Kindlin-2 promotes hepatocellular carcinoma invasion and metastasis by increasing Wnt/β-catenin signaling

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Abstract

**Background:** Kindlin-2 is a member of the focal adhesion protein family that regulates invasion and metastasis in multiple malignancies; however, little is known about the role of Kindlin-2 in hepatocellular carcinoma (HCC) progression.

**Methods:** Immunohistochemistry was used to investigate Kindlin-2 expression in 177 pairs of human HCC and adjacent liver tissue samples. The role of Kindlin-2 in the in vitro invasion and migration of HCC cell lines was evaluated in MHCC97H, LM3 and SMMC7721 cells. Microarray expression analysis was applied to explore the molecular mechanism through which Kindlin-2 promoted HCC progression. Quantitative real-time PCR and Western blotting were performed to verify the microarray results.

**Results:** High Kindlin-2 expression was found to significantly correlate with aggressive HCC clinicopathological features including tumor encapsulation, microvascular invasion, extrahepatic metastasis and poor prognosis. In vitro, Kindlin-2 knockout or knockdown inhibited HCC cell adhesion, migration and invasion, while ectopic Kindlin-2 expression promoted these processes. Importantly, Kindlin-2 activated Wnt/β-catenin signaling and increased β-catenin expression, especially levels of non-phosphorylated β-catenin, as well as two Wnt/β-catenin signaling pathway targets, Axin2 and MMP7. Kindlin-2 also induced a change in the expression profile of HCC cells, suggesting the cells underwent epithelial-mesenchymal transition. For example, the expression of the epithelial marker E-cadherin was downregulated, while the mesenchymal markers Vimentin, N-cadherin and Snail were upregulated.

**Conclusion:** Kindlin-2 promotes HCC invasion, metastasis and epithelial-mesenchymal transition through Wnt/β-catenin signaling.

**Keywords:** Hepatocellular carcinoma, Kindlin-2, Invasion, Metastasis, Wnt/β-catenin signaling
esophageal squamous cell carcinoma, pancreatic ductal adenocarcinoma, clear cell renal carcinoma, malignant mesothelioma and glioma [9–18]. In addition, Kindlin-2 has been identified as a prognostic biomarker for patients with HCC [19]; however, the specific role of Kindlin-2 in HCC progression remains unknown until now. Moreover, how Kindlin-2 participates in HCC invasion and metastasis needs to be elucidated.

In this study, we detected Kindlin-2 expression in 177 paired HCC and adjacent non-tumor tissue samples and found that high Kindlin-2 expression was significantly correlated with tumor encapsulation, microvascular invasion, extrahepatic metastasis and prognosis. Meanwhile, we experimentally determined that Kindlin-2 enhanced HCC cell adhesion and motility in vitro. Finally, we demonstrated that Kindlin-2 promoted HCC invasion and metastasis by enhancing epithelial-mesenchymal transition (EMT), which was activated by Wnt/β-catenin signaling.

**Methods**

**Patients and specimens**

Cohort 1 included 127 pairs of HCC and adjacent liver tissues (at least 2 cm away from the tumor, with microscopic confirmation of no tumor) collected from patients (110 males and 17 females, mean age 51 years) who underwent hepatic resection at the First Affiliated Hospital of Fujian Medical University (Fuzhou, China) between September 2001 and March 2011. These specimens were used for tissue microarray (TMA) construction. Additionally, 50 pairs of HCC and adjacent liver tissues (the same criteria as above) collected from patients (42 males and 8 females, mean age 59 years) who underwent hepatic resection at the Shengli Clinical Medical College of Fujian Medical University (Fuzhou, China) between August 2015 and August 2016 were enrolled into cohort 2. None of the patients received preoperative therapy. The diagnosis and identification of pathological factors of HCC were confirmed by two independent histopathologists. The size of specimens was divided into small HCC (≤ 3 cm) and non - small HCC (> 3 cm) according to the cut-off value of 3 cm [20]. The histological grade was classified into I to IV according to the Edmondson-Steiner grading system. Grade I – II stands for high differentiation while grade III – IV indicates low differentiation [21]. Liver cirrhosis, tumor encapsulation, microvascular invasion and metastasis was determined with histopathological diagnosis in combination with imaging examinations. The data of serum alpha - fetoprotein (AFP) and hepatitis B surface antigen (HBsAg), etc. was captured from the archived medical records. The study was approved by the Ethics Review Committee of Fujian Medical University (Reference No. SQ2015–036-01) and signed informed consent was obtained from each patient.

**Follow-up**

Follow-up was carried out for patients in cohort 1. Complete follow-up information for 125 patients was acquired until March 2014, and the median observation time was 22 months (range, 1–94 months). Overall survival (OS) was defined as the interval between hepatic resection and death or the last follow-up.

**Immunohistochemistry (IHC)**

All tissues were fixed in 10% neutral formalin, embedded in paraffin, and then 3-μm sections were prepared for IHC. Sections were stained using the EliVision™ plus two-step method (EliVision™ Super KIT9922, Maixin, Fuzhou, China). Mouse anti-Kindlin-2 monoclonal antibody was obtained from Millipore (MAB2617, Billerica, MA, USA) and used at a 1:100 dilution. Two pathologists, blinded to clinicopathological data, independently evaluated the immunostained sections. Staining intensity was scored as follows: 0, negative; 1, pale yellow; 2, medium yellow; and 3, tawny. The proportion of positive-stained cells was scored as follows: 0, ≤10%; 1, 11%–25%; 2, 26%–50%; 3, 51%–75%; and 4, ≥76%. We then multiplied intensity scores by proportion scores to obtain total scores. Cases with a total score of 0–3 were defined as low expression and those with a total score of 4–12 as high expression.

**Cell culture**

Human HCC cell lines LM3, MHCC97H and MHCC97L were purchased from the Liver Cancer Institute of Fudan University (Shanghai, China). The HepG2 cell lines were purchased from the American Tissue Culture Collection (Manassas, VA, USA). The SMMC7721 cell line and a normal liver cell line L-02 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco).

**Establishing stable cell lines with increased or decreased Kindlin-2 expression**

The Kindlin-2 open reading frame (Genebank: NM_006832) was cloned into the lentiviral vector GV358 (GENECHEM, Shanghai, China). Using the packaging plasmids pHelper 1.0 and pHelper 2.0 (GENECHEM), lentivirus expressing Kindlin-2 was generated and used to infect SMMC7721 cells. After 72 h infection, infected cells were cultured in DMEM containing 2 μg/ml puro-mycin to select cells stably expressing Kindlin-2. Cells that stably overexpressing Kindlin-2 were designated as SMMC7721/LV-K2, whereas control cells were named SMMC7721/LV-ctrl.
Three short-hairpin RNAs (shRNA) targeting Kindlin-2 and a negative control (shcontrol) were synthesized (Table 1) and cloned into the lentiviral vector GV248 (GENECHEM). The packaging of lentivirus expressing shRNA, infection of HCC cells and selection of stable knockdown cells were conducted as described above. Stable Kindlin-2-silenced cells were designated as LM3/LV-shK2, whereas control cells were named LM3/LV-shctrl. Kindlin-2 expression in all stable cell lines was verified by Western blotting analysis. knockdown cells were conducted as described above. Stable Kindlin-2-silenced cells were designated as LM3/LV-shK2, whereas control cells were named LM3/LV-shctrl. Kindlin-2 expression in all stable cell lines was verified by Western blotting analysis.

Establishing Kindlin-2 knockout cell lines via clustered regularly interspaced short palindromic repeats /CRISPR-associated protein 9 (CRISPR/Cas9) technology

Three single-guide RNA (sgRNA) sequences (Table 1) targeting exon 2 of Kindlin-2 were designed at http://crispr.mit.edu. CRISPR/Cas9-mediated Kindlin-2 knock-out was performed using the LentiCRISPRv2 lentiviral vector system (Addgene, Cambridge, MA, USA) following the protocol as described previously [22]. The primers used to verify clones with Kindlin-2 knockout are also presented in Table 1. Kindlin-2 protein levels in each clone were measured by Western blotting analysis.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). qRT-PCR assay was performed using the SYBR Master Mix (Takara, Shiga, Japan) on an ABI7500 (Applied Biosystems, Foster City, CA, USA). The primer sequences used are listed in Table 1. Relative gene expression was calculated using the 2^−ΔΔCt method.

Western blotting

Cell lysates were separated by 10% SDS-PAGE and transferred into polyvinylidene difluoride (PVDF) membranes (Millipore), which were incubated with antibodies specific to Kindlin-2 (MAB2617, Millipore; dilution 1:3000), E-cadherin (ab40772, Abcam, Cambridge, UK; 1:10,000), N-cadherin (ab76011, Abcam; 1:5000), Vimentin (5741, Cell Signaling Technology, Danvers, MA, USA; 1:1000), Snail (3879, Cell Signaling Technology; 1:1000), β-catenin (8480, Cell Signaling Technology; 1:1000), phospho-β-catenin (9561, Cell Signaling Technology; 1:1000), non-phospho-β-catenin (8814, Cell Signaling Technology; 1:1000), Axin2 (ab109307, Abcam; 1:1000), MMP7 (ab205525, Abcam; 1:1000) and GAPDH (ab8245, Abcam; 1:5000) overnight at 4 °C, followed by incubation with HRP-conjugated goat anti-mouse IgG antibody. Blots were visualized using an enhanced chemiluminescence kit and detected using QuantityOne software (Bio-Rad, Hercules, CA, USA).

Wound-healing assay

HCC cells were cultured in two wells of the Culture-Insert (Ibidi, Munich, Germany). When both wells were filled with adherent cells, a wound at approximately 500 μm was created by gently removing the Culture-Insert. Then cells were maintained in DMEM supplemented with 10% FBS and antibiotics. The cells were observed, and images were captured 0, 24 and 48 h after wound creation.

Adhesion assay

HCC cells were seeded onto 24-well plates pre-coated with 10 μg/ml Fibronectin or Collagen I (both from BD Biosciences, San Jose, CA, USA). Adherent cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Then the adherent cells were counted in 5 randomly selected visual fields at 200× magnification. Each experiment was independently repeated in triplicate.

Transwell migration and invasion assays

Migration and invasion assays were performed using uncoated or Matrigel-coated (Corning, Corning, NY, USA) transwell culture chambers (Millipore) according to the manufacturer’s instructions. The Matrigel membrane was stained using crystal violet, and migrating or invading cells were counted in 5 randomly selected visual fields.
fields at 200× magnification. Each experiment was independently repeated in triplicate.

**Gene expression microarray analysis**

The gene expression microarray analysis was performed on Kindlin-2 knockdown LM3 cells. Total RNAs extracted from LM3/LV-shK2 and LM3/LV-shctrl cells were linearly amplified, labeled with Cy3-UTP and purified. Prepared samples were hybridized to the Human Gene Expression 4 × 44 K Microarray v2 (Agilent Technologies, Santa Clara, CA, USA), scanned using the Agilent G2505C DNA Microarray Scanner, and then analyzed by Feature Extraction v11.0 and Agilent GeneSpring GX v12.1 software at KangChen Biotech Institute (Shanghai, China). The microarray data has been deposited into the NCBI Gene Expression Omnibus (GEO) database and is accessible through GEO Series accession number GSE97951.

**Transfection**

The siRNA targeting β-catenin and control siRNA were purchased from Cell Signaling Technology (Danvers, MA, USA). SMMC7721/LV-ctrl or SMMC7721/LV-K2 cells were transfected with siRNAs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols. Meanwhile, SMMC7721/LV-K2 cells were treated with 10 μM ICG-001 (Selleckchem, Houston, TX, USA), which antagonized Wnt/β-catenin/TCF-mediated transcription.

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**Fig. 1** Kindlin-2 expression is upregulated in HCC tissues and cell lines. **a, b** Immunohistochemical analysis of Kindlin-2 expression in a TMA of 127 pairs of HCC and adjacent liver tissues. Staining intensity is scored as: 3, tawny (left), 2, medium yellow (middle) and 1, pale yellow (right). **c** Immunohistochemical analysis of Kindlin-2 expression in another 50 pairs of HCC and adjacent liver tissues. Kindlin-2 expression is higher in tumor margins than central areas in some samples. ▲: tumor margin, ★: central area. **d** Kaplan-Meier analysis of overall survival for HCC patients in cohort 1 with high or low Kindlin-2 expression. HCC patients with high Kindlin-2 expression have shorter overall survival than those with low Kindlin-2 expression. Cases with IHC staining total scores, which is obtained through intensity score multiplying by proportion score, of 4–12 were defined as Kindlin-2 high expression and those with total scores of 0–3 as Kindlin-2 low expression. **e** Western blot analysis of Kindlin-2 expression in six human HCC cell lines and one normal liver cell line.
Statistical analysis

All statistical analyses were conducted with the statistical software SPSS version 19.0 (SPSS Inc., Armonk, NY, USA). All measured data were presented as mean ± standard deviation (SD) and were compared using Student’s *t*-test. Enumerated data were compared using the Kruskal – Wallis H(K) test. Survival rates were calculated using the Kaplan–Meier method, and comparisons of survival curves were performed by the log-rank test. Univariate and multivariate analyses were performed by log-rank test and the Cox proportional hazards regression model, respectively. *P* < 0.05 was defined as statistically significant.

### Table 2: Correlation Between Kindlin-2 Expression and Clinicopathological Characteristics of HCC in Cohort 1 and Cohort 2

| Clinicopathological variables | Cohort 1 | Cohort 2 |
|------------------------------|----------|----------|
|                              | n        | Kindlin-2 expression** | χ² | P value | n        | Kindlin-2 expression** | χ² | P value |
|                              |          | High(103) | Low(24) |          |          | High(31) | Low(19) |          |
| Sex                          |          |          |        |          |          |          |        |          |
| Female                       | 17       | 13       | 4      |          | 8        | 5        | 3      |          |
| Male                         | 110      | 90       | 20     | 0.273    | 0.602    | 42       | 26     | 16      | 0.001    | 0.975    |
| Age, years                   |          |          |        |          |          |          |        |          |
| ≤ 60                         | 111      | 88       | 23     |          |          | 36       | 23     | 13      |          |
| >60                          | 16       | 15       | 1      | 1.896    | 0.169    | 14       | 8      | 6       | 0.191    | 0.662    |
| Serum AFP, ng/ml             |          |          |        |          |          |          |        |          |
| ≤ 252                        | 51       | 40       | 11     |          |          | 18       | 10     | 8       |          |
| >252                         | 76       | 63       | 13     | 0.394    | 0.530    | 32       | 21     | 11      | 0.486    | 0.486    |
| HbsAg                        |          |          |        |          |          |          |        |          |
| Negative                     | 14       | 12       | 2      |          |          | 8        | 4      | 4       |          |
| Positive                     | 113      | 91       | 22     | 0.217    | 0.642    | 42       | 27     | 15      | 0.570    | 0.450    |
| Liver cirrhosis              |          |          |        |          |          |          |        |          |
| Absence                      | 23       | 19       | 4      |          |          | 14       | 10     | 4       |          |
| Presence                     | 104      | 84       | 20     | 0.041    | 0.839    | 36       | 21     | 15      | 0.719    | 0.396    |
| Tumor number                 |          |          |        |          |          |          |        |          |
| Single                       | 106      | 89       | 17     |          |          | 46       | 29     | 17      |          |
| Multiple                     | 21       | 14       | 7      | 3.394    | 0.065    | 4        | 2      | 2       | 0.260    | 0.610    |
| Maximal tumor size, cm       |          |          |        |          |          |          |        |          |
| ≤ 3                          | 10       | 6        | 4      |          |          | 7        | 3      | 4       |          |
| >3                           | 117      | 97       | 20     | 3.129    | 0.077    | 43       | 28     | 15      | 0.399    | 0.528    |
| Edmondson-Steiner grade      |          |          |        |          |          |          |        |          |
| I & II                       | 11       | 10       | 1      |          |          | 19       | 11     | 8       |          |
| III & IV                     | 116      | 93       | 23     | 0.750    | 0.387    | 31       | 20     | 11      | 0.215    | 0.643    |
| Tumor encapsulation          |          |          |        |          |          |          |        |          |
| Absence                      | 40       | 33       | 7      |          |          | 33       | 17     | 16      |          |
| Presence                     | 87       | 70       | 17     | 0.074    | 0.786    | 17       | 14     | 3       | 4.438    | 0.035    |
| Microvascular invasion       |          |          |        |          |          |          |        |          |
| Absence                      | 66       | 49       | 17     |          |          | 21       | 9      | 12      |          |
| Presence                     | 61       | 54       | 7      | 4.186    | 0.041    | 29       | 22     | 7       | 5.519    | 0.019    |
| Metastasis*                  |          |          |        |          |          |          |        |          |
| Absence                      | 9        | 4        | 5      |          |          | 12       | 3      | 9       |          |
| Intrahepatic presence        | 64       | 50       | 14     | 4.585    | 0.032    | 17       | 9      | 8       | 2.186    | 0.139    |
| Extrahepatic Presence        | 51       | 47       | 4      | 4.196    | 0.041    | 21       | 19     | 2       | 6.646    | 0.010    |

*Three patients’ metastatic status is uncertain in cohort 1 due to their rejection to the imaging examination. ** Cases with IHC staining total score, which was obtained through intensity score multiplying by proportion score, of 4–12 were defined as Kindlin-2 high expression and those with total score of 0–3 as Kindlin-2 low expression. All entries in boldface have significance (*P* < 0.05)
Results
Kindlin-2 expression is upregulated in HCC tissues and cells

IHC was used in a TMA that included 127 pairs of HCC and adjacent liver tissues to investigate Kindlin-2 expression in HCC. The results showed that Kindlin-2 was localized in the cytoplasm of HCC cell lines. High Kindlin-2 expression was found in 103 of 127 (81.1%) HCC tissues compared with 82 of 127 (64.6%) corresponding adjacent tissues (z = −3.280, P = 0.001) (Fig. 1a and b). Kindlin-2 upregulation in HCC was confirmed in an additional 50 pairs of HCC and adjacent liver tissues samples, which showed high Kindlin-2 IHC staining in 31 (62%) and 24 (48%) of cases, respectively (z = −2.646, P = 0.008). In addition, we found that the tumor margins exhibited higher Kindlin-2 expression than the central areas in 14 (28%) of these HCC samples (Fig. 1c). Next, Western blotting was performed to determine Kindlin-2 protein levels in HCC cell lines and a normal liver cell line. Compared with the normal liver cell line L-02, Kindlin-2 expression was increased in all six tested HCC cell lines. Notably, Kindlin-2 expression was higher in the highly metastatic LM3, MHCC97H and MHCC97L cells than in the low metastatic SMMC7721 and HepG2 cells. (Fig. 1e).

High Kindlin-2 expression correlates with aggressive clinicopathological features and poor prognosis of HCC

We next examined the correlations between Kindlin-2 expression and clinicopathological features of HCC patients in cohorts 1 and 2 as well as the OS of patients in cohort 1. High Kindlin-2 expression was found to significantly correlate with tumor encapsulation, microvascular invasion and extrahepatic metastasis (all P values < 0.05, Table 2). Kaplan–Meier analysis revealed that the HCC patients with high Kindlin-2 expression (39/102, 38.2%) had shorter OS than those with low Kindlin-2 expression (15/23, 65.2%, χ² = 4.595, P = 0.032) (Fig. 1d, Table 3). Multivariate analysis showed that high Kindlin-2 expression was an independent risk factor (P = 0.045, Table 4) for OS in HCC patients. Taken together, these data suggested that Kindlin-2 is involved in HCC metastasis and progression; furthermore, high Kindlin-2 expression may predict poor prognosis in HCC patients.

Kindlin-2 promoted HCC cell migration and invasion in vitro

To evaluate the role of Kindlin-2 in the invasion and migration of HCC cells, three stable HCC cell lines were established. According to the level of Kindlin-2 protein expression in different HCC cell lines (Fig. 1), we infected SMMC7721 cells (low Kindlin-2 expression, low metastatic potential) with lentiviruses that ectopically expressing Kindlin-2. LM3 cells (high Kindlin-2 expression, high metastatic potential) with shRNA-knockdown lentiviruses and MHCC97H cells (high Kindlin-2 expression, high metastatic potential) with sgRNA-Cas9 knockout lentiviruses. The level of Kindlin-2 expression was confirmed by Western blotting, and DNA sequencing was used to identify CRISPR/Cas9-induced genetic lesions (Fig. 2). Clone No. 5 of the MHCC97H stable cells, which had two bases missed in the Kindlin-2 open reading frame (Fig. 2d), was selected for further analyses and designated as MHCC97H/ko-K2, whereas infected MHCC97H cells without vector were designated as MHCC97H/wt-K2. The migration and invasion capacities of these cells were investigated using wound-healing and transwell assays. We found that Kindlin-2 upregulation significantly improved the wound healing ability of SMMC7721 cells and promoted cell migration and invasion through Matrigel, whereas Kindlin-2 knockdown or knockout inhibited the migration capacity of LM3 and MHCC97H cells (Fig. 3). These results suggested that Kindlin-2 promoted HCC migration and invasion in vitro.

Kindlin-2 activates Wnt/β-catenin signaling in HCC

We used microarray expression analysis to compare gene expression levels between Kindlin-2 knockdown and control cells. Our findings showed that Axin2, with a 4.49-fold change of, was significantly downregulated in LM3/LV-shK2 cells (Fig. 4a). Both qRT-PCR and Western blot analyses showed that Axin2 expression was decreased following Kindlin-2 knockdown or knockout but increased after Kindlin-2 overexpression (Fig. 4b and c), which was in agreement with the microarray results. Since Axin2 has been reported a target gene of Wnt/β-catenin signaling. We therefore examined the expression...
of β-catenin, a key molecule in the Wnt/β-catenin pathway, and MMP7, another target gene of Wnt/β-catenin signaling, in HCC cells. Western Blotting detected reduced expression of β-catenin and MMP7 protein in Kindlin-2 knockdown or knockout cells and elevated expression in Kindlin-2-overexpressing cells (Fig. 5a). In addition, non-phospho- rather than phospho-β-catenin was altered accordingly with changes in Kindlin-2 expression (Fig. 5b). These results demonstrated that Kindlin-2 activates Wnt/β-catenin signaling in HCC.

**Kindlin-2 promotes HCC EMT**

β-catenin is also an important marker of EMT, which endows cancer cells with migratory and invasive properties. To assess the effect of Kindlin-2 on EMT in HCC cells, Western blotting was performed to determine the expression of EMT biomarkers. Although Kindlin-2 knockdown did not change levels of the epithelial marker E-cadherin in LM3 cells, Kindlin-2 knockout led to E-cadherin upregulation in MHCC97H cells (Fig. 6a). In addition, either knockdown or knockout of Kindlin-2 resulted in the downregulation of the mesenchymal markers Vimentin, N-cadherin and Snail, while ectopic Kindlin-2 expression caused the opposite results in SMMC7721 cells (Fig. 6b). These results indicated that Kindlin-2 promotes EMT in HCC cells.

**Activation of Wnt/β-catenin signaling is required for kindlin-2-induced HCC cell migration and invasion**

To investigate whether β-catenin activation plays a critical role in Kindlin-2-induced HCC cell migration and invasion, SMMC7721 cells with stable ectopic Kindlin-2 expression were transiently transfected with β-catenin siRNA or treated with ICG-001, the inhibitor of Wnt/β-catenin signaling.
β-catenin/TCF-mediated transcription. Our findings showed that both β-catenin downregulation and ICG-001 treatment resulted in a decrease of Axin2 expression but did not affect Kindlin-2 expression (Fig. 7a), and ICG-001 treatment did not alter β-catenin expression (Fig. 7a). Additionally, Kindlin-2-induced migration and invasion was abolished by knockdown of β-catenin or ICG-001 treatment (Fig. 7b and c). These results indicated that β-catenin was a key molecular for Kindlin-2 activation of Wnt/β-catenin signaling. Wnt/β-catenin signaling was essential for Kindlin-2-induced HCC cell migration and invasion.

**Discussion**

It is clear that local invasion and distant metastasis are important hallmarks of advanced-staged carcinomas of epithelial origin. EMT, through which cancer cells acquire invasive and migratory abilities, has frequently been implicated in malignant progression. In this study, we demonstrated that Kindlin-2 was upregulated in both large-scale HCC clinical samples and HCC cell lines and was significantly correlated with aggressive clinicopathological features and poor prognosis. Moreover, we provided the first evidence that Kindlin-2 promoted the adhesion, migration, invasion, and invasion of HCC cells.
and EMT of HCC cells by activating Wnt/β-catenin signaling. Thus, Kindlin-2 may be a promising molecular marker for targeted therapy and prognostic evaluation.

It has been reported that Kindlin-2 is not or is only weakly expressed in ectoderm/endoderm-derived organs, but highly expressed in mesoderm-derived organs [23]. Interestingly, higher Kindlin-2 expression was detected in tumor tissues derived from the ectoderm or endoderm, including esophageal, gastric, lung, breast and pancreatic cancer [10, 12, 13, 18, 24]. In accordance with these observations, we detected an increase in Kindlin-2 expression in HCC cells which originate from the endoderm. Consistently, higher Kindlin-2 expression was found in both the HCC TMA and tissue samples than in the corresponding adjacent tissues.

Previous studies have verified the prominent role of Kindlin-2 in regulating the progression of some tumors. Aside from the study by Ge et al. [19], which suggested Kindlin-2 expression had prognostic value, there is no solid evidence for the effect of Kindlin-2 on HCC development. Using 177 HCC samples, our study confirmed that Kindlin-2 upregulation was associated with tumor encapsulation, microvascular invasion and extrahepatic metastasis. In addition, the highly metastatic LM3 and MHCC97H cell lines also exhibited relatively high Kindlin-2 expression. Consistent with previous studies, our findings showed that the HCC patients with high Kindlin-2 expression had shorter OS, suggesting high Kindlin-2 expression might be an independent risk factor for HCC. Our in vitro functional experiments showed that Kindlin-2 knockout or knockdown inhibited the adhesion, migration and invasion of HCC cells. Conversely, Kindlin-2 overexpression promoted the adhesion, migration and invasion of HCC cells. These results highlighted the role of Kindlin-2 in promoting HCC progression.

As an integrin coactivator that binds to the cytoplasmic tail of β-integrin, Kindlin-2 participates in a variety of physiological processes. The interaction between Kindlin-2 and integrin was first uncovered in studies of tumor invasion and metastasis. Kindlin-2 enhanced the
proliferation and adhesion of gastric cancer cells, and promoted invasion via the phosphorylation of integrin β1 and β3 [25]. Similarly, Kindlin-2 increased esophageal squamous cell carcinoma invasiveness through the integrin β1/PI3K/AKT pathway [26]. Along with integrin, Kindlin-2 forms functional complexes with YB-1, β-catenin, TCF4 and EGFR, to regulate the expression of genes associated with breast cancer and glioma progression [15, 24, 27]. Additionally, Kindlin-2 and oncogenes including GLI1 and TGFβ constitute a feedback loop in prostate cancer and pancreatic ductal adenocarcinoma [13, 28]. Thus far, there has been little knowledge pertaining to the mechanism by which Kindlin-2 promotes HCC invasion and metastasis. Thus, we performed a gene expression microarray analysis to identify targets or molecular signaling pathways that are activated in response to Kindlin-2 in HCC. Axin2 [29], a target gene of β-catenin, was found to be significantly downregulated in Kindlin-2 knockdown cells, which was confirmed by qRT-PCR and Western blotting. Next, β-catenin and MMP7, another downstream target of Wnt/β-catenin signaling [30], were also found to be downregulated in Kindlin-2 knockdown or knockout cells and upregulated in Kindlin-2-overexpressing cells as revealed by Western blotting. The β-catenin destruction complex comprised of GSK-3, Axin and APC can bind to and phosphorylate β-catenin, which is followed by its proteasomal degradation [31]. Therefore, only non-phosphorylated β-catenin is functional and can translocate into the nucleus and initiate target gene transcription. We found that non-phosphorylated, but not phosphorylated β-catenin, was altered in response to altered Kindlin-2 expression. These results suggest that Kindlin-2 activates Wnt/β-catenin signaling in HCC. Furthermore, silencing of β-catenin or pharmacological inhibition of Wnt/β-catenin/TCF-mediated transcription were both able to...
attenuate the effect of Kindlin-2 on HCC cells. These results suggest that β-catenin is required for Kindlin-2 promotion of migration and invasion in HCC cells. Meanwhile, Wnt/β-catenin signaling is essential for Kindlin-2-induced HCC progression.

β-catenin, which is a component of intercellular adherens junctions, links the cytoplasmic domain of cadherins to the actin cytoskeleton. Thus, when β-catenin translocates into the nucleus, the cadherin/β-catenin bond is dissociated, and intracellular adhesion is lost [32]. Our data showed no difference in E-cadherin expression between Kindlin-2 knockdown and control cells, however, Kindlin-2 knockout induced an increase in E-cadherin expression, while Kindlin-2 overexpression caused a decrease in E-cadherin expression indicating Kindlin-2 had an inhibitory effect on E-cadherin expression in HCC. These results also demonstrate that CRISPR/Cas9 technology can more effectively highlight the function of a gene than RNA interference. Loss of attachment to adjacent cells and reduced E-cadherin expression are typical alterations in the process of local invasion and distant metastasis of epithelial-originating carcinomas [33]. A deficiency of E-cadherin activity promotes EMT in cancer including HCC [34]. EMT is able to endow tumor cells with stem cell-like phenotype to increase the ability of migration/invasion [35, 36] and Wnt/β-catenin signaling was reporeted to have a close association with EMT [37]. The phenomenon of a cluster of tumor cells detaching from the mass and extending into the adjacent stroma at the invasive fronts of carcinomas provided morphological evidence for EMT [38]. Parts of solid carcinomas show an EMT expression profile such as loss of E-cadherin at the invasive fronts [39]. After discovering there was higher Kindlin-2 expression in the invasive margins of HCC tissues, we hypothesized that Kindlin-2 might promote EMT in HCC. Thus, we analyzed other key EMT biomarkers in HCC cells by Western blotting. Knockdown or knockout of Kindlin-2 caused a reduction in the expression of the mesenchymal markers N-cadherin, Vimentin and Snail, while ectopic Kindlin-2 expression caused the opposite results in SMMC7721 cells.

Fig. 6 Kindlin-2 promotes epithelial-mesenchymal transition in HCC cells. a Kindlin-2 knockout leads to upregulated expression of the epithelial marker E-cadherin in MHCC97H cells, while ectopic Kindlin-2 expression induced downregulated expression of E-cadherin in SMMC7721 cells. However, Kindlin-2 knockdown does not change the expression of E-cadherin in LM3 cells. b Knockout or knockdown of Kindlin-2 results in the downregulation of the mesenchymal markers vimentin, N-cadherin and Snail, while ectopic Kindlin-2 expression causes the opposite results in SMMC7721 cells.
markers in SMMC7721 cells. Together, these data support our hypothesis and show that Kindlin-2 promotes EMT in HCC.

**Conclusions**

In summary, the results of the present study demonstrate that high Kindlin-2 expression correlates with increased invasion and metastasis in vitro and with poor prognosis in HCC patients. In addition, our data indicate that Kindlin-2 promotes progression and EMT in HCC cells by increasing Wnt/β-catenin signaling.

**Abbreviations**

Cas 9: CRISPR-associated protein 9; CRISPR: Clustered regularly interspaced short palindromic repeats; EMT: Epithelial-mesenchymal transition; HCC: Hepatocellular carcinoma; IHC: Immunohistochemistry; OS: Overall survival
survival; qRT-PCR: Quantitative real-time PCR; sgRNA: single-guide RNA; shRNA: Short hairpin RNAs; TMA: Tissue microarray

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Availability of data and materials
The microarray data has been deposited into the NCBI Gene Expression Omnibus (GEO) database and is accessible through GEO Series accession number GSE97951.

Authors’ contributions
LJ and LWS conceived and designed the study as well as performed the laboratory analysis. LJ, WLP and CYX performed the histopathological analysis. ZSB performed the microarray expression analysis. HAM and YYB contributed reagents, materials, and analysis tools. LJ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Ethics Review Committee of Fujian Medical University (Reference No. SQ2015–2015–1). Consent for publication obtained from each patient.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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