INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common hepatic malignancy and the second leading cause of cancer mortality worldwide, with a fairly high and increasing incidence, frequent relapse and poor prognosis.\(^1\) Current diagnostic modalities include ultrasound and α-fetoprotein, but both are costly and lack sensitivity in tumor detection.\(^2\) Unfortunately, due to the asymptomatic characteristic of HCC, it is usually diagnosed at late and advanced stages, for which there are no effective therapies. Therefore, more efficient biomarkers for early diagnosis and molecular targets for therapy for HCC urgently need to be explored.

Metabolic reprogramming is one of the central properties of cancer cells.\(^3\) Even under the oxygenated conditions, cancer cells predominantly rely on glycolysis rather than oxidative phosphorylation to generate adenosine triphosphate (ATP).\(^4,5\) This
phenomenon is referred to as the "Warburg effect" (aerobic glycolysis), which gives cancer cells a proliferative advantage and often results in increasing glucose uptake, ATP accumulation and lactate production in cancer cells. Accumulating studies have shown that normal differentiated hepatocytes do not generate energy through aerobic glycolysis in an oxygenated environment; however, extensive metabolic reprogramming occurs in HCC. Therefore, understanding this process is critical to identify novel targets for therapy of HCC.

Ezrin is a member of the ezrin-radixin-moesin (ERM) family that functions as a molecular crosslinker between actin filaments and proteins anchored in the cell membrane. Overexpression of ezrin is known to be a metastasis-related oncogene that is involved in adhesion of cells to the extracellular matrix, cell interactions, cellular motility and invasion promotion of cells. Furthermore, ezrin has already been verified as a potential biomarker in malignancies associated with tumor cell proliferation and metastasis, such as breast, thyroid and lung cancer. Our group also found that ezrin was upregulated in cervical cancer, and its expression was significantly associated with metastasis and poor prognosis. These already discovered functions of ezrin indicated that it played an indispensable role in cancer tumorigenesis and progression. In contrast, the role of ezrin in metastasis and glycolytic reprogramming of HCC remains unclear.

Herein, we sought to demonstrate that the overexpression of ezrin indicates high invasion and poor prognosis in HCC. Furthermore, functional experiments validate that the expression of ezrin significantly correlates with HCC cell proliferation, metastasis and angiogenesis. In addition, we demonstrate that ezrin promotes tumor cell glycolysis, thereby promoting hepatocarcinogenesis and progression.

2. MATERIALS AND METHODS

2.1. Ethic statement

Our study adhered with the Helsinki Declaration and was ratified by the Human Ethics Committee and the Research Ethics Committee of Yanbian University Medical College. Patients were informed that the excised samples stored by the hospital would possibly be used to identify information/images (if applicable) for scientific research and publication, and their privacy was guaranteed to be protected. Subsequent survival data was retrospectively collected through analysis of medical records.

2.2. Clinical specimens

From 2006 to 2009, Shanghai Outdo Biotech and the tissue bank of Yanbian University Medical College selected 120 patients at random who underwent routine treatment and collected paraffin-embedded HCC tissues. The patients met rigorous follow-up criteria. Clinical pathological parameters were examined, including age, gender, tumor size, clinical stage, venous infiltration, α-fetoprotein (AFP) level, hepatitis B surface antigen (HBsAg) status and 5-year survival.

The ages of patients ranged from 33 to 73, with an average age of 51.8 years. The male-to-female ratio was 110:10. In accordance with the eighth edition of the American Joint Committee on Cancer, 55 of 120 HCC specimens were determined as early stage (I-II) and 65 as late stage (III-IV). The median survival time was 34.5 months. In addition, 71 cases from adjoining nontumor tissues were obtained from the margin of the cancer resection in these patients. The inclusion and selection criteria of patients, including no other treatment intervention, were received before admission, and all patients met the indications for surgical resection. All patients had a Karnofsky score of over 70, no mental disorder or abnormal cognitive function, and could effectively cooperate with verbal communication. All patients were followed up for more than 2 years after surgery.

2.3. Cell culture and transfection

The HCC cell lines (HepG2, Huh7, Hep3B and Sk-Hep1) purchased from the ATCC were cultured in DMEM medium (Gibco), which was supplemented with 10% FBS and appropriate antibiotics, and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Two kinds of ezrin siRNA (si-Ezrin-1, si-Ezrin-2) (RIBOBIO, China) were purchased. In light of the knockdown effect, control siRNA, si-Ezrin-1 and si-Ezrin-2 were applied in this research. The sequences of si-Ezrin-1 and si-Ezrin-2 were 5’-AAGGAAUCCUUAGCGAUGAGA-3’ and 5’-GGGCAAGTTCTACCTGAAG-3’. HCC cells were transfected with 30 nM of siRNA by adding Lipofectamine 3000 (Invitrogen), as stated in the manufacturer’s protocol. The pDONR223 plasmid was constructed with ezrin cDNA (You Biosciences) and the corresponding empty vector was transfected into HCC cells (Huh7 and Hep3B) using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s instructions.

2.4. Western blot

RIPA buffer containing a mixture of protease and phosphatase inhibitors was used to lyse cells. Then proteins (equal quantities) were loaded onto SDS-PAGE gels and transferred to a PVDF membrane (Millipore). After blocking with 5% fat-free milk, the mixture was incubated at 4°C with the primary antibody overnight and then with HRP-coupled secondary antibody (Millipore). Enhanced chemiluminescence (Millipore) was used to detect protein signals.

2.5. Reagents

Antibodies against β-actin, ZO-1, E-cadherin, Snail, Slug and Vimentin were purchased from Cell Signaling Technology (Boston). Antibodies against HK2, PKL, GLUT2, LDH and VEGF were purchased from Santa Cruz Biotechnology. MMP-2 and MMP-9 was purchased from Affinity (Cincinnati).
2.6 | Immunohistochemistry

For immunohistochemical studies, tissues were deparaffinized using xylene, while rehydrated in graded alcohol washes. Antigen retrieval was performed by placing the slides in sodium citrate buffer at 80°C for 40 minutes. After rinsing with PBS three times, the endogenous peroxidase was blocked with 3% H₂O₂ for 15 minutes at room temperature (RT). The slides were subsequently incubated with the indicated antibody (1:200 dilution) at 4°C overnight, followed by incubation with the secondary antibody at RT for 1 hour; DAB was immunostained and hematoxylin was counterstained.

2.7 | Evaluation of immunohistochemistry staining

The ezrin staining of HCC tissue sections was judged using a couple-scoring system, combining staining intensity and area extent. The intensity of staining was graded as: 0, no obvious staining; 1, weak staining; 2, moderate staining; 3, strong staining. The area extent was graded as (proportion of positive cells): 0, none or <5% positive cells; 1, 5%-25% positive cells; 2, 26%-50% positive cells; 3, >50% positive cells. The total scores were divided into low or high expression groups by multiplying the intensity score and the ratio of the positive cells score. 0–1 was negative (−), 2–4 was weak positive (+), 5–7 was moderate positive (++) and ≥8 was strong positive (+++). Tissue sections scored as "++" and "+++" were considered as strong positives (high-level expression) of ezrin. Two pathologists scored all the specimens in a blinded manner. The final score established for cases with discrepancies was determined by reassessment by both pathologists with a double-headed microscope.

2.8 | Immunofluorescence

Adherent cells in 6-well plates culture slides were fixed with 4% paraformaldehyde for 15 minutes; 0.5% Triton X-100 (CWBiO, China) was used to permeablishe the cells, which were blocked with 3% BSA for 2 hours. Cells were incubated with primary antibody in 3% BSA at 4°C overnight, washed in PBS three times and incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG secondary antibody (Invitrogen) for 1 hour; nuclei were stained with DAPI solution and then captured by fluorescence microscope (Leica SP5II).

2.9 | Colony formation assay

Cells (5000 cells) were seeded in 6-well plates in triplicate and incubated. After 2 weeks, cells were fixed by 4% paraformaldehyde and stained with hematoxylin for 20 minutes. Then, stained cells were washed with PBS for 30 minutes. Colonies (containing >50 cells) were counted directly and images were captured.

2.10 | MTT assay

Approximately 5000 cells per well were seeded into 96-well plates. MTT was then added (100 μL per well) at 0, 24, 48 and 72 hours, respectively. The MTT was removed from the wells after 4 hours and 100 μL DMSO was added to each well. The relative number of viable cells was assessed by measuring absorbance.

2.11 | Wound healing assay

Cells were seeded in a 6-well plate and cultured overnight. Sterile 200-μL pipette tips were used to create wounds through the monolayer; pre-warmed PBS was then used to wash the plates to remove cellular debris. Cell migration was monitored at 0, 12 and 24 hours, respectively, and images were captured using a microscope.

2.12 | Transwell assay

The migration assay was performed with 8-μm polyethylene terephthalate (PET) film using a 24-well Millicell culture insert (EMD Millipore). Then, 3 × 10⁵ cells were planted in the upper insert with FBS-free medium, while media containing 10% FBS was added to the outside insert as a chemotactractor. The cells were incubated at 37°C for 24 hours, and then fixed by 4% paraformaldehyde and stained with hematoxylin. Images were captured using a microscope (Olympus BX53).

2.13 | Matrigel tube formation assay

HUVEC were cultured in a 96-well plate at 37°C, coated with Matrigel (BD Biosciences) in cell culture medium at 4°C. The plate with Matrigel was allowed to solidify for 4 h at 37°C before cell seeding. The conditioned medium was collected from supernatant fluid of treated HCC cells, and then filtered using a 0.45-μm filter. HUVEC in 150 μL conditioned medium diluted at 2:1 in cell culture medium were seeded at 2 × 10⁴ cells in each well. After incubation at 37°C for 4 hours, the formation of capillaries-like structures was captured under microscope.

2.14 | Glucose uptake, lactate, adenosine triphosphate and pyruvic acid production assays

The media from cultured cells was used for measuring glucose uptake, lactate, ATP and pyruvic acid with a Glucose Uptake Colorimetric Assay Kit (Rongsheng Biotech), a Lactate Colorimetric Assay Kit (Nanjing Jianseng Bioengineering Institute), an ATP assay kit (Nanjing Jianseng Bioengineering Institute) and a Pyruvic Acid Assay Kit (Nanjing Jianseng Bioengineering Institute) according to the manufacturer’s instructions.
2.15 | Oxygen consumption rate and extracellular acidification rate

The Seahorse XFp Analyzer (Agilent) was used to detect the cellular oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). On the first day, experimental and control cells were seeded into Seahorse XFp cell culture microplates and the XFp sensor cartridges (Agilent) were hydrated. At least five replicates were performed for the measurement of each group. On the following day, for OCR detection, microplates were incubated with basic culture medium (17 mmol/L glucose, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, pH7.4) for 1 hour prior to the assay. OCR was measured with sequential injection of oligomycin, FCCP and rotenone/antimycin (final concentration: 1.5, 1 and 0.5 µmol/L, respectively). For ECAR detection, microplates were incubated with basic culture medium (containing 1 mmol/L L-glutamine, without Glucose) for 1 hour prior to the assay. ECAR was measured with sequential injection of glucose, oligomycin and 2-deoxyglucose (final concentration: 10, 1 and 50 mmol/L, respectively).

2.16 | Statistical analysis

The data analysis was performed using SPSS 25.0 software and GraphPad Prism 8.0 software. The t-test for independence means was used for group comparisons. Kaplan-Meier analysis was used to calculate the survival curves. Univariate and multivariate hazard ratios of study variables were examined using the Cox proportional hazards regression model. Group comparisons for continuous data were done by Mann-Whitney U-test or one-way ANOVA. Biochemical experiments were performed in triplicate and at least three independent experiments were evaluated. A value of $P < 0.05$ was considered statistically significant (Table 1).

3 | RESULTS

3.1 | Ezrin expression was upregulated in hepatocellular carcinoma

We used The Cancer Genome Atlas (TCGA) database to confirm the role of ezrin in HCC, and noted that the mRNA expression of ezrin was meaningfully elevated in HCC tissues compared with that observed in normal hepatic tissues (Figure 1A), and its expression was related to stage (http://gepia.cancer-pku.cn) (Figure 1B). Using immunohistochemistry, we examined the expression of ezrin protein in 120 HCC tissues and 71 normal tissues (Figure 1C). The results indicated that the protein expression level of ezrin was significantly upregulated in HCC tissues. Here, 103 of 120 HCC tissues (positive rate: 85.8%) and 21 of 71 non–tumor tissues (positive rate: 29.6%) were found to be positive for ezrin expression ($P < 0.05$). Furthermore, clinicopathological analysis showed that the expression of ezrin was positively correlated with tumor differentiation ($P = 0.040$), tumor size ($P = 0.038$) and clinical stage ($P = 0.037$) but not with age, lymph node metastasis and venous infiltration (Table 2, Figure 1D-G).

3.2 | Ezrin was strongly associated with poor prognosis in hepatocellular carcinoma

Next, we interrogated publicly available data and found that ezrin expression was strongly correlated with overall survival (OS), relapse-free survival and histological stage III in patients with HCC (http://kmplot.com/analysis/) (Figure 2Aa-c). Kaplan-Meier survival analysis showed similar results: that HCC patients with higher ezrin expression had meaningfully shorter survival time. In addition, compared with lower ezrin expression patients, patients with well or moderate differentiation status (both $P = 0.000$), early clinical stages ($P = 0.000$) and large or small tumor size ($P = 0.000$, $P = 0.001$, respectively), had significantly curtailed OS (Figure 2Ba-f). Further analysis of the Cox proportional hazards model revealed that the tumor size and differentiation status of ezrin expression were correlated with OS rates. Moreover, multivariate Cox analysis confirmed that ezrin expression could be a significant independent prognostic marker in HCC (Table 3).

3.3 | Ezrin significantly promoted hepatocellular carcinoma cell proliferation and colonization

To explore the biological functions of ezrin in HCC progression, HCC cell lines Hep3B and Huh7 were transfected with non–targeting siRNA as si-control or two different ezrin-specific lentiviral si-RNA. The expression of ezrin was analyzed by western blot. As shown in Figure 3A, ezrin was significantly depleted by si-Ezrin sequencez #1 and #2. We also constructed stable overexpression of ezrin in Hep3B and Huh7 cells at the same time (Figure 3B). Next, cell proliferation capacity and clonogenicity were detected

| Diagnosis | Number of cases | Ezrin protein expression | Positive rate | Strongly positive rate |
|-----------|----------------|--------------------------|---------------|------------------------|
| Normal    | 71             | − 50 18 3 0              | 29.6%         | 4.2%                   |
| HCC       | 120            | 17 47 35 21              | 85.8%**       | 46.7%**                |

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HCC, hepatocellular carcinoma.

* $P < 0.05$ and ** $P < 0.01$: compared with normal hepatic tissues.

TABLE 1 Ezrin protein expression in HCC

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by MTT, EdU and colony formation assays, and the results showed that loss of ezrin inhibited HCC cell proliferation and clonogenicity, while ezrin overexpression elevated the cell proliferation and clonogenicity (Figure 3C-E).

3.4 | Ezrin promoted hepatocellular carcinoma cell metastasis through epithelial-to-mesenchymal transition progression

Subsequently, we concentrated on the functionalities of ezrin in the migration of HCC cells. As expected, the migration capacities of HCC cells were significantly regulated by ezrin, as shown in the wound healing and transwell assays (Figure 4A,B). For further investigation of its metastatic mechanism, we analyzed publicly available data in the KEGG pathway; the results showed that ezrin participated in HCC cells tight junction, which was closely related to EMT (Figure 4C). Furthermore, positive relationships between ezrin mRNA and Vimentin or Snail mRNA expression were discovered in TCGA (Figure 4D). Therefore, we hypothesized that the ezrin may regulate HCC cell metastasis through EMT. To assess the interaction of ezrin in the EMT progression of HCC cells, western blot was used to image the change in...
EMT markers. Expression of epithelial markers (E-cadherin and ZO-1) was upregulated. Adversely, mesenchymal markers (Vimentin, Snail and Slug) were downregulated, which were paralleled with the change of ezrin expression level (Figure 4E). Immunofluorescence staining further verified our findings (Figure 4F). These data implied that ezrin expression was closely related to EMT progression in HCC.

3.5 | Ezrin promoted angiogenesis in hepatocellular carcinoma cells

As a crucial factor for cancer metastasis and progression, angiogenesis is involved in hepatocarcinogenesis.17,18 According to the aforementioned results, ezrin was closely related to the VEGF signal pathway (Figure 4C). In addition, the mRNA level of ezrin was positively associated with VEGF, MMP-2 and MMP-9, which took part in angiogenesis (Figure 5A). To further explore the effect of ezrin in angiogenesis, we performed in vitro tube formation experiments using HUVEC. As Figure 5B demonstrates, the microtubule formation capacity of HUVEC was decreased in ezrin-depleted cells, while increased in ezrin overexpression cells. Western blot indicated that ezrin depletion downregulated the VEGF, MMP-2 and MMP-9 expression level, while the contradictory effect was shown in ezrin overexpression cells. Altogether, our data suggested that ezrin promoted HCC angiogenesis (Figure 6C).

3.6 | Ezrin promoted cell proliferation, migration and epithelial-to-mesenchymal transition progression through the Warburg effect in hepatocellular carcinoma

Tumorigenesis and cancer progression accompanied an alteration of glucose metabolism. Aberrant cancer metabolism is involved in the process of cancer migration, invasion and metastasis by modulating the EMT.19,20 We questioned whether ezrin was important in glycolysis of HCC. According to the results of the LinkedOmics analysis (http://www.linkedomics.org/), ezrin was significantly connected with biological regulation and metabolic processes (Figure S1A-D). Moreover, the progression of glycolysis metabolism in tumor cells was mainly related to the increased expression or activity of key enzymes in glycolysis, such as HK2.21 We found positive relationships between ezrin and HK2, and other glycolytic enzymes LDHA, PFKL mRNA expression in TCGA (Figure 6A). Furthermore, the results of the kits revealed that glucose consumption, lactate production, ATP and pyruvic acid levels were obviously raised in ezrin overexpression cells but were reduced in ezrin-depleted cells (Figure 6B, Figure S4A,B). To provide further evidence, we identified that HK2, PFKL and LDH, and the glucose transporter GLUT2 were upregulated in HCC cells with ezrin overexpression and were downregulated with ezrin depletion (Figure 6C). Measuring the extracellular acidification rate (ECAR) and the OCR with the Seahorse metabolic analyzer revealed marked reductions in glycolytic function in Huh7 cells upon ezrin depletion (Figure S2A,B). Several studies
WANG et al. have reported that 2-DG, the inhibitor of glycolysis, is useful in tumor treatment by attacking anaerobic cells. To further investigate whether ezrin mediated HCC cell proliferation, migration and the EMT in a manner dependent on the glycolytic pathway, HCC cells were treated with 2-DG for 24 hours. We found that 2-DG significantly inhibited cell proliferation and migration capacities, as determined by colony formation assay, MTT and migration assays (Figure 6D-G). Moreover, 2-DG reversed the ezrin-induced upregulation of Vimentin, MMP9, Snail and Slug as well as the downregulation of E-cadherin and Zo-1 (Figure 6H). Taken together, these results demonstrated that ezrin promoted cell proliferation, migration and EMT progression, initiating glycolytic metabolism reprogramming in HCC.

4 | DISCUSSION

The cytoskeletal organizer ezrin, which is the principal member of the ERM family, has a primary role in tumor development. Previous studies found that ezrin was positively associated with malignancy in a series of tumors and its expression has also been related to poor prognosis in several cancers. Kong et al. reported that the high expression of ezrin is closely linked to poor differentiation, late stage and lymph node metastasis in cervical cancer, which suggests that ezrin is a potential biomarker for predicting clinical prognosis. In accordance with the results of previous studies, we found that ezrin was obviously overexpressed in HCC, and was significantly correlated with patients’ survival and clinical results. Moreover, ezrin overexpression contributed
to poor differentiation, later clinical stage and shortened OS. A similar trend was found in pancreatic cancer and melanomas, supporting our findings. Therefore, the ezrin expression level can predict HCC patients' prognosis, and has the potential to be an efficient tool for the appropriate management of personalized therapy.

Cancer cell proliferation and migration underlie development and metastatic dissemination, which are major problems in cancers. Substantial evidence reveals that ezrin plays an important role in the development and metastatic dissemination of malignant tumors. Zhang et al. reported that the S-Nitrosylation of ezrin promotes non-small-cell lung carcinoma cell proliferation and metastasis, facilitating mechanical transduction from the cytoskeleton to the membrane. Based on the abovementioned studies, we considered whether ezrin may be involved in cell growth and migration in HCC. To confirm this hypothesis, our group performed colony formation, MTT and migration assays. The results verified that depletion of ezrin weakens the proliferation and migration capacities of HCC cells. Moreover, with a high propensity to metastasize cancer, the EMT process is critical in cancers. A recent study indicated that ezrin knockdown restricted actin filament remodeling, hence inhibiting lung adenocarcinoma cell metastasis during EMT. According to the effect of ezrin on EMT progression in tumors, we observed a change in EMT markers in HCC cells transfected with or without ezrin. Western blot revealed that ezrin significantly downregulated the expression level of epithelial markers but upregulated mesenchymal markers, accelerating HCC metastasis. These results had a similar trend to our previous studies in breast cancer cells in which we found that ezrin was involved in cell proliferation, tumorigenesis and EMT progression.

Another crucial factor in tumor growth is angiogenesis, which is necessary for cancer metastasis, progression and hepatocarcinogenesis. We found that ezrin knockout cells strikingly disrupted the ability of HUVEC tube formation. Western blot demonstrated that the marker of angiogenesis VEGF was downregulated. In addition, angiogenesis influences the capacity of tumor cells to attain endothelial cell behavior that could evoke vascular networks in cancer, promotes EMT phenotype for tumor cells and facilitates the cancer cell's stemness. We found that angiogenesis contributed to HCC progression, ezrin depletion significantly inhibited the expression of MMP2 and MMP9, while overexpression enhanced MMP2 and MMP9 expression. These findings suggested that ezrin may contribute to angiogenesis in vitro and even enhance HCC progression.

A cohort of cancers displays an aerobic glycolytic phenotype that often correlates with tumor progression and poorer clinical results, especially in HCC. Due to the high proliferation capacity of HCC, adapted metabolic mechanisms were needed to satisfy its urgent demand for nutrients. However, the role of ezrin in HCC aerobic glycolysis is yet to be established. We first found that ezrin was related to metabolic processes by searching a public database. Furthermore, ezrin increased glucose consumption, lactate production, ATP levels and pyruvic acid production in HCC, which were changed significantly with the Warburg effect. More importantly, ECAR and OCR measurements showed that glycolysis flux and OxPhos-dependent ATP generation were decreased in ezrin-depleted cells, revealing that ezrin might regulate HCC metabolic processes. Mounting research has revealed that cancer cells can remodel their metabolism to an anomalous state to favor the proliferation and metastasis.

### TABLE 3

Univariate and multivariate survival analyses (Cox regression model) of various factors in 174 HCC patients

| Characteristics | B     | SE    | Wald  | 95% CI      | P-value |
|-----------------|-------|-------|-------|-------------|---------|
| Ezrin           | 1.620 | 0.324 | 25.026| 2.679       | 0.000*  |
| Age             | 0.473 | 0.318 | 2.205 | 0.860       | 0.342   |
| Gender          | -0.417| 0.439 | 0.902 | 0.278       | 0.342   |
| Differentiation | 0.810 | 0.299 | 7.343 | 1.251       | 0.007** |
| Tumor size      | 0.702 | 0.301 | 5.432 | 1.118       | 0.020   |
| Clinical stage  | 0.739 | 0.393 | 3.540 | 0.970       | 0.060   |

| Characteristics | B     | SE    | Wald  | 95% CI      | P-value |
|-----------------|-------|-------|-------|-------------|---------|
| Age             | 0.060 | 0.347 | 0.030 | 0.538       | 0.863   |
| Gender          | -0.256| 0.457 | 0.314 | 0.316       | 0.575   |
| Tumor size      | 0.612 | 0.328 | 3.486 | 0.970       | 0.062   |
| Differentiation | 0.076 | 0.257 | 0.087 | 0.651       | 0.769   |
| Clinical stage  | -0.149| 0.543 | 0.076 | 0.297       | 0.783   |
| Venous infiltration | 0.254 | 0.482 | 0.277 | 0.501       | 0.599   |
| Ezrin           | 1.753 | 0.176 | 17.454| 2.536       | 0.000** |

B, Coefficient; CI, confidence interval; HCC, hepatocellular carcinoma; SE, standard error; Wald, Wald statistic.

*Significant difference.
FIGURE 3  Ezrin promoted hepatocellular carcinoma (HCC) cell proliferation and colonization. A, B. Ezrin expression level in the constructed Hep3B and Huh7 cells was examined by western blot. β-actin was used as a loading control. C-E, Cell proliferation and colonization was confirmed by MTT (C), colony (D) and EdU assays, ×100 (E) in the constructed cells.
FIGURE 4 Ezrin promoted hepatocellular carcinoma (HCC) cell metastasis via epithelial-to-mesenchymal transition (EMT) progression. A, B, Wound healing assay (A) and transwell assay (B) were used to examine cell migration capacity of ezrin in Hep3B and Huh7 cells. C, Ezrin-related KEGG pathways in HCC tissue in String database. D, Analysis of the correlation between Ezrin expression levels and Vimentin or Snail in 421 LiHC samples. E, EMT markers were confirmed by western blot in the constructed Hep3B and Huh7 cells. β-actin was used as the loading control. F, Immunofluorescence staining for ezrin and EMT markers in the constructed Hep3B and Huh7 cells (×400)
It is reported that overexpression of hexokinase 2 (HK2), the key enzymes in glycolysis, can induce HCC development by promoting glycolysis.\textsuperscript{31} Wong's group found that re-expression of miR-122 in HCC cell lines decreased pyruvate kinase M2 (PKM2) expression, lessened glucose uptake in vitro and subdued tumor growth of HCC in vivo.\textsuperscript{32} However, the question of whether ezrin influences HCC cell proliferation and metastasis by reprogramming of glycolytic metabolism remained largely unclear. Therefore, we blocked the glycolytic pathway with specific inhibitor 2-DG; the results of the functional experiments showed that 2-DG significantly attenuated ezrin-enhanced proliferation, migration and EMT progression in HCC cells. These data were in accord with the concept that augmented glycolysis is beneficial to the maintenance of malignant phenotypes of cancer cells, suggesting that ezrin plays positive roles in HCC mostly or partially throughout glycolysis promotion.

Our study revealed that ezrin was overexpressed in HCC tissues in relation to normal tissues. We supplied a conceptual framework to illustrate how ezrin regulated glycolysis in HCC cells. A, Analysis of the correlation between ezrin expression level and VEGFA, MMP2 or MMP9 in 421 LiHC samples. B, Matrigel tube formation assay was performed in Hep3B and Huh7 cells. C, Western blot analysis of VEGF, MMP2 and MMP9 in the constructed Hep3B and Huh7 cells. β-actin was used as the loading control.

**FIGURE 5** Ezrin promotes angiogenesis in hepatocellular carcinoma (HCC) cells. A, Analysis of the correlation between ezrin expression level and VEGFA, MMP2 or MMP9 in 421 LiHC samples. B, Matrigel tube formation assay was performed in Hep3B and Huh7 cells. C, Western blot analysis of VEGF, MMP2 and MMP9 in the constructed Hep3B and Huh7 cells. β-actin was used as the loading control.
FIGURE 6 Ezrin promoted cell proliferation, migration and epithelial-to-mesenchymal transition (EMT) progression through the Warburg effect in hepatocellular carcinoma (HCC). A, Analysis of the correlation between ezrin expression levels and HK2, LDHA, PFKL and GLUT2 in 421 LIHC samples. B, Cell culture media were collected to measure glucose consumption (a and d), and lactate production (b and e) and cellular ATP levels (c and f) were measured. C, The expression of metabolic enzymes (HK2, PFKL and LDH) and the glucose transporters GLUT2 was determined by western blot. β-actin was used as a loading control. D-F, Representative images showing the colony formation (D), MTT assay (E) and EdU assay (×100) (F) of Hep3B and Huh7 cells overexpressing ezrin or treated with 2-DG. G, Transwell assay of treated cells, stained with crystal violet. (H) Western blot analysis of EMT markers in treated cells. β-actin was used as the loading control.
cells and linked the molecular mechanisms of reprogrammed metabolism to proliferation and metastasis. Hence, our study linked ezrin to HCC progression and established ezrin as a promising biomarker for clinical prognosis and a novel therapeutic target in HCC (Figure 7C).

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CONFLICT OF INTEREST
The authors confirm that there are no conflicts of interest.

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REFERENCES
1. Tang A, Hallouch O, Chernyak V et al. Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis. *Abdom Radiol*. 2018;43:13–25.
2. Greten TF, Malek NP, Schmidt S et al. Diagnosis and treatment of hepatocellular carcinoma. *Z Gastroenterol*. 2013;51:1269–1326.
3. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab*. 2016;23:27–47.
4. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009;324:1029–1033.
5. Tekade RK, Sun X. The Warburg effect and glucose-derived cancer theranostics. *Drug Discov Today*. 2017;22:1637–1653.
6. Hay N. Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? *Nat Rev Cancer*. 2016;16:635–649.
7. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer*. 2011;11:85–95.
8. Li Q, Pan X, Zhu D et al. Circular RNA MAT2B promotes glycolysis and malignancy of hepatocellular carcinoma through the miR-338-3p/PKM2 axis under hypoxic stress. *Hepatology*. 2019;70:1298–1316.
9. Chen Z, Zuo X, Zhang Y et al. MIR-3662 suppresses hepatocellular carcinoma growth through inhibition of HIF-1α-mediated Warburg effect. *Cell Death Dis.* 2018;9:549.
10. Bretscher A, Reczek D, Berrymar M. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. *J. Cell. 1997;110:3011–3018.
11. Feohon RG, McClatchey AI, Bretscher A. Organizing the cell cortex: the role of ERM proteins. *Nat Rev Mol Cell Biol*. 2010;11:276–287.
12. Bretscher A, Edwards K, Feohon RG. ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol*. 2002;3:586–599.
13. Elliott BE, Meens JA, SenGupta SK et al. The membrane cytoskeletal crosslinker Ezrin is required for metastasis of breast carcinoma cells. *Breast Cancer Res.* 2005;7:365–373.
14. Lathika LM, Nair JKKM, Saritha VN et al. Role of phospho-ezrin in differentiating thyroid carcinoma. *Sci Rep*. 2019;9:6190.
15. Li Q, Gao H, Xu H, Wang X, Pan Y, Hao F et al. Expression of ezrin correlates with malignant phenotype of lung cancer, and in vitro knockdown of ezrin reverses the aggressive biological behavior of lung cancer cells. *Tumour Biol*. 2012;33:1493–1504.
16. Kong J, Di C, Piao J et al. Ezrin contributes to cervical cancer progression through induction of epithelial-mesenchymal transition. *Oncotarget*. 2016;7:19631–19642.
17. Sato Y. Molecular diagnosis of tumor angiogenesis and anti-angiogenic cancer therapy. *Int J Clin Oncol*. 2003;8:200–206.
18. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med*. 2011;17:1395–1370.
19. Beyoglu D, Idle JR. The metabolomic window into hepatobiliary disease. *J Hepatol*. 2013;59:842–858.
20. Dasgupta S, Putluri N, Long W et al. Coactivator SRC-2-dependent metabolic reprogramming mediates prostate cancer survival and metastasis. *J Clin Invest*. 2015;125:1174–1188.
21. Robey RB, Hay N. Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. *Oncogene*. 2006;25:4683–4696.
22. Wang Z, Wang N, Chen J et al. Emerging glycolysis targeting and drug discovery from Chinese medicine in cancer therapy. *Evid Based Complementary Altern Med*. 2012;2012:873175.
23. McClatchey AI. Merlin and ERM proteins: unappreciated roles in the role of ERM proteins. *Nat Rev Mol Cell Biol*. 2002;3:586–599.
24. Li Q, Gao H, Xu H et al. Expression of ezrin correlates with malignant phenotype of lung cancer, and in vitro knockdown of ezrin reverses the aggressive biological behavior of lung cancer cells. *Tumour Biol*. 2012;33:1493–1504.
25. Kai M, Mousa SA. The role of angiogenesis in cancer treatment. *Breast Cancer Res Ther*. 2015;16:187–197.
26. Zhang YX, Xu JT, You XC et al. Inhibitory effects of hydrogen on proliferation and migration of vascular smooth muscle cells via down-regulation of mitogen/activated protein kinase and ezrin-radixin-moesin signaling pathways. *Chin J Physiol*. 2016;59:46–55.
27. Li Q, Gao H, Xu H et al. Expression of ezrin correlates with malignant phenotype of lung cancer, and in vitro knockdown of ezrin reverses the aggressive biological behavior of lung cancer cells. *Tumour Biol*. 2012;33:1493–1504.
28. Li Nan, Kong Jienan, Lin Zhenhua et al. Ezrin promotes breast cancer progression by modulating AKT signals. *Br J Cancer*. 2019;120:703–713.
29. Rajabi M, Mousa SA. The role of angiogenesis in cancer treatment. *Biomedicines*. 2017;5:34.
30. Hendrix MJ, Seftor EA, Hess AR et al. Vasculogenic mimicry and tumor-cell plasticity: lessons from melanoma. *Nat Rev Cancer*. 2003;3:411–421.

31. DeWaal D, Nogueira V, Terry AR et al. Hexokinase-2 depletion inhibits glycolysis and induces oxidative phosphorylation in hepatocellular carcinoma and sensitizes to metformin. *Nat Commun*. 2018;9:10.

32. Pan C, Wang X, Shi K et al. MiR-122 reverses the doxorubicin-resistance in hepatocellular carcinoma cells through regulating the tumor metabolism. *PLoS ONE*. 2016;11:e0152090.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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