad Software, Inc.). Unpaired Student's t-test was used for the comparison of parameters between two groups. Survival analyses were performed using Kaplan-Meier curves and survival was compared using the log-rank test. Data are presented as the means ± standard deviations. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

CLEC4M overexpression in unpaired non-tumor liver tissue samples is compared to HCC tissue samples. CLEC4M mRNA expression levels were collected from four published HCC datasets in the Oncomine™ database. CLEC4M mRNA levels were significantly higher in the unpaired non-tumor liver tissue samples compared with HCC tissue samples in all datasets ($P<0.05$; Fig. 1).

CLEC4M overexpression is associated with a favorable prognosis. Data from the KMplot™ database demonstrated the

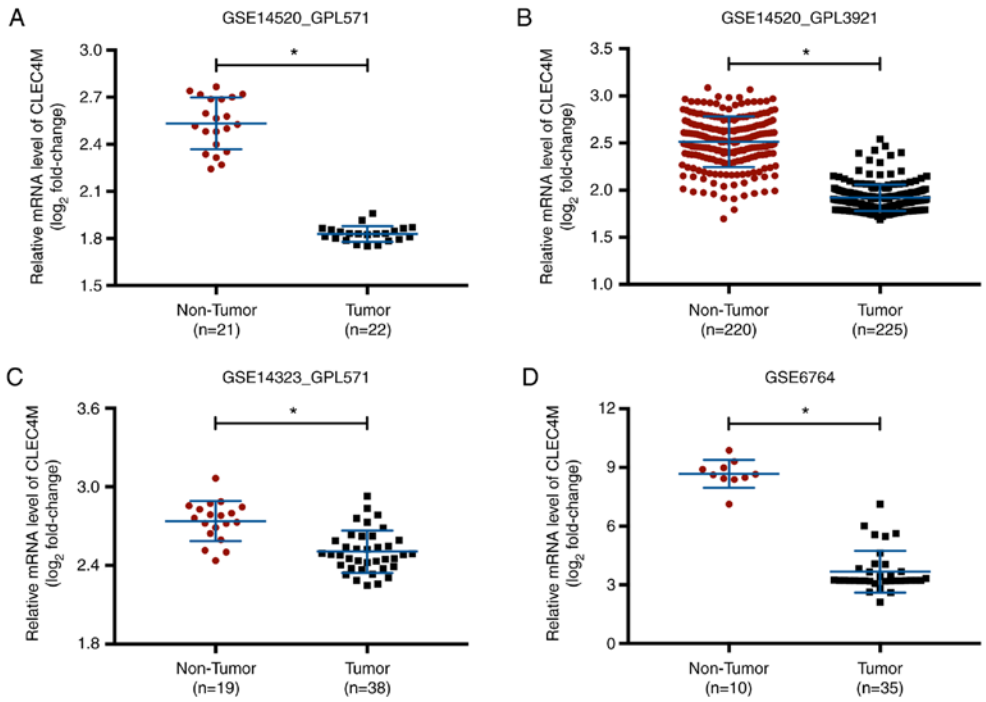

Figure 1. CLEC4M overexpression in unpaired non-tumor liver tissue samples is compared with HCC tissue samples. CLEC4M mRNA expression levels in unpaired non-tumor liver and hepatocellular carcinoma tumor tissue samples were examined in the (A) GSE14520_GPL571, (B) GSE14520_GPL3921, (C) GSE14323_GPL571 and (D) GSE6764 datasets from the Oncomine™ database. *P<0.05. CLEC4M, C-type lectin domain family 4 member M; HCC, hepatocellular carcinoma.

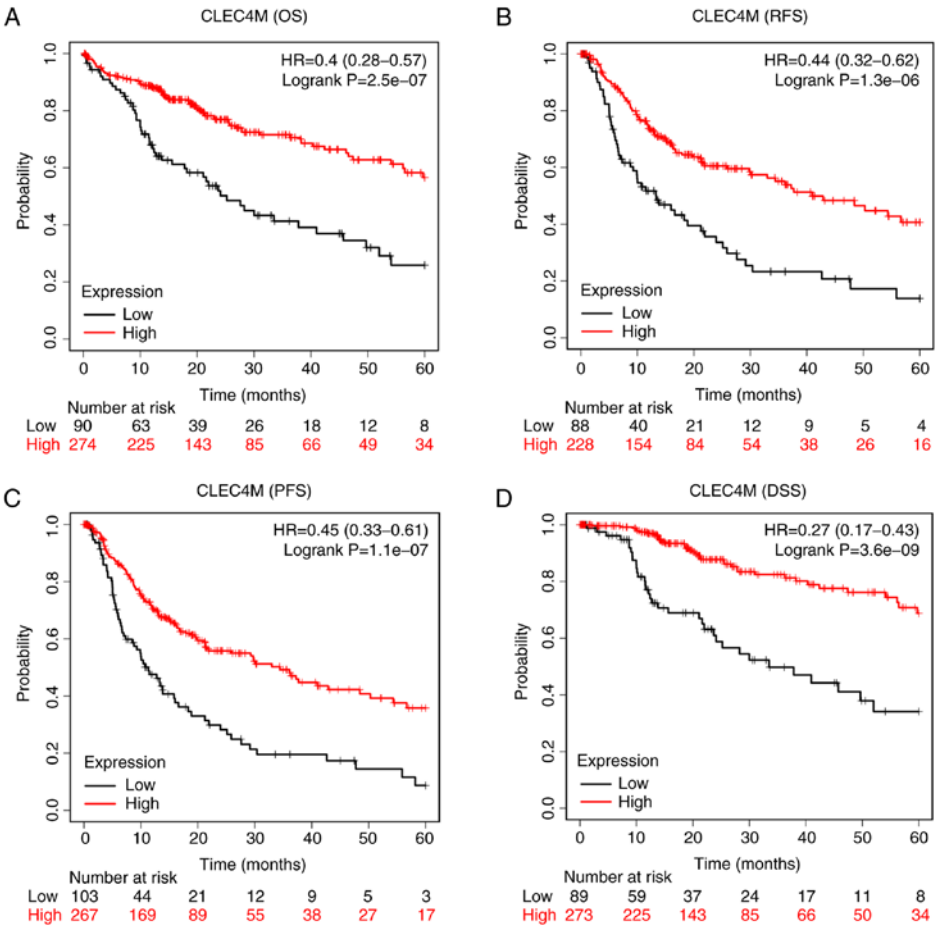

Figure 2. CLEC4M overexpression is associated with favorable prognosis. The association of CLEC4M expression with 5-year (A) OS, (B) RFS, (C) PFS and (D) DSS was determined by analyzing patients with hepatocellular carcinoma included in the KMplot™ database. CLEC4M, C-type lectin domain family 4 member M; OS, overall survival; RFS, relapse-free survival; PFS, progression-free survival; DSS, disease-specific survival; HR, hazard ratio.

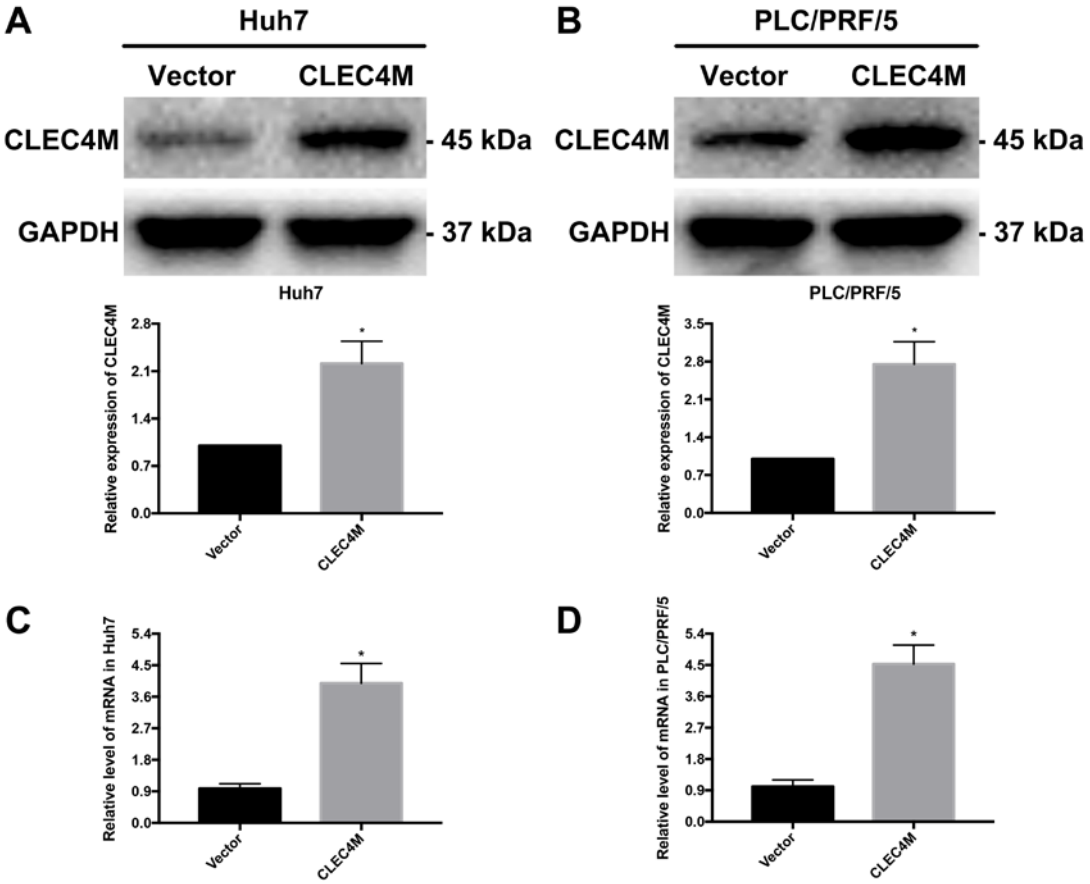

Figure 3. Construction of HCC cell lines with stable overexpression of CLEC4M. CLEC4M protein levels were assessed in infected (A) Huh7 and (B) PLC/PRF/5 cells using western blotting. CLEC4M mRNA levels were assessed in infected (C) Huh7 and (D) PLC/PRF/5 cells via reverse transcription-quantitative PCR analysis. Data are presented as the mean ± standard deviation. *P<0.05. HCC, hepatocellular carcinoma; CLEC4M, C-type lectin domain family 4 member M; Vector, negative control.

CLEC4M overexpression group had prolonged OS [P<0.05; hazard ratio (HR), 0.4; 95% confidence interval (CI), 0.28-0.57; Fig. 2A] and RFS (P<0.05; HR, 0.44; 95% CI, 0.32-0.62; Fig. 2B) times compared with the low-expression group. Similar results were reported for PFS and DSS. The CLEC4M overexpression group had longer PFS (P<0.05; HR, 0.45; 95% CI, 0.33-0.61; Fig. 2C) and DSS (P<0.05; HR, 0.27; 95% CI, 0.17-0.43; Fig. 2D) times compared with the low-expression group.

Construction of HCC cell lines with stable overexpression of CLEC4M. The liver cancer cell lines exhibited significant increases in CLEC4M expression at the protein (P<0.05; Fig. 3A and B) and mRNA (P<0.05; Fig. 3C and D) levels in the CLEC4M overexpression group compared with the vector group, indicating that CLEC4M was successfully overexpressed. Stable CLEC4M-overexpressing HCC cell lines were used for subsequent experiments.

CLEC4M overexpression inhibits the proliferation of HCC cells. Whether CLEC4M influences the viability of proliferating HCC cell lines was examined. Huh7 (P<0.05; Fig. 4A) and PLC/PRF/5 (P<0.05; Fig. 4B) cell viability was significantly reduced by CLEC4M overexpression, as evidenced by CCK-8 assays. Additionally, HCC cell proliferation was evaluated using EdU assays. Huh7 cell proliferation was significantly decreased by CLEC4M overexpression (P<0.05; Fig. 4C), as was PLC/PRF/5 cell proliferation (P<0.05; Fig. 4D).

CLEC4M overexpression induces apoptosis in HCC cells. Additionally, whether CLEC4M overexpression induced apoptosis in HCC cell lines was examined. The results revealed that CLEC4M overexpression significantly triggered apoptosis in Huh7 (P<0.05; Fig. 5A) and PLC/PRF/5 (P<0.05; Fig. 5B) cells.

CLEC4M overexpression inhibits the JAK1/STAT3 pathway. Since the JAK1/STAT3 pathway has been revealed to serve a critical role in the growth of malignant cells (20,21), the impact of CLEC4M on the JAK1/STAT3 pathway was assessed. The results demonstrated that the protein levels of p-JAK1 and p-STAT3 in Huh7 (P<0.05; Fig. 6A) and PLC/PRF/5 (P<0.05; Fig. 6B) cells were significantly reduced in the CLEC4M overexpression group compared with the vector group. These results indicated that CLEC4M overexpression inhibited the JAK1/STAT3 pathway, which may inhibit HCC progression.

Discussion

It is critical to investigate the genes responsible for HCC progression and elucidate the molecular pathogenesis of HCC. The present study primarily investigated the biological

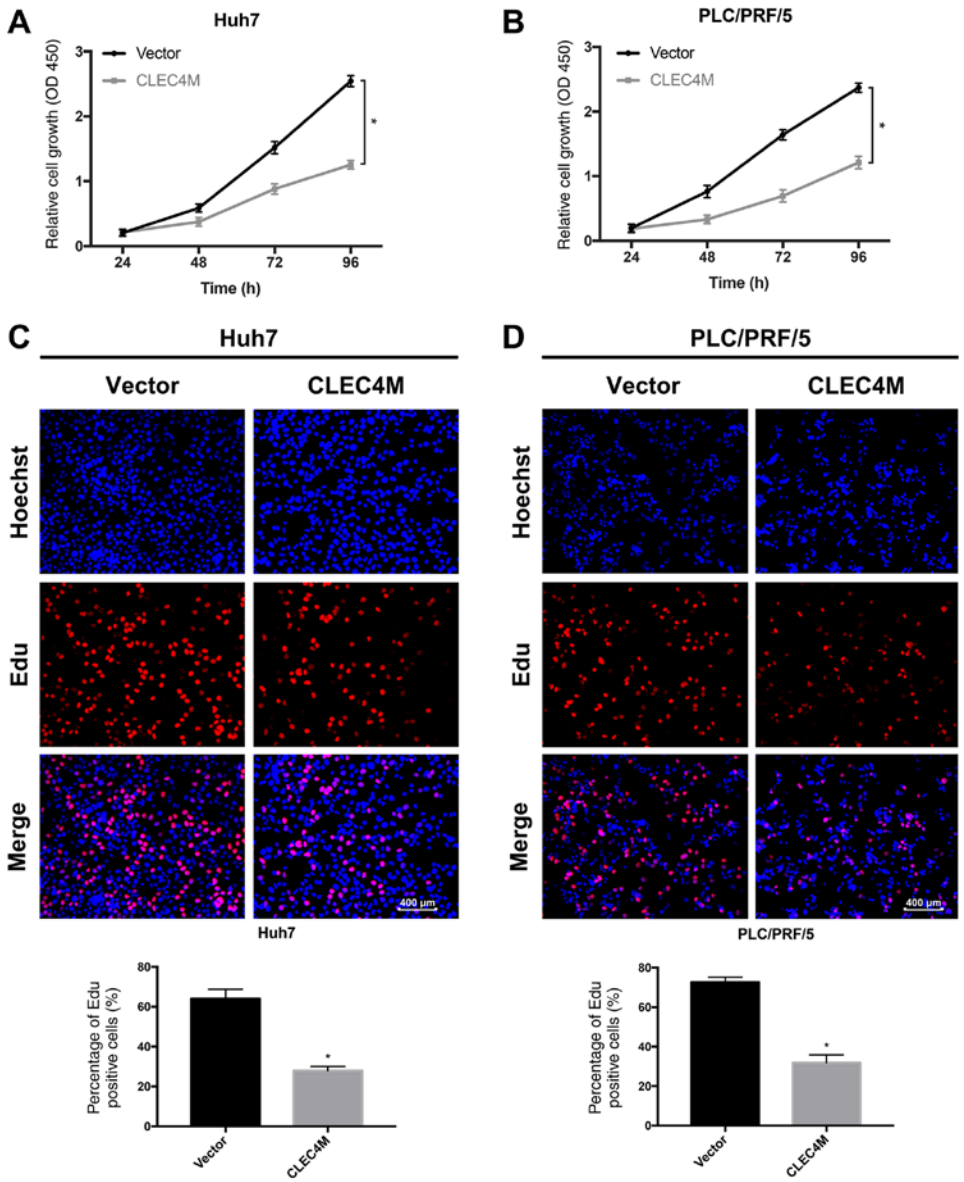

Figure 4. CLEC4M overexpression inhibits the proliferation of HCC cells. The viability of infected (A) Huh7 and (B) PLC/PRF/5 cells was assessed using a Cell Counting Kit-8 assay. The proliferation of infected (C) Huh7 and (D) PLC/PRF/5 cells was evaluated with an EdU assay. Data are presented as the mean ± standard deviation. *P<0.05. CLEC4M, C-type lectin domain family 4 member M; HCC, hepatocellular carcinoma; EdU, 5-Ethynyl-2'-deoxyuridine; OD, optical density; Vector, negative control.

functions of CLEC4M on HCC and the results revealed significantly decreased expression of CLEC4M in HCC tissue samples compared with unpaired non-tumor liver tissue samples. Furthermore, CLEC4M overexpression was associated with favorable patient OS, RFS, PFS and DSS. Moreover, the results for the Huh7 and PLC/PRF/5 cell lines confirmed that CLEC4M inhibited proliferation and enhanced apoptosis in HCC cells, at least in part via the JAK1/STAT3 pathway. These results indicated that the favorable clinical outcomes of patients with HCC and increased CLEC4M expression may result from proliferation inhibition and apoptosis enhancement.

C-type lectin CLEC4M is mainly localized in the endothelial cells of the liver, lungs and lymph nodes (6). Although the clinical significance of CLEC4M in HCC has been investigated previously, this previous study was limited to OS (22) and the other biological functions of CLEC4M in the context of HCC remain unclear. Similar to a previous result (22), prognostic analysis of patients with HCC observed that those with higher expression of CLEC4M exhibited prolonged OS, RPS, PFS and DSS compared with patients with lower expression in the current study. These results indicated that CLEC4M may lead to improved clinical outcomes in patients with HCC. Due to these results, the role of CLEC4M in HCC was further investigated. The results revealed that CLEC4M overexpression significantly inhibited proliferation in the HCC cell lines and enhanced cell apoptosis, indicating a new tumor-suppressive effect of CLEC4M in HCC. These results were similar to those reported by Liu *et al* (10), who reported low CLEC4M expression in serum samples from patients with lung cancer (10). Moreover, lymphoid tissue samples from patients with non-Hodgkin's lymphoma have been demonstrated to be negative for CLEC4M by immunohistochemistry (23). However, the results of these studies are in contrast to previous research results for colon cancer (8) and gastric cancer (9). This may be due to tumor

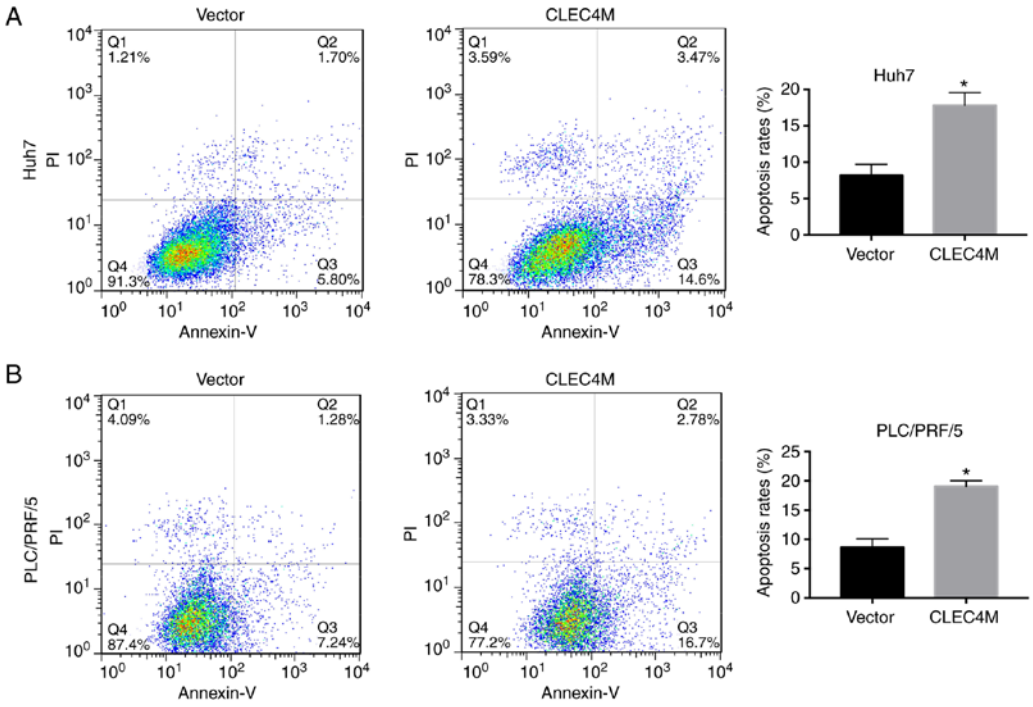

Figure 5. CLEC4M overexpression induces apoptosis in HCC cells. Apoptosis in infected (A) Huh7 and (B) PLC/PRF/5 cells was assessed via flow cytometric assays. Data are presented as the mean ± standard deviation. *P<0.05. CLEC4M, C-type lectin domain family 4 member M; HCC, hepatocellular carcinoma; Vector, negative control; PI, propidium iodide; Q, quadrant.

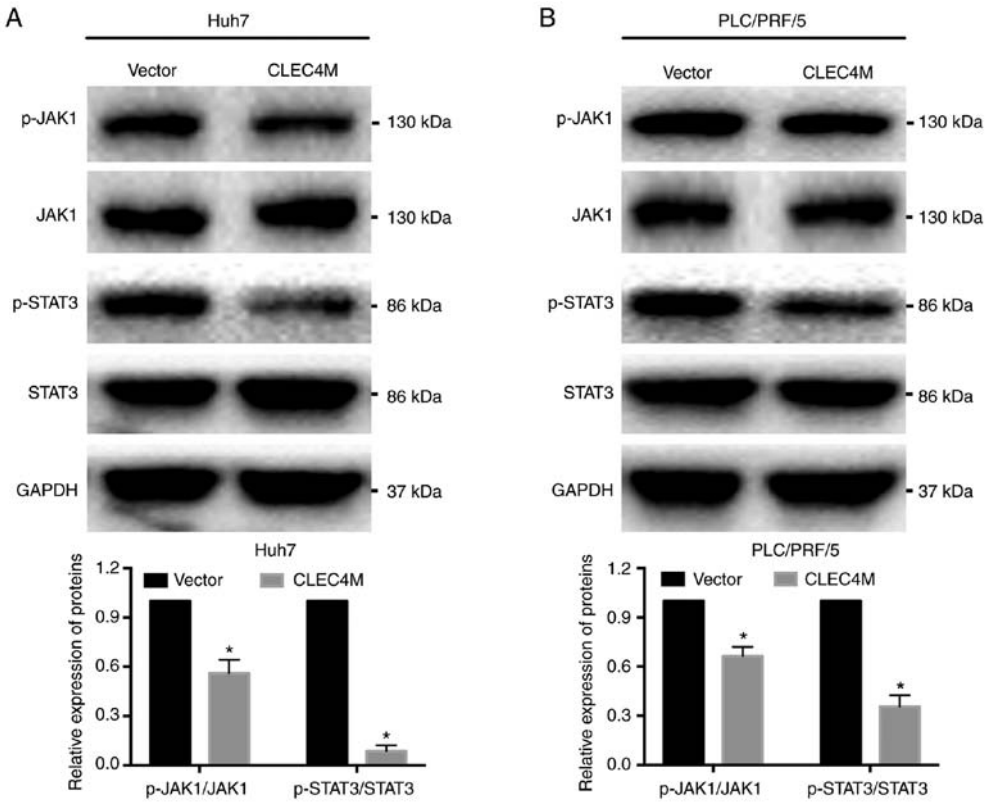

Figure 6. CLEC4M overexpression inhibits the JAK1/STAT3 pathway. p-JAK1, JAK1, p-STAT3, STAT3 and GAPDH protein levels were assessed in infected (A) Huh7 and (B) PLC/PRF/5 via western blotting. Data are presented as the mean ± standard deviation. *P<0.05. CLEC4M, C-type lectin domain family 4 member M; p-, phosphorylated; JAK1, Janus kinase 1; STAT3, signal transducer and activator of transcription 3; Vector, negative control.

complexity and the functions of CLEC4M varying among different tumor types and samples (24).

Reportedly, among the JAK/STAT family members, JAK1/STAT3 serves crucial roles in numerous biological

processes, including cell growth, apoptosis, migration and invasion (25-27). For instance, miR-34e was revealed to suppress HCC cell proliferation and invasion by regulating the JAK1/STAT3 pathway (12) and inhibition of the JAK1/STAT3 pathway suppressed HCC cell growth *in vivo* (11). Thus, the JAK1/STAT3 pathway may be a promising molecular target for the treatment of HCC. The results of the present study demonstrated that CLEC4M overexpression inhibited the JAK1/STAT3 pathway in HCC. Specifically, CLEC4M overexpression inhibited JAK1 and STAT3 phosphorylation in HCC cells. Therefore, CLEC4M may hinder HCC progression by inhibiting the JAK1/STAT3 pathway. However, the present study also has some limitations; for example, whether CLEC4M overexpression has the same tumor suppressor function *in vivo* remains unknown and further animal experiments may better reflect the role of CLEC4M in HCC.

In conclusion, the results of the present study reported that CLEC4M overexpression was associated with a favorable HCC prognosis and described a novel role for CLEC4M in HCC. Moreover, although no detailed mechanism was demonstrated in this study, the possible process by which JAK1/STAT3 signaling was regulated by CLEC4M was also elucidated. These results revealed that CLEC4M overexpression contributed to the inhibition of HCC progression and that CLEC4M may be a novel HCC biomarker that modulates JAK1 and STAT3.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KG designed the present study. QY and KG performed the experiments and analyzed the data. QY wrote the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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