




## ORIGINAL ARTICLE

# Ezrin promotes hepatocellular carcinoma progression by modulating glycolytic reprogramming

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## Abstract

Ezrin, one of the ezrin-radixin-moesin (ERM) proteins, is involved in the formation of cell membrane processes and has been implicated in the promotion of cancer proliferation and metastasis. However, the possible role of ezrin in hepatocellular carcinoma (HCC) metastasis and glycolysis reprogramming has remained unclear. In this study, we found that ezrin was upregulated in HCC tissues, and its overexpression was linked with HCC patients' aggressive tumor characteristics and poor prognosis. Functional experiments further revealed that ezrin overexpression promoted HCC cell proliferation, epithelial-to-mesenchymal transition (EMT) progression and angiogenesis. In addition, by measuring glucose consumption, lactate production, ATP levels and the expression of glucose metabolism-related markers in HCC cells, we investigated whether ezrin regulated glucose metabolism. Moreover, 2-deoxy-D-glucose (2-DG) affected ezrin-mediated proliferation, migration and EMT of HCC cells, which suggested that ezrin may, at least in part, promote HCC progression by regulating glycolysis reprogramming. Based on our results, we proposed that ezrin was involved in HCC progression and may be a valid prognostic marker.

## KEYWORDS

angiogenesis, EMT, Ezrin, glycolytic reprogramming, hepatocellular carcinoma, prognostic value

## 1 | 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.<sup>1</sup> Current diagnostic modalities include ultrasound and  $\alpha$ -fetoprotein, but both are costly and lack sensitivity in tumor detection.<sup>2</sup> 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.<sup>3</sup> Even under the oxygenated conditions, cancer cells predominantly rely on glycolysis rather than oxidative phosphorylation to generate adenosine triphosphate (ATP).<sup>4,5</sup> This

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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.<sup>6,7</sup> 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.<sup>8,9</sup> 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.<sup>10,11</sup> 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.<sup>12</sup> Furthermore, ezrin has already been verified as a potential biomarker in malignancies associated with tumor cell proliferation and metastasis, such as breast,<sup>13</sup> thyroid<sup>14</sup> and lung cancer.<sup>15</sup> Our group also found that ezrin was upregulated in cervical cancer, and its expression was significantly associated with metastasis and poor prognosis.<sup>16</sup> 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,  $\alpha$ -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 non-tumor 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% CO<sub>2</sub>. 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'-GGGCCAAGTCTACCCTGAAG-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  $\beta$ -actin, ZO-1, E-cadherin, Snail, Slug and Vimentin were purchased from Cell Signaling Technology (Boston). Antibodies against HK2, PFKL, 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% H<sub>2</sub>O<sub>2</sub> 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 (CW BIO, China) was used to permeabilize 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,  $3 \times 10^5$  cells were planted in the upper insert with FBS-free medium, while media containing 10% FBS was added to the outside insert as a chemoattractant. 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-μmol/L filter. HUVEC in 150 μL conditioned medium diluted at 2:1 in cell culture medium were seeded at  $2 \times 10^4$  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 Jiancheng Bioengineering Institute), an ATP assay kit (Nanjing Jiancheng Bioengineering Institute) and a Pyruvic Acid Assay Kit (Nanjing Jiancheng 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/anti-mycin (final concentration: 1.5, 1 and 0.5  $\mu\text{mol/L}$ , 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://kmpplot.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

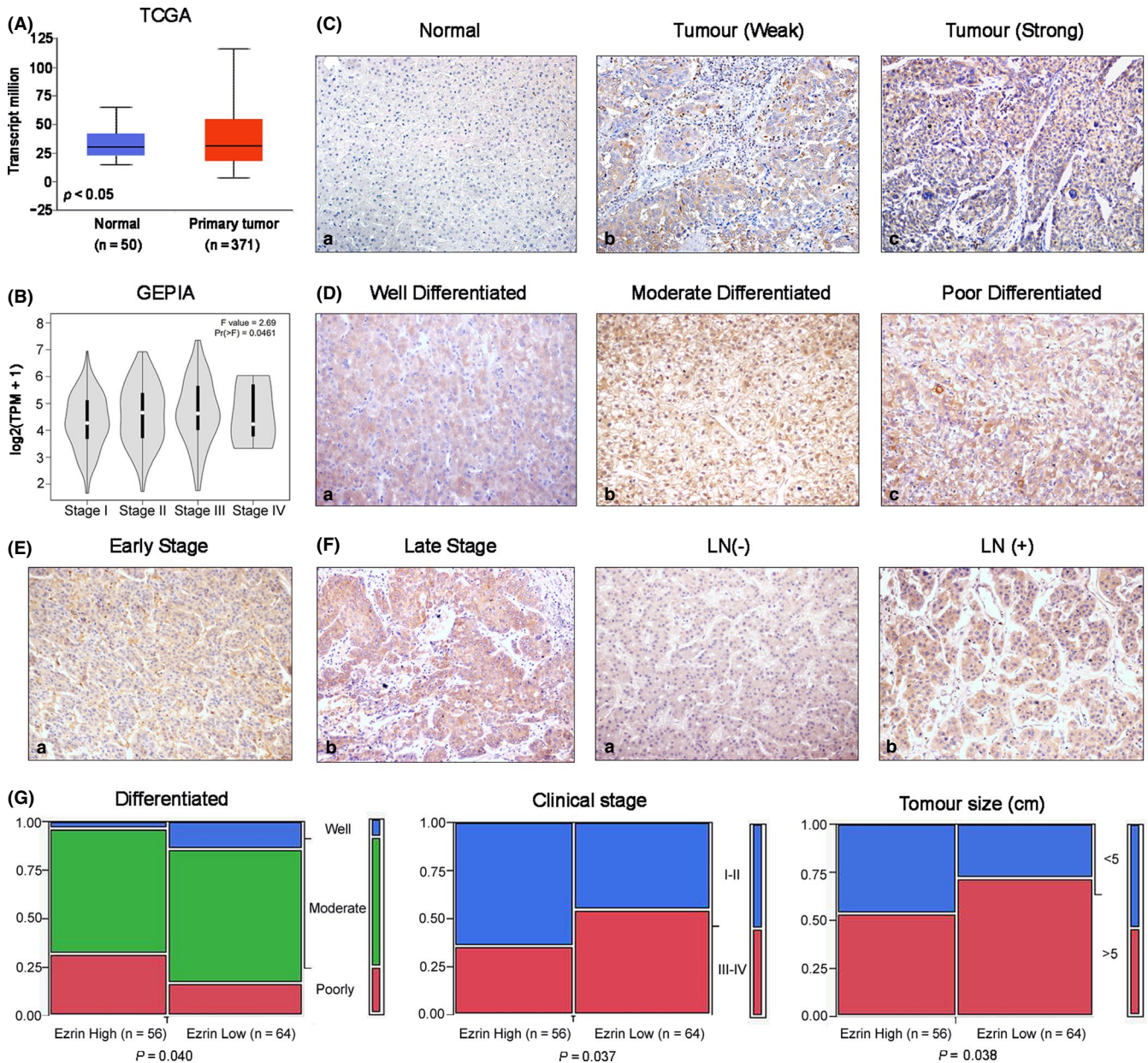
To explore the biological functions of ezrin in HCC progression, HCC cell lines Hep3B and Huh7 were transfected with non-targeting siRNA as si-control or two different ezrin-specific lentiviral si-RNA. The expression of ezrin was analyzed by western blot. As shown in Figure 3A, ezrin was significantly depleted by si-Ezrin sequence #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%**

**TABLE 1** Ezrin protein expression in HCC

HCC, hepatocellular carcinoma.

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



**FIGURE 1** Ezrin expression was significantly upregulated in hepatocellular carcinoma (HCC). A, Expression of ezrin in The Cancer Genome Atlas (TCGA). B, Analysis of ezrin expression by stage. C, Ezrin expression in normal hepatic tissues (a) and HCC tissues (b, c) as examined by immunohistochemistry (IHC) ( $\times 200$ ). Representative examples of ezrin staining are shown. D-F, Representative IHC images of ezrin expression in different clinicopathological features of HCC

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

Variables	Number of cases	Tiam1 strongly positive cases (%)	$\chi^2$	P-value
Age				
<50	47	17 (34.0)	3.420	0.064
≥50	73	39 (53.4)		
Tumor size				
<5	76	30 (39.5)	4.309	0.038 <sup>*</sup>
≥5	44	26 (59.1)		
Clinical stage				
0-II	55	20 (36.4)	4.331	0.037 <sup>*</sup>
III-IV	65	36 (55.2)		
Differentiation				
Well differentiated	11	2 (18.2)	6.439	0.040 <sup>*</sup>
Moderately differentiated	80	36 (45.0)		
Poorly differentiated	29	18 (62.1)		
Lymph node metastasis				
Positive	17	8 (47.1)	0.001	0.972
Negative	103	48 (46.6)		
Venous infiltration				
Positive	109	52 (45.9)	0.302	0.583
Negative	11	4(54.5)		

\* $P < 0.05$  and \*\* $P < 0.01$ .

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.

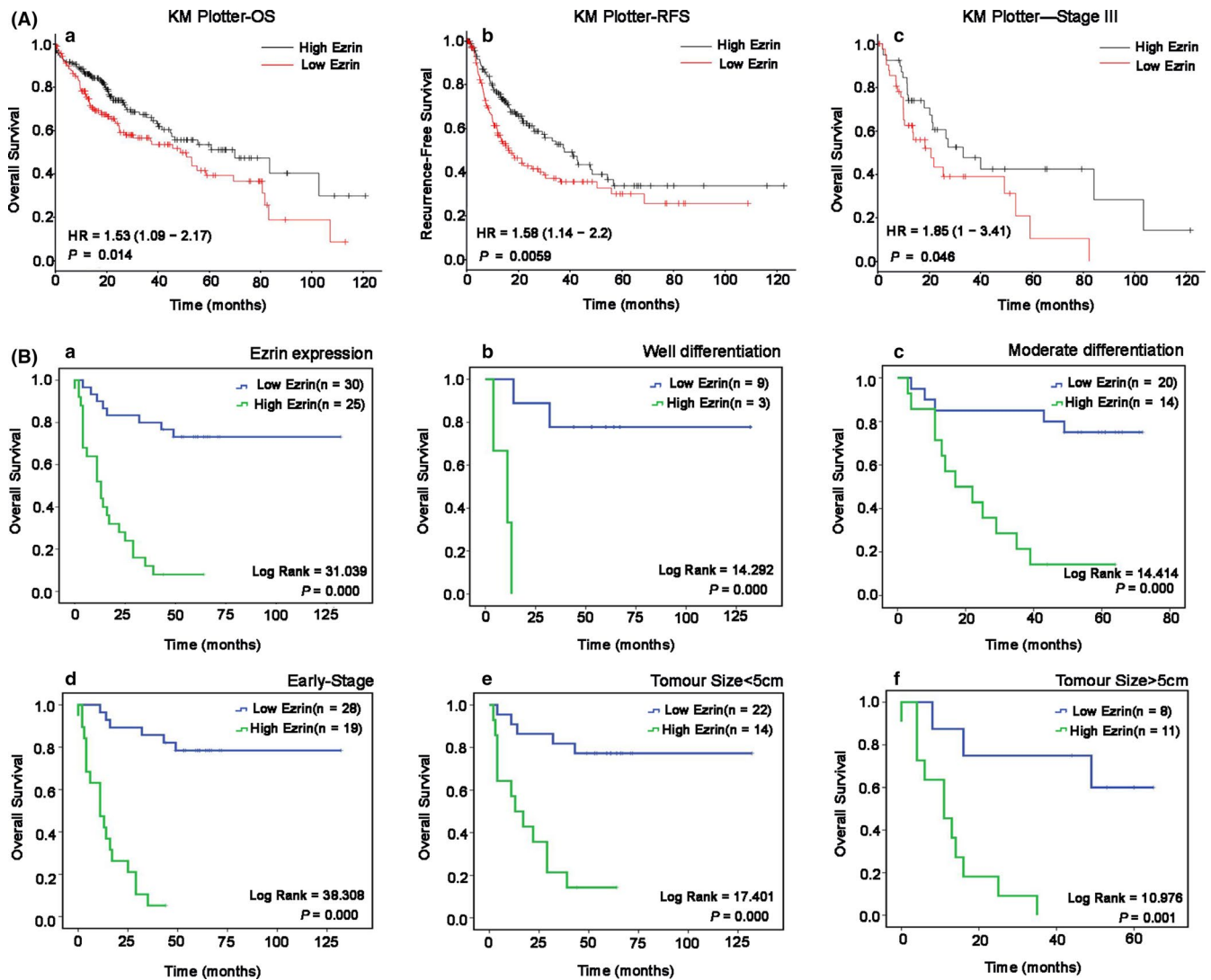
### 3.5 | Ezrin promoted angiogenesis in hepatocellular carcinoma cells

As a crucial factor for cancer metastasis and progression, angiogenesis is involved in hepatocarcinogenesis.<sup>17,18</sup> 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).

**TABLE 2** Correlation between ezrin expression and the clinicopathological features of HCC

### 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.<sup>19,20</sup> 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.<sup>21</sup> 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



**FIGURE 2** Ezrin expression was associated with poor outcome in hepatocellular carcinoma (HCC). A, The implication of ezrin for survival of patients with HCC was determined in Kaplan-Meier Plotter. The influence of ezrin on patients' overall survival (OS), relapse-free survival (RFS) and histological stage III with HCC. B, HCC patients with high ( $n = 25$ ) vs low ( $n = 30$ ) levels of ezrin in all (a), high ( $n = 3$ ) vs low ( $n = 9$ ) levels of well differentiation status (b), high ( $n = 14$ ) vs low ( $n = 20$ ) levels of moderate differentiation status (c), high ( $n = 19$ ) vs low ( $n = 28$ ) levels of early clinical stage (d), and high ( $n = 22$ ) vs low ( $n = 14$ ) levels of tumor size less than 5 cm and high ( $n = 22$ ) vs low ( $n = 14$ ) levels of tumor size more than 5 cm (e, f) were all plotted using the Kaplan-Meier method

have reported that 2-DG, the inhibitor of glycolysis, is useful in tumor treatment by attacking anaerobic cells.<sup>22</sup> 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.<sup>23</sup> 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*<sup>16</sup> 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

Characteristics	B	SE	Wald	95% CI		P-value
				Lower	Upper	
Univariate survival analyses						
Ezrin	1.620	0.324	25.026	2.679	9.536	0.000*
Age	0.473	0.318	2.205	0.860	2.996	0.138
Gender	-0.417	0.439	0.902	0.278	1.559	0.342
Differentiation	0.810	0.299	7.343	1.251	4.038	0.007**
Tumor size	0.702	0.301	5.432	1.118	3.640	0.020*
Clinical stage	0.739	0.393	3.540	0.970	4.518	0.060
Multivariate survival analyses						
Age	0.060	0.347	0.030	0.538	2.097	0.863
Gender	-0.256	0.457	0.314	0.316	1.897	0.575
Tumor size	0.612	0.328	3.486	0.970	3.507	0.062
Differentiation	0.076	0.257	0.087	0.651	1.786	0.769
Clinical stage	-0.149	0.543	0.076	0.297	2.495	0.783
Venous infiltration	0.254	0.482	0.277	0.501	3.315	0.599
Ezrin	1.753	0.176	17.454	2.536	13.137	0.000**

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

\*Significant difference.

to poor differentiation, later clinical stage and shortened OS. A similar trend was found in pancreatic cancer<sup>24</sup> and melanomas,<sup>25</sup> 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.

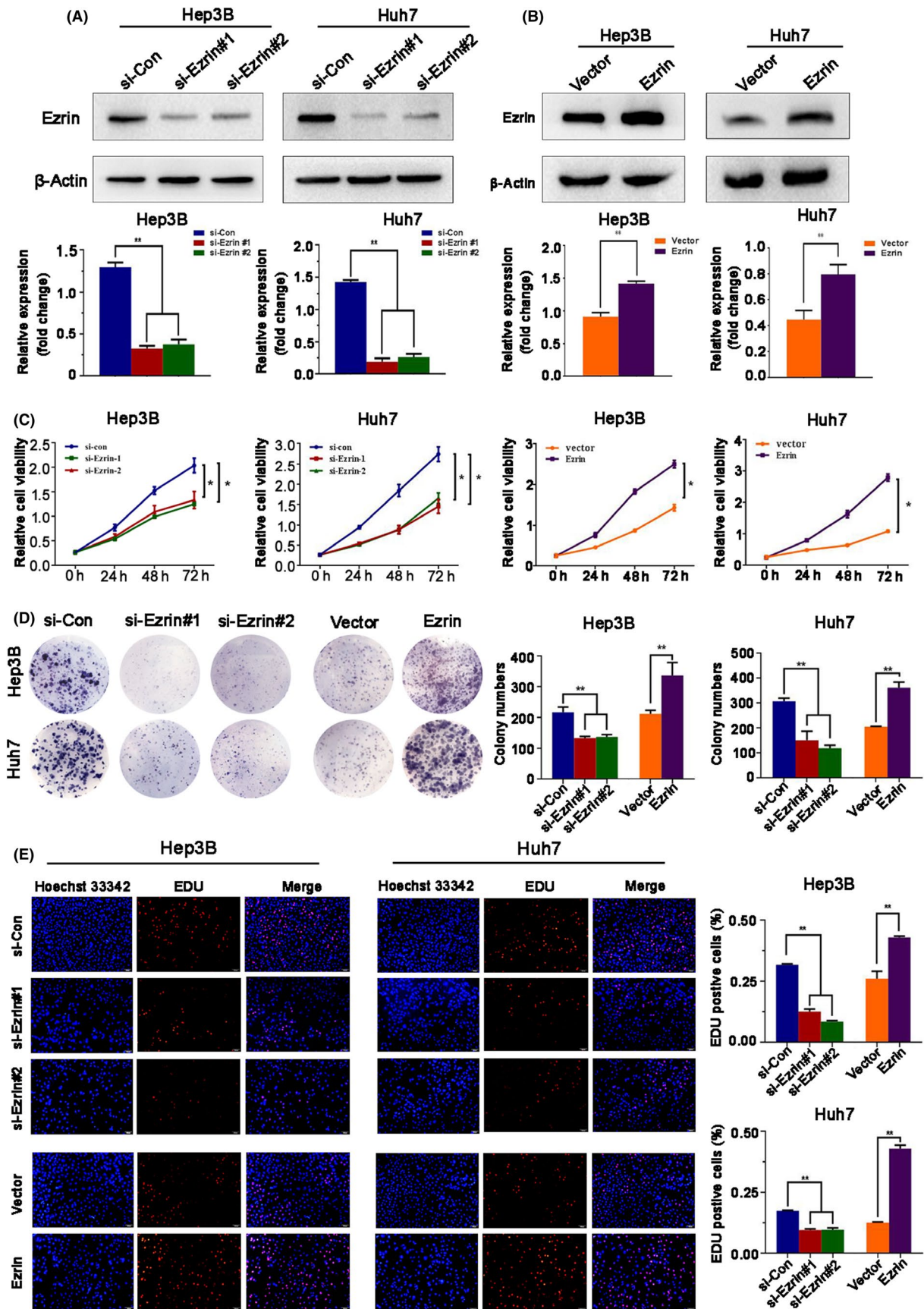
Cancer cell proliferation and migration underlie development and metastatic dissemination, which are major problems in cancers. Substantial evidence reveals that ezrin plays an important role in the development and metastatic dissemination of malignant tumors. Zhang *et al*<sup>26</sup> reported that 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.<sup>27</sup> According to the effect of ezrin on EMT progression in tumors, here, 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.<sup>28</sup>

**TABLE 3** Univariate and multivariate survival analyses (Cox regression model) of various factors in 174 HCC patients

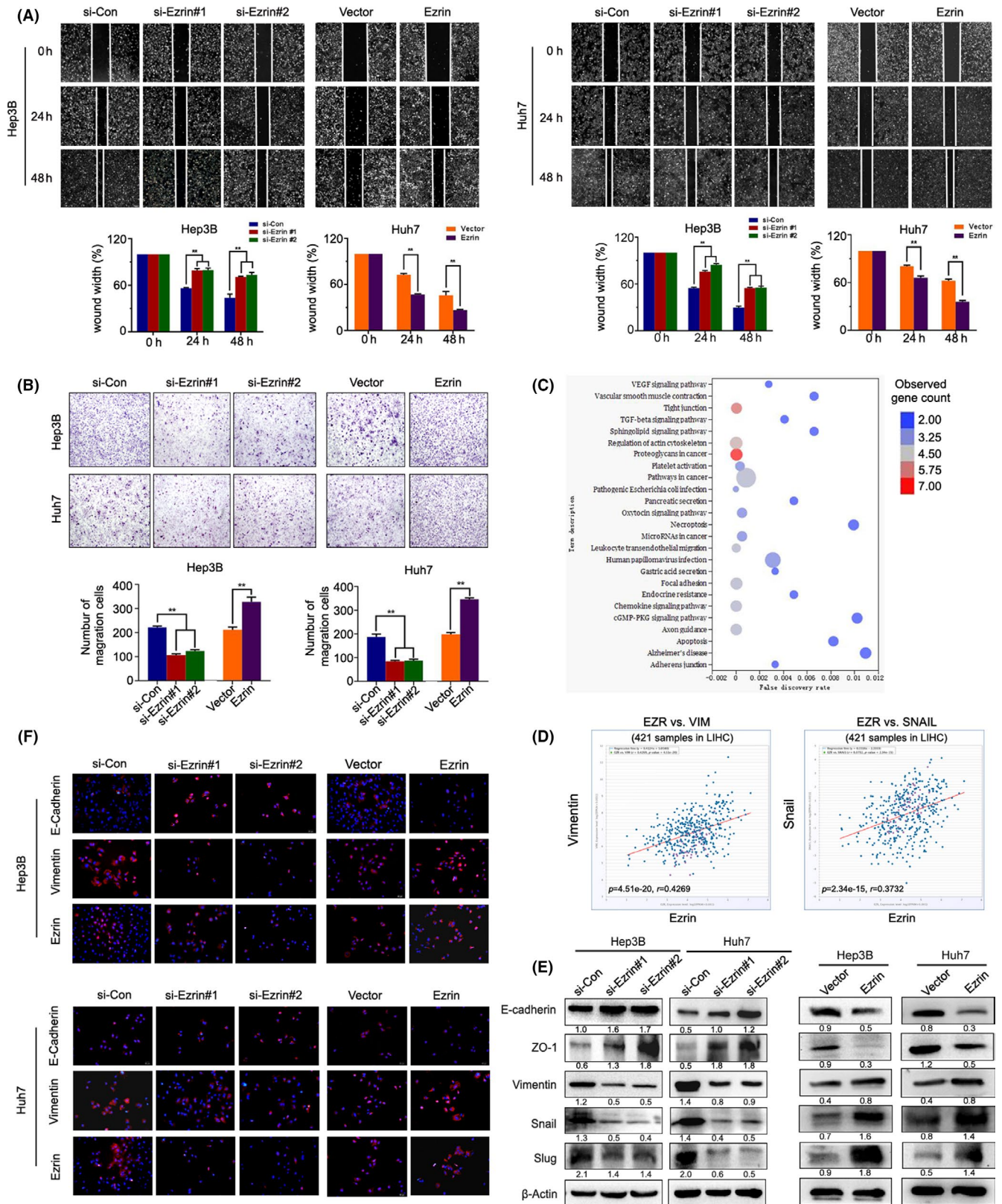
Another crucial factor in tumor growth is angiogenesis, which is necessary for cancer metastasis, progression and hepatocarcinogenesis.<sup>29</sup> We found that ezrin knockout cells strikingly disrupted the ability of HUVEC tube formation. Western blot demonstrated that the marker of angiogenesis VEGF 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.<sup>30</sup> 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.

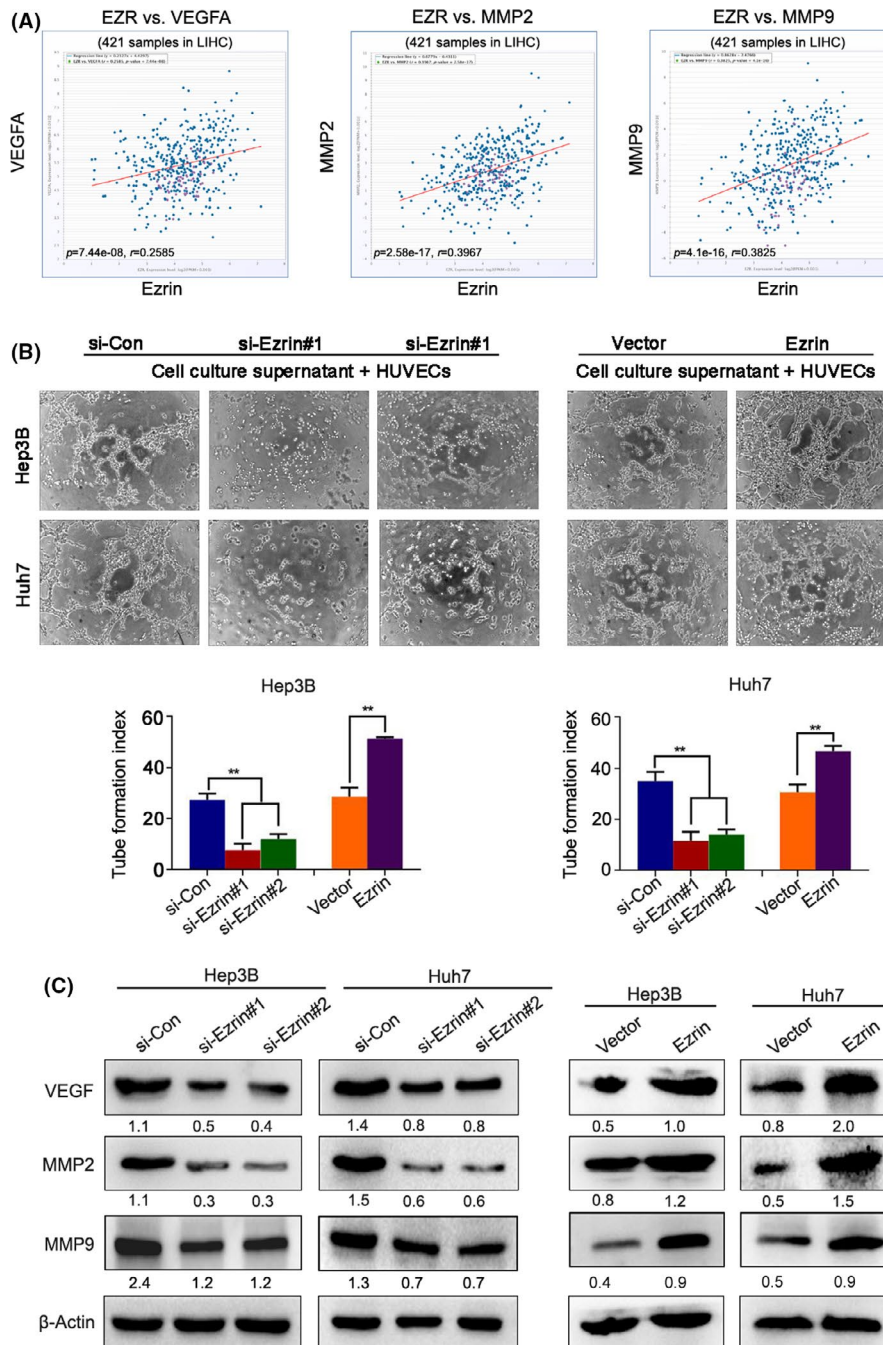




**FIGURE 3** Ezrin promoted hepatocellular carcinoma (HCC) cell proliferation and colonization. A, B, Ezrin expression level in the constructed Hep3B and Huh7 cells was examined by western blot.  $\beta$ -actin was used as a loading control. C-E, Cell proliferation and colonization was confirmed by MTT (C), colony (D) and EdU assays,  $\times 100$  (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.  $\beta$ -actin was used as the loading control. F, Immunofluorescence staining for ezrin and EMT markers in the constructed Hep3B and Huh7 cells ( $\times 400$ )

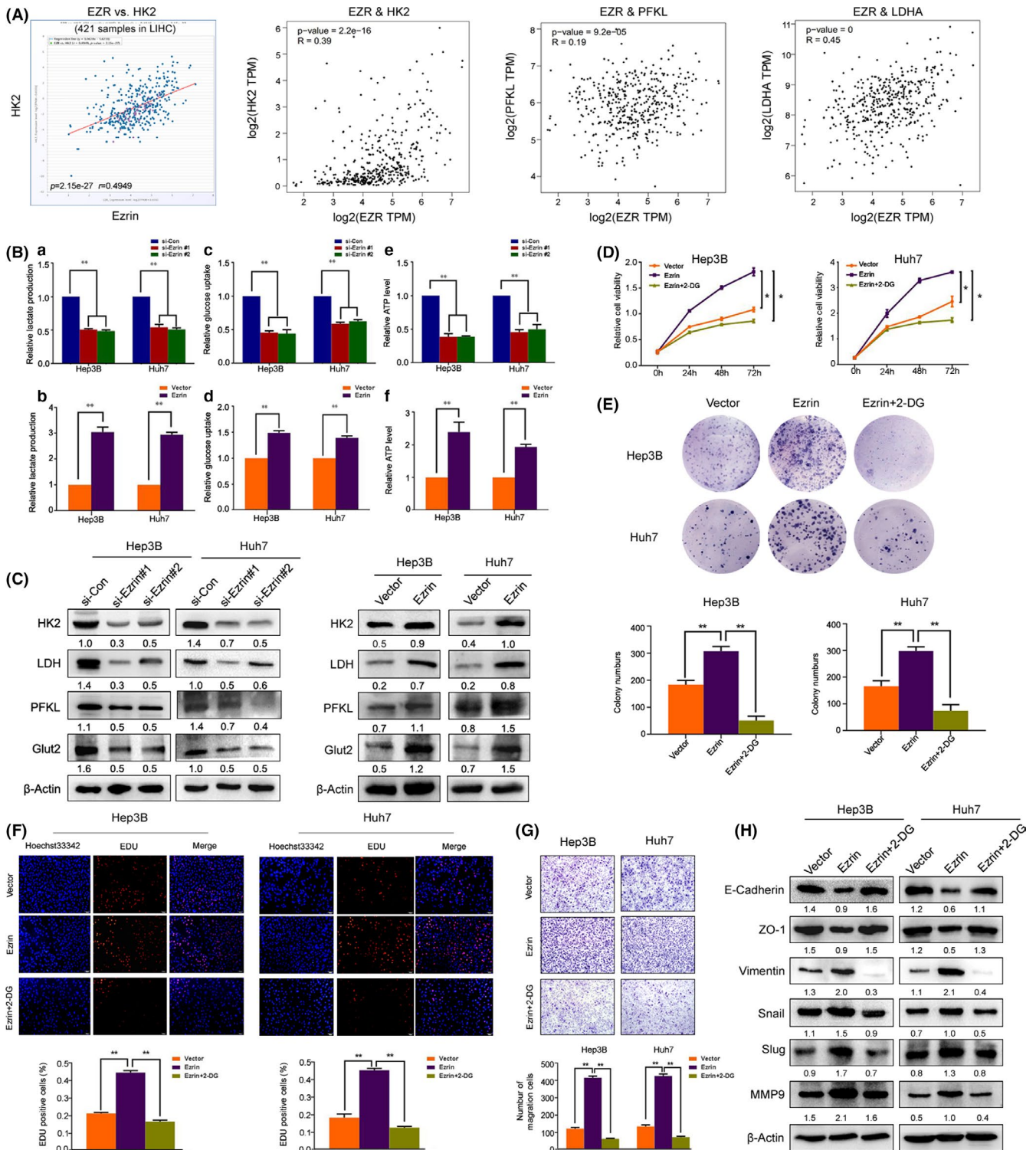


**FIGURE 5** Ezrin promotes angiogenesis in hepatocellular carcinoma (HCC) cells. A, Analysis of the correlation between ezrin expression level and VEGFA, MMP2 or MMP9 in 421 LIHC samples. B, Matrigel tube formation assay was performed in Hep3B and Huh7 cells. C, Western blot analysis of VEGF, MMP2 and MMP9 in the constructed Hep3B and Huh7 cells.  $\beta$ -actin was used as the loading control

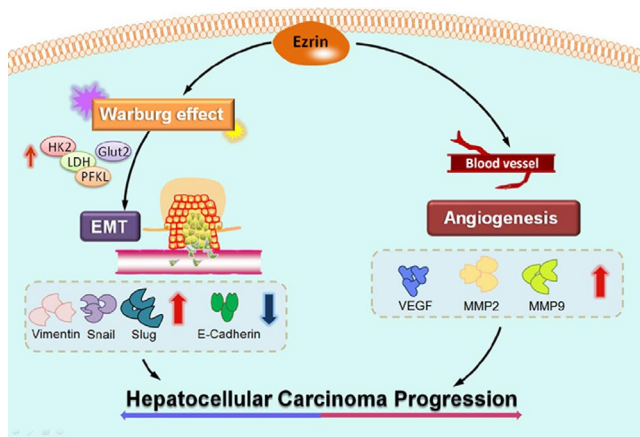
It is reported that overexpression of hexokinase 2 (HK2), the key enzymes in glycolysis, can induce HCC development by promoting glycolysis.<sup>31</sup> Wong's group found that re-expression of miR-122 in HCC cell lines decreased pyruvate kinase M2 (PKM2) expression, lessened glucose uptake in vitro and subdued tumor growth of HCC in vivo.<sup>32</sup> 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



**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.  $\beta$ -actin was used as a loading control. **D-F**, Representative images showing the colony formation (**D**), MTT assay (**E**) and EdU assay ( $\times 100$ ) (**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.  $\beta$ -actin was used as the loading control



**FIGURE 7** Schematic model showed the role of ezrin in the regulation of epithelial-to-mesenchymal transition (EMT) progression through glucose metabolism and angiogenesis in hepatocellular carcinoma (HCC)

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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#### SUPPORTING INFORMATION

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