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ORIGINAL ARTICLE

WAVE3 Induces EMT and Promotes Migration and Invasion in Intrahepatic Cholangiocarcinoma

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Abstract

Background Wiskott–Aldrich syndrome protein family verprolin-homologous protein 3 (WAVE3) plays a critical role in cancer progression and metastasis. However, the specific role of WAVE3 in intrahepatic cholangiocarcinoma (ICC) has not been studied.

Aims This study aimed to explore the role and mechanism of WAVE3 in the progression and metastasis of ICC.

Methods The expression of WAVE3 in ICC tissues and adjacent non-cancerous tissues was detected by immunohistochemistry. Western blot analysis was utilized to detect the expression of WAVE3 in ICC cells. A transwell assay was used to assess the potential for migration and invasion. The expression of WAVE3 in CC-LP-1 cells was knocked down by small interfering RNA (siRNA) interference.

Results The expression of WAVE3 in ICC tissues was significantly higher than that in adjacent non-cancerous tissues. The overall survival was lower in the subgroup of ICC patients with higher WAVE3 expression compared to the subgroup with a lower level of WAVE3 expression. WAVE3 expression was an adverse prognostic factor for ICC patients. CC-LP-1 cells expressed higher levels of WAVE3 protein compared to RBE cells and human intrahepatic biliary epithelial cells, which correlated with greater migration and invasion capabilities compared with the RBE cells. After the transfection of CC-LP-1 cells with WAVE3 siRNA, the level of WAVE3 protein was significantly decreased, accompanied by a marked reduction in migration, invasion and proliferation. Moreover, after the knockdown of WAVE3 expression in CC-LP-1 cells, the protein levels of Slug and Vimentin were significantly decreased, while that of E-cadherin was significantly increased.

Conclusions WAVE3 may represent a new adverse prognostic factor for patients with ICC. This protein

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enhances migration and invasion capabilities in ICC, most likely through the induction of an epithelial-mesenchymal transition.

Keywords WAVE3 · Intrahepatic cholangiocarcinoma · Migration · Invasion · Epithelial-mesenchymal transition

Introduction

Intrahepatic cholangiocarcinoma (ICC), comprising less than 10 % of all bile duct malignancies [1], is characterized by the early invasion of surrounding tissues and lymphatic metastasis. Although surgery and curative liver transplantation are options for select patients, the 5-year survival rate remains particularly low. Of note, the morbidity and mortality of ICC have been increasing worldwide in recent years [2]. Metastasis and recurrence are the most common causes of death in patients with ICC. The treatment of tumor progression and metastasis has become a worldwide challenge. New insights into the biologic processes associated with ICC and the identification of novel biomarkers are urgently required for ICC management. To date, several studies have attempted to reveal the molecular mechanism underlying the malignant characteristics of ICC. Numerous molecules and signaling pathways, including matrix metalloproteinases (MMPs) [3], integrin α6 [4], CXC chemokine receptor type 4 (CXCR4) [5], S100A4 [6], the Wnt pathway [7], and the PI3K-Akt pathway [8], have been identified in the invasion and metastasis of ICC.

Wiskott–Aldrich syndrome protein family verprolin-homologous protein 3 (WAVE3), an actin cytoskeleton remodeling protein that was first found in Wiskott–Aldrich syndrome in 1993, is highly expressed in advanced stages of various malignancies and influences tumor cell invasion. WAVE3 and other members of the WAVE family share a tripartite VCA (verprolin homology, cofilin homology and acidic) C-terminal domain. The activation of WAVE proteins leads to the exposure of the VCA domain, which can bind to the Arp2/3 complex and initiate the rapid polymerization of actin filaments [9, 10], ultimately leading to cytoskeletal remodeling, which is necessary for cell motility and migration [11]. Sossey-Alaoui et al. [12] proposed that the WAVE3-p38 pathway contributes to breast cancer progression and metastasis. Fernando et al. [13] found that WAVE3 is pivotal in controlling the invasiveness of prostate cancer cells. Recently, Ji et al. [14] characterized WAVE3 as a biomarker for the progression and metastasis of hepatocellular carcinoma. Epithelial-mesenchymal transition (EMT) has a close relation with the migration and invasion of tumor cells. An increasing number of studies have shown that EMT is associated with the acquisition of malignant characteristics in ICC. Recently, WAVE3 has been reported to induce the EMT process in cancers [15]. Although the mechanism of WAVE3 in promoting tumor progression has been defined in some malignant diseases, the metastasis-promoting role and mechanism of WAVE3 in ICC remain unknown.

In the present study, we investigated the relationship between WAVE3 dysregulation and the migration and invasion of ICC. First, we compared the expression of WAVE3 between ICC tissues and adjacent normal cholangial tissues via immunohistochemistry. Next, we used small interfering RNA (siRNA) to knock down WAVE3 expression to investigate the function of WAVE3 in the migration and invasion of the ICC cell line.

Materials and Methods

Patients and Samples

A total of 96 pairs of ICC tissues and ICC-adjacent non-cancerous samples were collected from surgically treated patients in The First Affiliated Hospital, Sun Yat-Sen University from January 2008 to September 2010. In this study, ICC patients who received chemotherapy or radiotherapy before surgery or who suffered from other cancers were excluded. The general conditions and clinicopathologic features of the ICC patients are presented in supplementary Table S1. The tumor-node-metastasis staging was assessed according to the Cancer Staging Manual (the 7th edition) of the American Joint Committee on Cancer. The survival period was defined as the period from the date of surgery to the date of the last follow-up (or death). Through July 2015, we obtained follow-up survival information by telephone from 72 of the 96 ICC patients after their operation. The remaining 24 patients were lost to follow-up due to the lack of contact. The 5-year overall survival rate was 7 %, and the median survival time was 7.3 months (range 2–81 months). The Institute Research Ethics Committee of The First Affiliated Hospital, Sun Yat-Sen University approved this study.

Immunohistochemistry

An immunohistochemistry assay was conducted on 5-mm-thick formalin-fixed and paraffin-embedded sections. First, the paraffin section was baked in an oven at 60 °C for 2 h. The tissue section was then immediately deparaffinized with xylene and rehydrated in varying concentrations of alcohol. The high-temperature and high-pressure method for antigen retrieval was performed in saline sodium citrate (pH 6.0) for 5 min. Then, 3 % H_2O_2 was utilized to block the endogenous peroxidase activity in the tissues at room temperature for 15 min. Next, the tissue section was

stained with anti-WAVE3 antibody (1:100, Abcam) at 4 °C overnight. After washing with phosphate buffered saline (PBS), the sections were incubated with the MaxVision™ HR-Polymer anti-Rabbit IHC Kit for 15 min at room temperature (Maixin Co., Fuzhou, China) and then were stained with the DAB Horseradish Peroxidase Color Development Kit (Maixin Co., Fuzhou, China) and counterstained with hematoxylin. According to both the area that was positive for staining and the intensity of the staining in the tissues, two experienced pathologists independently scored the magnitude of the staining. The area positive for staining was scored according to the following standard [16]: zero points indicates no tumor cells were positively stained; one point indicates <30 % of the area was positively stained tumor cells; two points indicates 30–60 % of the area was positively stained tumor cells; and three points indicates >60 % of the area was positively stained tumor cells. The intensity of the positive staining was scored according to the following standard: zero points indicates no positive staining; one point indicates the color was light yellow (weak staining); two points indicates the color was yellow–brown (moderate staining); and three points indicates the color was brown (strong staining). The final immunohistochemistry staining score was calculated by multiplying the staining intensity score by the score for the positively stained area. Ultimately, the expression of WAVE3 in the ICC tissues and ICC-adjacent non-cancerous samples was assessed according to the final immunohistochemistry staining score. Thus, the results could be classified into a score of 0, 1, 2, 3, 4, 6, or 9. The scores were then statistically analyzed, and the median of the scores, which was equal to four, was determined as the cutoff value. Therefore, a final immunohistochemistry staining score >4 was used to define the tissue as having a high WAVE3 expression, and ≤4 was defined as a low WAVE3 expression.

Cell Culture and Transfection

Two ICC cell lines (CC-LP-1 and RBE) and a non-neoplastic human intrahepatic biliary epithelial cell (HIBEC) line, which was derived from an explanted liver with hepatitis C virus-related cirrhosis [17], were purchased from the Chinese Academy of Sciences. Cells were maintained in RPMI-1640 supplemented with 10 % FBS (GIBCO), 100 U/ml penicillin and streptomycin at 37 °C in a humidified incubator with 5 % CO_2. All the cells used for experiments were in a logarithmic growth phase. For the transfection experiment, cells were seeded in six-well plates and transfected with WAVE3 siRNA or a negative control siRNA using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) following the manufacturer's instructions. The cells were cultured in an incubator, and 24 h later, the cellular proteins were extracted, or the transwell assays and plate clone-forming assay were performed. Three candidate WAVE3 siRNA sequences were used; the sequence that had the highest interference efficiency was chosen. The WAVE3 siRNA sequences included WAVE3-1 sense: GGU UUC AAA GAA CAG CAU UTT, WAVE3-2 sense: GCA AAC AUG CUG AAG ACA UTT, and WAVE3-3 sense: GCU CUG CCU GAA GGG AUU ATT; the negative control siRNA sequence was sense: UUC UCC GAA CGU GUC ACG UTT.

Western Blot Analysis

Western blotting was performed using antibodies directed against WAVE3 (1:500, Abcam), E-Cadherin (1:2000, Cell Signaling Technology), Vimentin (1:1000, Cell Signaling Technology), and Slug (1:500, Cell Signaling Technology). Cells were lysed with a radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology) and 1 mM PMSF (Cell Signaling Technology). A BCA Protein Assay Kit (Thermo Scientific pierce) was used to measure the total protein concentrations according to the manufacturer's instructions. Aliquots (30 μg) of cellular total proteins were resolved by SDS-PAGE (10 %) and then electrotransferred onto PVDF membranes. The primary antibodies were incubated at 4 °C overnight, and the secondary antibodies were added and incubated for 60 min at room temperature. Then, the protein-antibody complexes were detected by chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo, USA), according to the manufacturer's protocol (Applied Biosystems, MA, USA). GAPDH (Ray Antibody Bioteck, Beijing, China) was used as an internal control. All the band intensities were quantified using Image J software (National Institutes of Health, USA).

Transwell Migration and Invasion Assays

Transwell assays were conducted to detect tumor cell migration and invasion abilities. BD BioCoat Control Cell Culture Inserts were used to detect the migratory potential of ICC cells in a transwell migration assay. In the transwell invasion assay, a BD BioCoat BD Matrigel™ Invasion Chamber was utilized to detect the invasive potential. Each experiment was repeated three times under the same conditions. Briefly, 5×10^4 cells were seeded in the upper Boyden chambers (for migration) or the upper membranes (for invasion) of the cell culture inserts. After 24 h of culture in an incubator, the cells were washed with PBS and fixed in 4 % formaldehyde for 30 min. The residual cells in the upper chamber (for migration) or on the upper membrane (for invasion) were then wiped out carefully with a cotton swab. After the cells were washed again with

PBS, a DAPI staining solution (Beyotime Biotechnology, China) was used in the dark to stain the cells that adhered to the lower surface of the upper chamber (for migration) or the upper membrane (for invasion). Ten minutes later, an inverted microscope equipped with a digital camera was used to observe and count the tumor cells that were stained blue. Five random fields of vision at 200× magnification were selected and captured. The numbers of cells in the different groups were statistically analyzed.

Wound-Healing Assays

CC-LP-1, RBE and transfected cells were seeded into six-well plates at a density of 5×10^5 cells per well. The cells were cultured with RPMI-1640 medium containing 10 % FBS for 24 h, and a wound was then scratched into a cross using a 1-ml plastic pipette tip on the cell surface. The cells were washed with PBS three times to remove cell debris and then RPMI-1640 culture medium containing 1 % FBS was added to the plates. A region with a defined area within the scratch was imaged using an inverted microscope equipped with a digital camera at once (0 h) and after 24 h. The distance of the scratch was measured using Photoshop Creative Suite 5 software (Adobe Systems Inc., USA). The extent to which the wound had closed over 24 h was calculated and expressed as a percentage of the difference between 0 and 24 h.

Plate Clone-Forming Assay

We compared the proliferative potential of CC-LP-1 cells transfected with the WAVE3 siRNA and the negative control siRNA using a plate clone-forming assay. In this assay, cells were cultured with RPMI-1640 medium containing 10 % FBS in a six-well plate at a density of 200 cells per well. The culture medium was changed once every 4 days. After 12 days, the cells were fixed in 4 % formaldehyde and then stained with crystal violet. Finally, cell clones with diameters greater than 1 mm (50 cells/clone) were counted and analyzed.

Statistical Analysis

The data were representative of experiments that were repeated three times. All statistical analyses were conducted using SPSS for Windows, version 13.0 (SPSS, Inc., Chicago, IL, USA). The differences of continuous variables between groups were analyzed using ANOVA and Student's t test. The Chi square test was used to compare the differences of categorical variables. Non-normally distributed data were analyzed with the rank-sum test. The survival curve was calculated using the Kaplan–Meier method and was analyzed by the log-rank test. Univariate and multivariate analyses of independent prognostic factors were performed using the Cox proportional hazards regression model. In all the statistical analyses, a difference was regarded as statistically significant when $p < 0.05$.

Results

The Expression of WAVE3 in ICC Tissues and Adjacent Non-cancerous Tissues

The positivity for WAVE3 protein expression was distinctly located in the cytoplasm of cells in the ICC tissues (Fig. 1b, c, e, f), while low or scarce expression of WAVE3 was found in adjacent non-cancerous tissues (Fig. 1a, d). The immunostaining scores for WAVE3 protein expression in non-cancerous intrahepatic bile duct tissues were significantly lower than those in the ICC tissues ($p < 0.001$; Fig. 2a). Among the ICC cases, the WAVE3 levels were significantly higher in those ICC cases with lymphatic metastasis than those without this feature (Fig. 2b); likewise, the levels were higher in poorly differentiated adenocarcinomas than those in well- and moderately-differentiated adenocarcinomas (Fig. 2c).

The Expression of WAVE3 Is Associated with the Clinicopathological Features of ICC Patients

To investigate the role of WAVE3 expression in the aggressive progression of ICC, the correlation between WAVE3 expression and the clinicopathologic characteristics of the ICC patients was analyzed. The data in Table 1 clearly indicate that abnormally high expression of WAVE3 was associated with lymphatic metastasis ($p < 0.001$) and differentiation ($p < 0.001$). There was no obvious correlation between WAVE3 expression and gender ($p = 0.454$), age ($p = 0.102$), tumor stage ($p = 0.982$), distant metastasis ($p = 0.347$), clinical stage ($p = 0.079$), tumor size ($p = 0.213$), characteristics of the liver background ($p = 0.290$) or resection margin ($p = 0.670$). The median age was 57.5 years (31–75 years); the median tumor size was 6 cm (2–18 cm).

The Prognostic Value of WAVE3 Dysregulation for Patients with ICC

To clarify the prognostic value of WAVE3 in ICC patients, the relationship between WAVE3 expression and survival time was further investigated in 72 of the 96 ICC patients. Figure 3 demonstrates a significant difference in the overall survival between the high WAVE3 expression group (5-year overall survival rate = 3 %, median survival

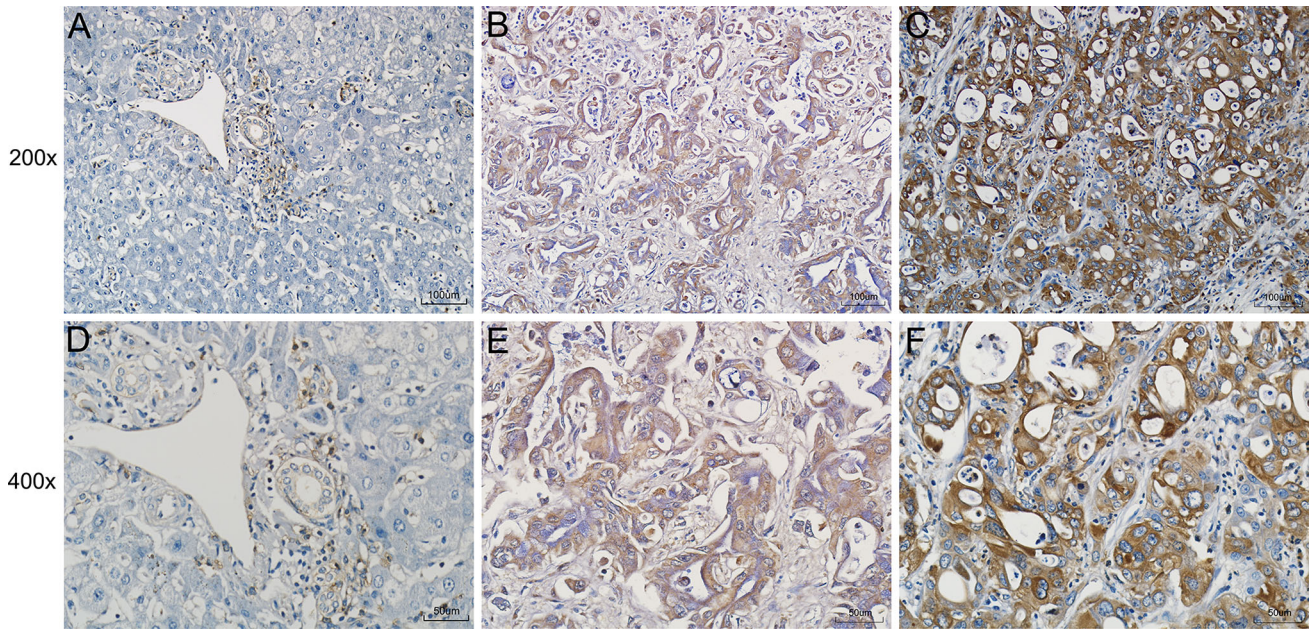

Fig. 1 The expression of WAVE3 in ICC tissues and adjacent non-cancerous tissues by immunohistochemistry. **a**, **d** Adjacent non-cancerous tissues. **b**, **e** ICC without lymphatic metastasis. **c**, **f** ICC with lymphatic metastasis. **a**–**c** Captured at ×200 magnification, and **d**–**f** Captured at ×400 magnification under a microscope. *Scale bar* ×200, 100 μm; ×400, 50 μm

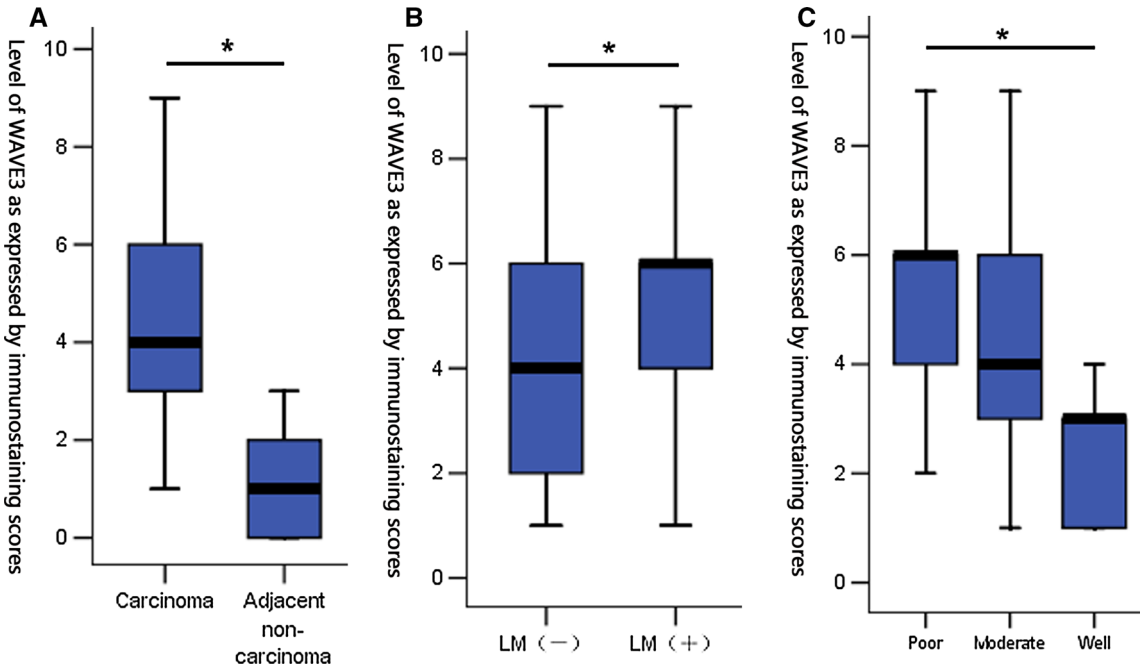

Fig. 2 Abnormal WAVE3 expression in the progression of ICC patients. Box plots are presented for comparing the expression of WAVE3 between ICC tissues and adjacent non-cancerous tissues (**a**), in ICC cases with or without lymphatic metastasis (LM) (**b**), and in different tumor differentiation (**c**). The *p* values were analyzed by Mann–Whitney *U* and Kruskal–Wallis tests. The *boxes* represent the 25th to 75th percentile of the observations, and the median is indicated by the *lines in the middle of the box*. $*p < 0.05$

time = 5.8 months) and the low WAVE3 expression group (5-year overall survival rate = 13 %, median survival time = 9 months; $p = 0.001$). Moreover, we utilized univariate and multivariate analyses to estimate the impact of WAVE3 expression and clinicopathological factors on the prognosis of patients with ICC. The clinical parameters for the univariate analysis were composed of gender, age, T classification, lymphatic metastasis, distant metastasis,

Table 1 Association of WAVE3 expression with clinicopathologic characteristics of ICC patients

Characteristics	WAVE3 expression		p value[a]
	High	Low	
Gender			
Male	26	32	0.454
Female	20	18	
Age			
≤57	27	21	0.102
>57	19	29	
Tumor stage			
T1	21	22	0.982
T2	12	12	
T3	5	6	
T4	8	10	
Lymphatic metastasis			
Negative	14	36	<0.001
Positive	32	14	
Distant metastasis[b]			
Negative	32	39	0.347
Positive	14	11	
Clinical stage			
I	5	11	0.079
II	5	8	
III	1	5	
IV	35	26	
Differentiation			
Poor	26	12	<0.001
Moderate	20	27	
Well	0	11	
Tumor size			
≤6	21	15	0.213
>6	11	15	
Characteristics of liver background			
PSC	3	8	0.290
HCV-related cirrhosis	2	5	
Others[c]	8	6	
None	33	31	
Resection margin			
R0	34	35	0.828
R1	7	10	
R2	5	5	

[a] Chi-square test

[b] Before surgery, 25 patients did not have distant metastasis. Ten patients were found to have distant metastasis during surgery and underwent palliative surgery (resection margin was R2). In the other 15 patients, distant metastasis was detected during the follow-up period

[c] Others contain hepatolithiasis, schistosomiasis, HBV-related hepatitis and steatohepatitis

clinical stage, tumor cell differentiation, tumor size, characteristics of the liver background and resection margin. When the p value of any of the above influential factors in the univariate analyses was less than 0.1, the factors were then brought into the multivariate analysis. Regarding the 5-year overall survival rate, the univariate and multivariate analyses both demonstrated that WAVE3 expression ($p = 0.002$) and resection margin ($p = 0.016$) were significant adverse prognostic factors for ICC patients, while distant metastasis ($p = 0.052$) tended to be an adverse prognostic factor (shown in Table 2). These results reveal that the expression of WAVE3 in ICC has a significant correlation with the prognosis.

WAVE3 Overexpression Is Involved in the Migration and Invasion of ICC Cell Lines

To explore the relationship between WAVE3 and migration and invasion abilities, we first utilized a western blot assay to detect the expression of WAVE3 in ICC cell lines (CC-LP-1 and RBE) and the normal bile duct epithelial cell line (HIBEC). As demonstrated in Fig. 4a, the protein levels of WAVE3 in RBE cells and HIBECs were significantly lower than that in CC-LP-1 cells. Compared with RBE cells and HIBECs, CC-LP-1 cells exhibited higher migration, invasion and proliferation abilities, as reflected by the wound healing assay (Fig. 4b), transwell migration

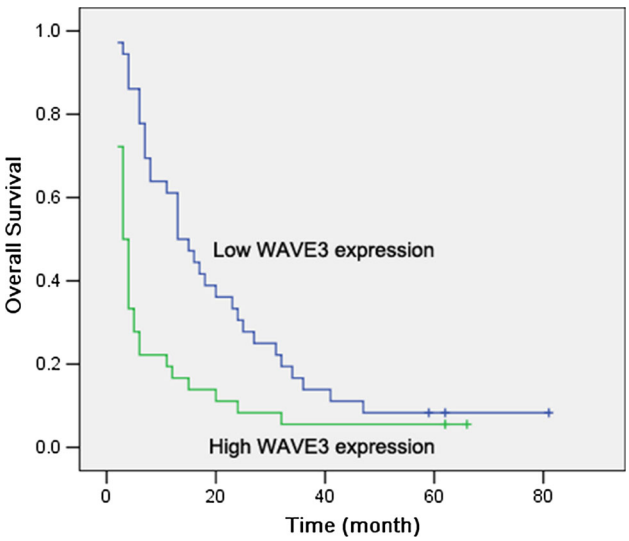

Fig. 3 The role of WAVE3 expression on prognostic implication in ICC patients. Kaplan–Meier plots of 5-year overall survival in low WAVE3 expression groups and high WAVE3 expression groups. The differences in overall survival rate were statistically significant ($p < 0.05$)

Table 2 Univariate and multivariate analyses for 5-year overall survival in ICC patients

Overall survival	Univariate analysis		Multivariate analysis	
	p value	R°egression coefficient (SE)	*p* value	Relative risk (95 % CI)
Gender	0.903	0.031 (0.252)	–	–
Age	0.349	−0.231 (0.246)	–	–
WAVE3 expression	0.001	0.798 (0.251)	0.002	2.511 (1.417–4.452)
T	0.534	0.067 (0.107)	–	–
N	0.002	0.795 (0.263)	0.991	–
M	0.013	0.651 (0.263)	0.052	1.800 (0.996–3.253)
Clinical stage	0.006	0.344 (0.125)	0.290	–
Differentiation	0.142	0.257 (0.175)	–	–
Tumor size	0.222	−0.369 (0.302)	–	–
Characteristics of liver background	0.349	0.110 (0.117)	–	–
Resection margin	0.004	0.512 (0.178)	0.016	1.556 (1.087–2.227)

Once the *p* value of factors was less than 0.1 in the univariate analyses, the factors were then brought into the multivariate analyses

and invasion assays (Fig. 4c, d), as well as the plate clone-forming assay (Fig. 4e). We also found that the expression levels of Slug, Vimentin and ZEB1, EMT-related proteins, were higher in CC-LP-1 cells than in RBE cells and HIBECs, while the expression of E-cadherin was lower in the CC-LP-1 cells than in the RBE cells and HIBECs (Fig. 4a).

Suppression of WAVE3 Inhibits ICC Cell Migration, Invasion and Proliferation Capabilities In Vitro

To further study the effect of WAVE3 on promoting migration and invasion in ICC cells, the expression of WAVE3 in the CC-LP-1 cell line was knocked down by transfection with WAVE3 siRNA. In this assay, three different sequences of WAVE3 siRNA (Si-1, Si-2, Si-3) were used. Ultimately, Si-2 was chosen as the best siRNA. The protein level of WAVE3 was significantly decreased in the CC-LP-1 cells after WAVE3 siRNA interference (Fig. 5a). Following gene silencing of WAVE3, the CC-LP-1 cells showed changes in morphology (Fig. S3). The cells in the NC siRNA group had a spindle-like morphology (Fig. S3A and C), whereas the cells in the WAVE3 siRNA group showed a cobblestone-like morphology (Fig. S3B and D). These data suggest that an increase in the expression of WAVE3 is associated with mesenchymal morphology. CC-LP-1 cells transfected with WAVE3 siRNA displayed significantly decreased migration (Fig. 5b, c), invasion (Fig. 5d) and proliferation abilities (Fig. 5e). Moreover, after knockdown of the expression of WAVE3 in the CC-LP-1 cells, several EMT-related proteins such as Slug, Vimentin and ZEB1 were significantly decreased, and the protein levels of E-cadherin were significantly increased (Fig. 5a).

Discussion

Although a certain amount of progress has been made in treating ICC in recent years, most patients have an incurable condition. In our study, the 5-year overall survival rate of ICC patients was 7 %, and the median survival time was 7.3 months. Both the overall 5-year survival rate and the median survival time are lower than that in Spolverato's study [18], which reported 22 % and 27 months, respectively. The causes may include the areas and ethnic variations to some extent; moreover, the R0 rate, which is an important prognostic factor following surgical resection in our study (71.8 %), was quite lower than that in Spolverato's study (81.2 %). In addition, their sample size (584 patients) was much larger than that in our study (96 patients). A better understanding of the molecular mechanisms underlying invasion and progression is urgently required in the development of new, targeted therapies for ICC patients who lose the opportunity for surgical treatment. Consequently, this need demands that we address the factors underlying migration and invasion. In this study, we identified and functionally characterized WAVE3 as an important factor in the development and progression of ICC. First, we detected and observed that the expression of WAVE3 in ICC tissues was significantly higher than that in adjacent non-cancerous tissues. Additionally, we found that high levels of WAVE3 in ICC cases were closely associated with the malignant behavior of ICC. We also determined that WAVE3 overexpression has poor prognostic

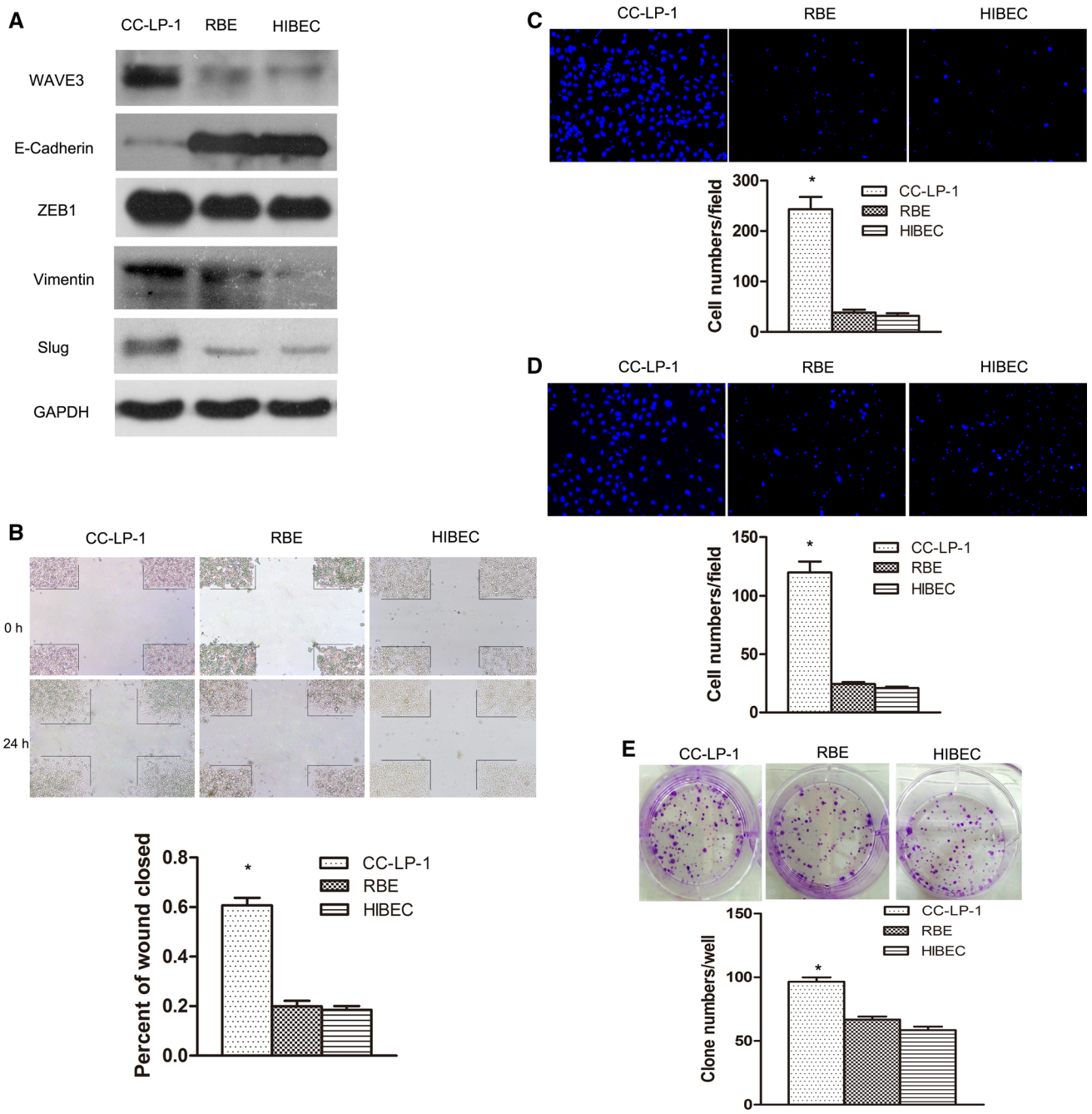

Fig. 4 WAVE3 overexpression is involved in the migration, invasion and proliferation of ICC cell lines. **a** Western blotting showed the expression of WAVE3 protein and the expression of epithelial-mesenchymal transition (EMT) markers (E-cadherin, ZEB1, Vimentin and Slug) in CC-LP-1, RBE and HIBEC cells. The migration potential of ICC cells and HIBEC cells was detected by the wound healing assay (**b**) and transwell migration assay (**c**). The invasion potential of ICC cells and HIBEC cells was detected by the transwell invasion assay (**d**). The proliferation capabilities of ICC cells and HIBEC cells were detected by the plate clone-forming assay (**e**). CC-LP-1 cells displayed significantly higher migration, invasion and proliferation abilities than RBE and HIBEC cells. $*p < 0.05$

significance for ICC patients. To our knowledge, this is the first report indicating that WAVE3 plays a vital role in the progression and metastasis of ICC. Moreover, we demonstrated in vitro that the increased level of WAVE3 promoted the migration, invasion and proliferation abilities of ICC cells. Collectively, our results suggest that WAVE3 may serve as a predictive marker for a case of ICC with high invasion and metastatic potential.

WAVE3, one of the members of the Wiskott–Aldrich syndrome protein family, has the function of controlling

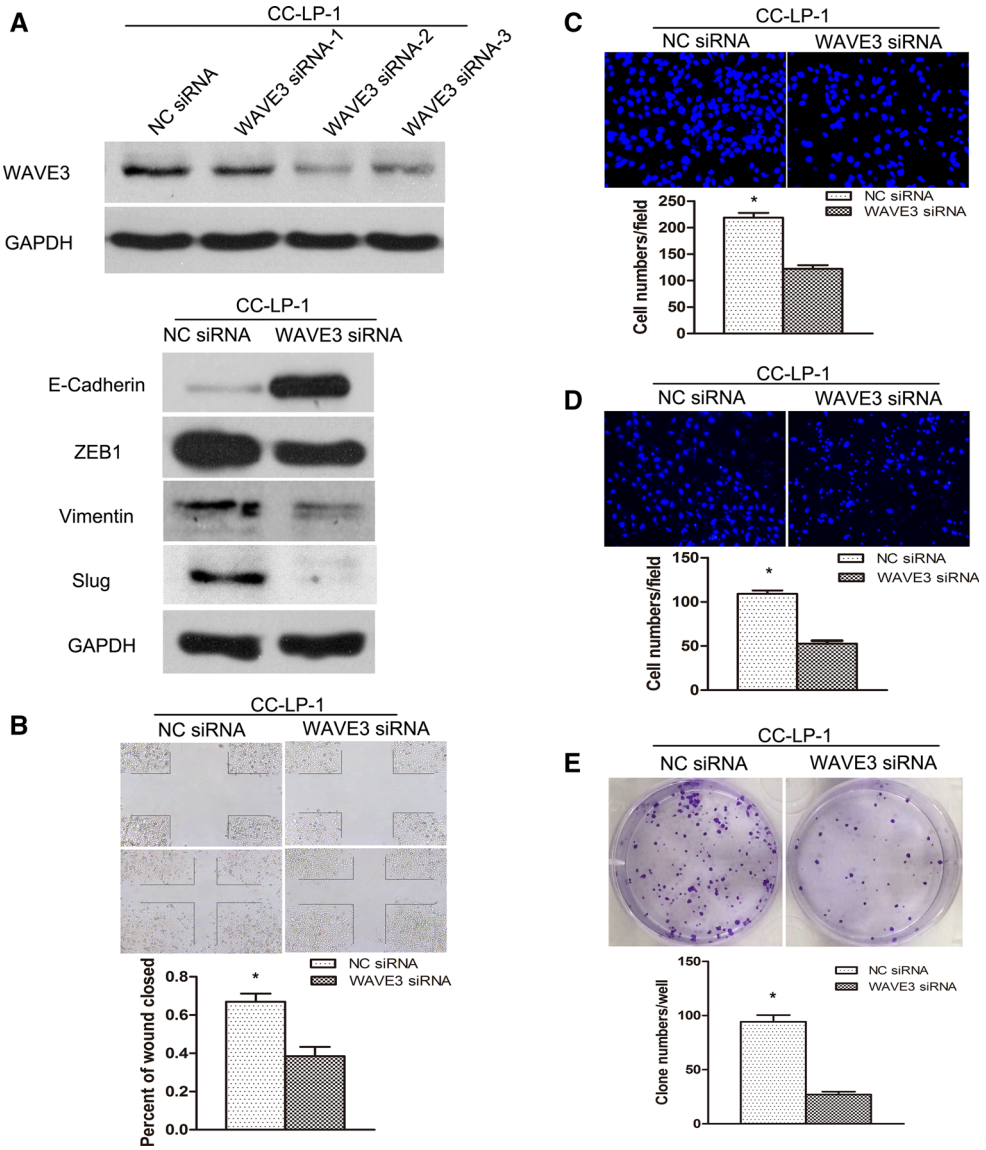

Fig. 5 WAVE3 knockdown suppressed the migration, invasion and proliferation capabilities of ICC cells in vitro. **a** The WAVE3 protein level was significantly decreased in the WAVE3 siRNA-transfected CC-LP-1 cells compared to the negative control siRNA (NC siRNA)-transfected CC-LP-1 cells. Moreover, CC-LP-1 cells with WAVE3 knockdown displayed decreased ZEB1, Vimentin, and Slug protein levels and increased E-cadherin protein levels. WAVE3 knockdown prominently reduced the migration (**b**, **c**) and invasion (**d**) potentials of CC-LP-1 cells. **e** The clone-forming abilities of the CC-LP-1 cells were dramatically restrained after transfection with the WAVE3 siRNA. $*p < 0.05$

cytoskeletal organization and actin polymerization. Even more important, WAVE3 may have a crucial effect on certain cellular processes [19], for example, cell proliferation, migration and motility. In recent years, the role of WAVE3 in promoting the migration and invasion of cancer cells has been the focus of an increasing number of researchers. WAVE3 acts as one of the candidate tumorigenesis genes located in Xp11.22 and has been detected in a multitude of human tumor tissues and cell lines. Several reports have demonstrated that the overexpression of WAVE3 is associated with the invasion and metastasis of various cancer cells. In breast cancer, WAVE3 regulates cell motility and invasion [20, 21]; knockdown of WAVE3 leads to reduced ZEB1 levels and increased miR-200 and E-cadherin levels, resulting in the loss of invasion potential [22]. WAVE3 has a significant value for assessing prognosis in colorectal cancer [23] and non-small cell lung cancer [24]. WAVE3 has also been reported to promote gastric cancer cell migration and invasion by functioning in EMT via the upregulation of Snail [15]. Teng et al. [25] demonstrated that the inactivation of the WAVE3 gene could suppress the tumorigenicity and metastasis of prostate cancer; furthermore, these authors also found that the invasion phenotype was accompanied by the down-regulation of MMP 9. Taylor et al. [26] found that WAVE mediated the initiation of EMT programs stimulated by TGF-β in triple-negative breast cancer cells. Similarly, in the present study, we also found that the increased expression of WAVE3 is associated with migration and invasion in ICC cells. Moreover, we demonstrated that the knockdown of WAVE3 by siRNA could restrain the migratory, invasive and proliferative abilities of ICC cells.

Collectively, all of the above results disclose that WAVE3 plays a critical role in the migration, invasion and proliferation of cancers.

The EMT is a highly conserved process in which polarized, immotile epithelial cells lose adherent and tight junctions and become migratory mesenchymal cells [27]. Cancer progression is associated with the EMT process, which enhances local invasion and metastasis [28]. The Slug, Vimentin, ZEB1 and E-cadherin proteins are all involved in the EMT process. During ICC progression, in the EMT process, aberrant biliary epithelial cells lose adherence and gain mesenchymal potential; these cells then have greater migratory and invasive capabilities. Recently, several studies have shown that the EMT is associated with the acquisition of malignant characteristics in ICC. In Yang's study [29], EMT plays a critical role in the migration and invasion of ICC cells. The EMT process is associated with tumor progression and a poor outcome in patients with intrahepatic cholangiocarcinoma [30, 31]. WAVE3 has been reported to induce EMT in cancers. Yue et al. [15] found that WAVE3 could promote cancer cell migration and invasion by functioning in EMT via the upregulation of Snail. A previous report [32] showed that the forced expression of the WAVE3 gene in non-invasive T47D cells leads to disruption of E-cadherin-based cell–cell adhesion. In Teng's study, the WAVE3 influence on ZEB1 activation was found to lead to the loss of cell surface E-cadherin in breast cancer cells, which is consistent with its ability to profoundly influence invasion. While ZEB1 [33] is a transcriptional repressor of E-cadherin, Gu et al. [34] found that the overexpression of Vimentin and reduced expression of E-cadherin were correlated with poorly differentiated ICC; these authors further noted that EMT phenotypes can be useful markers for the prediction of ICC patient outcomes. In accordance with these previous reports, we also determined that WAVE3 was closely associated with the EMT because of the phenomenon that WAVE3 knockdown resulted in the degradation of Vimentin, Slug and ZEB1 and an increase of E-Cadherin in ICC cell lines. Moreover, in our study, following gene silencing of WAVE3, the morphology of CC-LP-1 cells changed from spindle-like to cobblestone-like.

Our data demonstrated that WAVE3 plays a significant role in the progression and metastasis of ICC, and the overexpression of WAVE3 promotes the migration and invasion of ICC cells by inducing the EMT process. Nevertheless, there are some limitations in our study. First, the effect of WAVE3 on promoting the migration and invasion of ICC cells was studied only in vitro; animal experiments were not conducted. In a previous study [35], Teng et al. used the zebrafish model to simulate an in vivo experiment and demonstrated that the WAVE3-gene-deficient tumor cells had significantly reduced metastatic potential compared to the high-WAVE3-expressing cells. Second, EMT-related proteins consist of more than Vimentin, Slug, ZEB1 and E-cadherin; the Snail and N-cadherin proteins are also EMT markers. In this study, we detected the protein levels of Vimentin, Slug, ZEB1 and E-cadherin to determine the EMT process. In summary, in this study, we first revealed the exceptional overexpression of WAVE3 both in ICC tissues and cell lines. In addition, a high expression of WAVE3 was found to be closely associated with the malignant characteristics of ICC. We noted that WAVE3 could be used as an independent prognostic factor for patients with ICC. Moreover, we illustrated that WAVE3 elevates the migratory, invasive and proliferative abilities of ICC cells. Therefore, our data suggest a novel mechanism for ICC progression: WAVE3 overexpression promotes migration and invasion in ICC. WAVE3 may thus provide a theoretical basis for the targeted therapy of ICC.

Compliance with ethical standards

Conflict of interest The authors of this manuscript have no financial arrangements to disclose.

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