RETRACTED ARTICLE: MicroRNA-3194-3p inhibits metastasis and epithelial-mesenchymal transition of hepatocellular carcinoma by decreasing Wnt/β-catenin signaling through targeting BCL9

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ABSTRACT

Local and systemic metastasis of hepatocellular carcinoma (HCC) causes the poor prognosis and increasing evidence confirms that aberrant miRNAs were involved in cancer progression. However, the expression and mechanisms of a specific miR-3194-3p in HCC remains unknown. In this research, we demonstrated that miR-3194-3p, significantly down-regulated in HCC tissues and cell lines, was associated with metastasis and recurrence of HCC. Notably, gain- and loss-of-function assays demonstrated that miR-3194-3p inhibited the migration, invasion and epithelial-mesenchymal transition (EMT) of HCC cells in vitro and in vivo. BCL9, up-regulated in HCC tissues, was a direct downstream target of miR-3194-3p and mediated the functional influence of miR-3194-3p. Most importantly, miR-3194-3p exerted its function by regulating β-catenin pathway. Moreover, miR-3194-3p and BCL9 expression were markedly correlated with adverse clinical features and poor prognosis of HCC patients. We showed that hypoxia was responsible for the down-expression of miR-3194-3p in HCC. Also, the promoting effects of hypoxia on metastasis and EMT of HCC cells were reversed by miR-3194-3p. Altogether, our study suggested that miR-3194-3p inhibits HCC EMT via decreasing Wnt/β-catenin signaling through targeting BCL9 and might be a therapeutic target for HCC.

Abbreviations: 3’-UTR 3’: untranslated region; BCL9: B cell lymphoma 9; EMT: epithelial-mesenchymal transition; H&E: hematoxylin and eosin; HCC: hepatocellular carcinoma; IF: immunofluorescence; IHC: immunohistochemistry; miRNAs: microRNAs; qRT-PCR: real-time quantitative reverse transcription polymerase chain reaction; TNM: tumor-node-metastasis

Background

Hepatocellular carcinoma (HCC) is one of the common malignancies and the second leading cause of cancer-related mortality worldwide [1,2]. For the high prevalence of hepatitis B strongly predisposes individuals into developing chronic liver disease, and subsequently, HCC, the incidence of HCC is particularly high in China [3]. Although superior improvements in therapeutic strategy for HCC have undergone, the long-term survival of HCC patients remains poor due to high rates of recurrence and metastasis [4,5]. Therefore, understanding the molecular mechanisms underlying cancer progression may contribute to the discovery of more effective intervention for HCC treatment.

MicroRNAs (miRNAs), a group of evolutionarily conserved small non-coding RNA, inhibit translation or induce mRNA degradation by binding to the 3’-untranslated regions (3’-UTR) of target genes [6,7]. As post-transcriptional regulators, miRNAs play critical roles in physiological and pathological processes, including cell differentiation, proliferation, apoptosis, migration, and invasion [8]. Accumulating evidence confirmed that miRNAs play a role as promising therapeutic and prognostic biomarkers in HCC diagnosis and treatment [9]. Nevertheless, underlying molecular mechanisms and function of miR-3194-3p in HCC remain unknown.

Epithelial-to-mesenchymal transition (EMT) contribute to cancer invasion and metastasis by transformation of adherent and polarized epithelial cells, disruption of cell-cell adhesion, into an invasive mesenchymal cell phenotype [10]. EMT is marked of losing epithelial adhesion and cytoskeletal markers, such as E-cadherin, meanwhile, the obtaining of migratory mesenchymal symbols, such as N-cadherin and Vimentin [11,12]. Increasing studies reveal that EMT is a main cause for HCC invasion and metastasis [13]. However, the association between miR-3194-3p and EMT in HCC is poorly investigated.

In the present study, we demonstrated that down-regulated miR-3194-3p was associated with adverse
prognostic features of HCC patients. miR-3194-3p inhibited migration, invasion and EMT progression of HCC cells in vitro and in vivo. Notably, B-cell lymphoma 9 (BCL9) was a direct target of miR-3194-3p and mediated the function of miR-3194-3p in HCC cells. Besides, miR-3194-3p, BCL9 and their combination were valuable predictors for the prognosis of HCC patients.

Materials and methods

Clinical samples

Patients’ tumor tissues and corresponding adjacent non-tumor tissues were obtained from 129 patients in our department between January 2008 and December 2011 after the informed consent was obtained from all patients. They did not receive any therapy before surgery. Xi’an Jiaotong University Ethics Committee approved the research on the basis of Declaration of Helsinki.

Cell culture

The normal immortalized human hepatocyte LO2 and a panel of HCC cells (Hep3B, HepG2, Huh7, MHCC-97H, and SMMC-7721) (Chinese Academy of Sciences, Shanghai, China) were maintained in DMEM (Invitrogen, Carlsbad, USA) containing 10% FBS (Gibco, Grand Island, USA) in 37°C with 5% CO2. Cells were classified into several groups under different treatments. The transfection was performed according to the instructions of lipofectamine 2000 (#11668019, Invitrogen, CA, USA). All the sequence details were shown in Supporting Figure 1.

RNA extraction and qRT-PCR

qRT-PCR was conducted as reported previously [14,15]. All RNA was extracted based on the protocol of TRizol reagent (Invitrogen, Carlsbad, CA, USA). qPCR primer against miR-3194-3p (HmiRQP0106), U6 (HmiRQP9002), GAPDH (HQ006940) and BCL9 (HQ016366) were purchased from Genecopoeia (Guangzhou, China).

Immunohistochemical staining (IHC)

The sections were dewaxed, dehydrated, and rehydrated. BCL9 (1:300, Abcam, USA) was applied and covered the sections, which were incubated at 4°C overnight. Then, applied the biotinylated secondary antibodies (Goldenbridge, Zhongshan, China) according to SP-IHC assays. The detailed experiment was conducted similar to the previously reported [16].

Western blot analysis

We separated proteins by SDS–PAGE and transferred proteins to PVDF membranes. Detailed experiment was performed similar to the previously reported [17–19]. E-cadherin (Mouse Monoclonal, 13–5700), N-cadherin (Mouse Monoclonal, MA1-91128), Vimentin (Mouse Monoclonal, MA5-11883), beta-catenin (Rabbit Monoclonal, 84805) and BCL9 (Rabbit Polyclonal, PA5-51747) antibodies were purchased from CST Signaling Company. GAPDH (Rabbit Monoclonal, ab181602) and HIF-1a (Rabbit Monoclonal, ab179483) were purchased from Abcam Company.

Luciferase reporter assays

The 3′-UTR sequence of BCL9, together with a corresponding mutated sequence within the predicted target sites, were synthesized and inserted into the pmiR-GLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA). The assays were carried out as described previously [14,20].

Cell migration and invasion analyses

To evaluate cell migration and invasion, we used 2 × 10⁴ transfected cells suspended in 150 μL serum-free DMEM medium into the upper chamber of Matrigel-uncoated and -coated transwell inserts (8 μm pore size; Millipore), and 550 μL 20% FBS DMEM medium was placed in the lower chamber. After 24 h incubation, fixed in 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet dye for 15 min, cells on the inner layer were softly removed with a cotton swab and counted at five randomly selected views and the average cell number per view was calculated.

In vivo experiments

Four-week-old male BALB/c nude mice (Centre of Laboratory Animals, The Medical College of Xi’an Jiaotong University, Xi’an, China) were randomized into two groups (n = 6) and either MHCC-97H-miR-3194-3p or MHCC-97H-miR-control cells (1 × 10⁶); Hep3B-anti-miR-3194-3p or Hep3B-anti-miR-NC were injected into the tail veins for the establishment of pulmonary metastatic model. Mice were sacrificed 10 weeks’ post-injection and examined microscopically by H&E staining for the development of lung metastatic foci. Accounting of lung-metastasis nodules was finished in 100-fold high-power field of microscope in sections after stain. Animals were housed in cages under standard conditions. All in vivo protocols were approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University.

Statistical analysis

Performing at least three independent replicates, we collected the presented data as the mean ± SD. To evaluate the statistical significance, we operated SPSS software, 16.0 (SPSS, Inc, Chicago, IL, USA) and Graphpad Prism 6.0 (CA, USA) by two-tailed Student t-test, Pearson’s correlation analysis, Kaplan–Meier method, and the log-rank test. Differences were defined as p < .05.
Results

Reduced miR-3194-3p expression confers metastasis and recurrence of HCC

To determine the expression level of miR-3194-3p in HCC tissues, we performed qRT-PCR and found that miR-3194-3p expression was significantly lower in human HCC tissues than in adjacent non-tumor tissues \((p < .05, \text{Figure 1}(A))\), which was clarified in TCGA database and shown in Supporting Figure 2A. We selected the HCC tissues with intrahepatic metastasis and venous infiltration as aggressive HCC tissues and found that miR-3194-3p was obviously decreased in aggressive HCC tissues compared to non-aggressive tissues \((p < .05, \text{Figure 1}(B))\). Moreover, miR-3194-3p was down-regulated in tumor tissues from patients with recurrence compared to tissues without recurrence \((p < .05, \text{Figure 1}(C))\). In addition, miR-3194-3p was significantly down-regulated in several HCC cell lines as compared with hepatic normal cell line LO2 \((p < .05, \text{Figure 1}(D))\). The significant down-regulation of miR-3194-3p in HCC tissues and cell lines supports its role as a tumor suppressor in HCC and confers metastasis and recurrence of HCC.

miR-3194-3p inhibits HCC migration and invasion in vitro

Systemic metastasis of HCC is the culprit of the poor prognosis of HCC patients [21]. To investigate the biological function of miR-3194-3p on HCC, we stably overexpressed miR-3194-3p in MHCC-97H and knocked down in Hep3B cells. As determined by qRT-PCR, we confirmed that miR-3194-3p was effectively up-regulated in MHCC-97H cells \((p < .05, \text{Figure 2}(A))\) or down-regulated miR-3194-3p in Hep3B cells \((p < .05, \text{Figure 2}(C))\). As examined by Matrigel-coated (for invasion) and -uncoated (for migration) transwell assays, miR-3194-3p over-expression significantly inhibited the migration and invasion of MHCC-97H cells \((p < .05, \text{Figure 2}(B))\), whereas miR-3194-3p knock-down obviously increased the number of migrated and invaded Hep3B cells \((p < .05, \text{Figure 2}(D))\). These data suggested that miR-3194-3p could regulate HCC cell migration and invasion and may exert an anti-metastatic effect on HCC.

miR-3194-3p inhibits epithelial-to-mesenchymal transition of HCC cells

EMT has been identified as a key modulator in the initiation of metastasis progression of HCC. To elucidate the potential role of miR-3194-3p in HCC metastasis, we performed Western blotting and IF to confirm that miR-3194-3p overexpression increased the EMT marker in MHCC-97H cells \((p < .05, \text{respectively, Figure 3}(A,C))\). By contrast, miR-3194-3p knockdown decreased E-cadherin and increased N-cadherin and Vimentin expression in Hep3B cells \((p < .05, \text{respectively, Figure 3}(B,D))\). Furthermore, we also investigated the correlation between miR-3194-3p and EMT markers in HCC tissues. We demonstrated that E-cadherin expression in high miR-3194-3p group HCC tissues was higher than that in low expressing cases. Conversely, the expression of Vimentin in the high miR-3194-3p group was markedly lower than that in low expression group \((p < .05, \text{respectively, Figure 3}(E))\).
Therefore, our results suggest that miR-3194-3p inhibits EMT progress of HCC cells.

**BCL9 is direct downstream target of miR-3194-3p in HCC**

We used the target algorithm (TargetScan and miRNAda) to predict candidate targets of miR-3194-3p and found BCL9 3’-UTR contained the binding site of miR-3194-3p (Figure 4(A)). To facilitate that BCL9 was a direct target of miR-3194-3p, we performed luciferase reporter assay to show that over-expression of miR-3194-3p significantly inhibited luciferase activity of wild-type (wt) BCL9 3’-UTR, compared with mutant-type (mt) BCL9 3’-UTR (p < .05, Figure 4(B)). By contrast, miR-3194-3p knockdown promoted the luciferase activity of wt BCL9 3’-UTR (p < .05, Figure 4(B)) but had no change on mt BCL9 3’-UTR. Furthermore, miR-3194-3p overexpression significantly inhibited BCL9 mRNA and protein levels in MHCC-97H cells, while the down-regulated of miR-3194-3p obviously increased BCL9 mRNA and protein expression in Hep3B cells (p < .05, Figure 4(C,D)). In addition, our results showed that the miRNA and protein of BCL9 in the miR-3194-3p high-expressing tumor tissues were significantly lower than those in the miR-3194-3p low-expressing tumor tissues (p < .05, respectively, Figure 4(E,F)). Notably, an obvious inverse correlation between the levels of miR-3194-3p and BCL9 mRNA was confirmed by Spearman’s correlation analysis in HCC tissues (p < .05, Figure 4(G)). Altogether, we demonstrated that miR-3194-3p directly binds to BCL9 3’-UTR and regulates its expression in HCC cells.

**BCL9 is up-regulated in HCC tissues and promotes migration and invasion of HCC cells**

To determine the potential role of BCL9 in HCC, we performed qRT-PCR and WB to show that the BCL9 mRNA and protein expression was higher in HCC than adjacent non-tumor (p < .05, Figure 5(A,B) and Supporting Figure 2(B)). To confirm the functional effects on HCC, we established stable BCL9 overexpression or knockdown cells (p < .05, Figure 5(C)) by lentiviral transduction. Transwell assays showed that BCL9 over-expression significantly promoted cell migration and invasion of Hep3B cells (p < .05, Figure 5(D)). In contrary, BCL9 knockdown inhibited cell migration and invasion in MHCC-97H cells (p < .05, Figure 5(E)). In addition, WB confirmed that BCL9 alteration obviously regulated EMT process (p < .05, Figure 5(F)). Therefore, we demonstrated that alteration of BCL9 could mimic miR-3194-3p-induced function on HCC cells.
Restoration of BCL9 reverses the biological effects of miR-3194-3p on HCC cells

To further illustrate that miR-3194-3p affects the migration and invasion of HCC cells was mediated by BCL9, we restored BCL9 in miR-3194-3p-overexpressing MHCC-97H cells ($p < .05$, Figure 6(A)). BCL9 restoration abolished the inhibitory effects of miR-3194-3p on migration and invasion of MHCC-97H cells ($p < .05$, respectively, Figure 6(B)). By contrast, BCL9 knock-down by a specific siRNA in miR-3194-3p-suppressive Hep3B cells significantly reversed the miR-3194-3p knockdown effects on Hep3B cells ($p < .05$, respectively, Figure 6(C,D)). In addition, alteration of BCL9 could rescue the effects of miR-3194-3p on EMT process ($p < .05$, respectively, Figure 6(E,F)). These results confirm that BCL9 is the functional mediator of miR-3194-3p in HCC cells.

miR-3194-3p suppressed the metastatic capacity of HCC cells in vivo

To confirm the effects of miR-3194-3p in vivo, we used lateral veins injection model to construct a lung metastasis model. We demonstrated that miR-3194-3p over-expression group showed fewer and smaller foci in the lungs of nude mice via microscopic evaluation ($p < .05$, Figure 7(A)). Moreover, we confirmed that the lung sections of miR-3194-3p over-expression group showed decreased expression of BCL9 (Figure 7(B)). In contrary, miR-3194-3p knockdown in Hep3B cells led to a significant increase of lung metastasis nudes and BCL9 expression ($p < .05$, Figure 7(C,D)). In addition, to confirm if miR-3194-3p regulates EMT of HCC cells in vivo, we found that miR-3194-3p over-expression increased E-cadherin expression and inhibited Vimentin expression (Figure 7(B)).
Figure 4. BCL9 is a direct target of miR-3194-3p in HCC cells. (A) miR-3194-3p and its putative binding sequence in the 3'-UTR of BCL9. The mutant binding site was generated in the complementary site for the seed region of miR-3194-3p. (B) miR-3194-3p significantly suppresses the luciferase activity that carried wild-type (wt) but not mutant (mt) 3'-UTR of BCL9. Anti-miR-3194-3p led to a notable increase in the luciferase activity of wt 3'-UTR of BCL9. (C) qRT-PCR analysis of BCL9 mRNA expression in MHCC-97H cells with miR-3194-3p or miR-control vector transfection and Hep3B cells with anti-miR-3194-3p or anti-miR-NC vector transfection. n = 6 repeats with similar results. (D) Over-expression of miR-3194-3p reduced the expression of BCL9 protein in MHCC-97H cells and knockdown of miR-3194-3p increases the level of BCL9 protein in Hep3B cells. (E) The expression of BCL9 mRNA in miR-3194-3p high-expressing tumors was significantly lower than that in miR-3194-3p low-expressing tumors. (F) A significantly inverse correlation between miR-3194-3p and BCL9 protein expression was observed in HCC tissues, as determined by Western blot. (G) A significant inverse correlation between miR-3194-3p and BCL9 was observed in HCC tissues.

Figure 5. BCL9 was up-regulated in HCC and regulated the migration and invasion in HCC. (A, B) The BCL9 mRNA and protein level was up-regulated in HCC tissues. (C) Hep3B and MHCC-97H cells that were transfected with corresponding vectors were subjected to WB for BCL9 expression. (D) Over-expression of BCL9 promoted cell proliferation, migration and invasion in Hep3B cells. (E) Down-regulation of BCL9 inhibited cell migration and invasion in MHCC-97H cells. (F) Western blot analysis of EMT markers expression in the over-expression or knock-down of BCL9 n= six independent experiments. *p < .05.
However, miR-3194-3p knockdown showed opposite effects (Figure 7(D)). Altogether, these results suggest that miR-3194-3p restrain metastatic behaviors and EMT phenotype of HCC by regulating BCL9 in vivo.

**Clinical significance of miR-3194-3p and BCL9 expression for HCC patients**

To further confirm the role of miR-3194-3p and BCL9 in HCC patients, we divided all HCC patients into high and low group according to the median value of miR-3194-3p and BCL9 expression. As shown in Table 1, we demonstrated that miR-3194-3p down-regulation was significantly associated with tumor-node-metastasis (TNM) stage(III + IV, p = .007), venous invasion (p = .009) and multiple tumor nodes (p = .015), while BCL9 over-expression was correlated with venous invasion (p = .013) and advanced TNM stage (p = .003). Data suggest that ectopic expressions of miR-3194-3p and BCL9 is associated with adverse prognostic features of HCC patients. Notably, poorer overall survival (OS) and disease-free survival (DFS) have been tested by Kaplan–Meier survival curves in low miR-3194-3p of HCC patients, while BCL9 high expressing patients conferred an obviously worse OS and DFS (<.05, respectively, Figure 8(A–D)). With combination analysis, the data showed that patients with low miR-3194-3p and high BCL9 expression had the worst OS and DFS (<.05, respectively, Figure 8(E,F)). These data suggest that combination of
miR-3194-3p and BCL9 is a potential biomarker for the clinical outcome of HCC patient.

**miR-3194-3p regulates the wnt/β-catenin pathway through BCL9**

Previous studies confirmed that the β-catenin/BCL9 complex plays an important role in cancer development and progression [22]. Therefore, we further performed WB and found that miR-3194-3p over-expression significantly decreased the β-catenin expression, whereas miR-3194-3p knockdown increased the β-catenin level (p < .05, Figure 9(A)). Altogether, these results suggested miR-3194-3p regulated Wnt/β-catenin signaling through BCL9.

**Hypoxia condition induced miR-3194-3p down-regulation and mediates the effects of hypoxia on HCC metastasis**

Hypoxia, an important feature of tumor microenvironments, have been recognized as a critical regulator of cancer metastasis [23]. Previous study confirmed that BCL9, which was a
downstream target of miR-3194-3p in this research, could be regulated by hypoxia [24]. Therefore, we try to explore whether miR-3194-3p could be regulated by hypoxia and its role in hypoxia. Hypoxia condition significantly increased HIF-1α expression in Hep3B cells (p < .05, Figure 9(B)) and led to decrease of miR-3194-3p expression (p < .05, Figure 9(C)). Furthermore, miR-3194-3p over-expression abolished the promoting effects of hypoxia on migration and invasion of Hep3B cells (p < .05, Figure 9(D)). Similarly, the positive effects of hypoxia on EMT process were reversed by miR-3194-3p restoration in Hep3B cells (p < .05, Figure 9(E)). Altogether, these results indicated that hypoxia-induced miR-3194-3p loss play a key role in migration, invasion, and EMT events of HCC cells.

Discussion

Accumulating evidence reported that aberrant miRNAs act as oncogenes or tumor suppressors in the cancer initiation, development, and progression [15,25]. New studies have established the potential usefulness of miRNAs as therapeutic molecules against cancers [26]. In this study, we propose a novel role for miR-3194-3p in coordinating the BCL9 expression in HCC cells and tissues. Being the first report, we demonstrated that miR-3194-3p was significantly down-regulated in HCC tissues and cell lines. The tissues with metastatic and recurrent phenotype showed reduced miR-3194-3p expression. These results strongly suggest that miR-3194-3p is a tumor suppressor in HCC and plays a pivotal role in the progression of HCC. Metastasis, a major reason that leads to the dismal prognosis of HCC [27], has been reported by numerous studies, which confirm that miRNAs are identified as regulators in metastasis of HCC [28]. To this end, we explored whether miR-3194-3p was involved in the progression of HCC by regulating the metastasis of HCC. In this study, gain- and loss-of-function experiment demonstrated that miR-3194-3p over-expression inhibited the migration and invasion of HCC cells while miR-3194-3p knockdown increased these metastatic behaviors in vitro and in vivo. EMT has been recognized as an important process in the invasion and metastasis of HCC [29]. We confirmed that miR-3194-3p inhibited EMT process of HCC cells. In patients’ tissues, we also showed that miR-3194-3p was inversely associated with EMT markers. These findings indicated that miR-3194-3p inhibits metastasis of HCC by regulating EMT and potentially serve as a therapeutic target of HCC metastasis.

B cell lymphoma 9 (BCL9), a component of aberrantly activated Wnt signaling, is an important contributing factor to tumor progression [30]. Despite overwhelming evidence highlighted the clinical implication of BCL9 in cancers [31,32], here we confirmed that BCL9 was a direct downstream target of miR-3194-3p and mediated the biological effects of miR-3194-3p on HCC. miR-3194-3p negatively regulated BCL9 mRNA and protein expression level in HCC, which we confirmed that was up-regulated in HCC tissues and promoted the migration and invasion of HCC cells. Moreover, miR-3194-3p was inversely correlated with the expression of BCL9 in tissues. Previous studies reported that dysregulated BCL9 expression is an activator of Wnt pathway. In our study, we discovered that miR-3194-3p restrained the activation of Wnt/β-catenin signaling.

In clinical roles, we confirm that miR-3194-3p and BCL9 could serve as valuable biomarkers for prognostic prediction. We found that low miR-3194-3p expression and high BCL9 were significantly associated with poor prognostic features of HCC patients. Nevertheless, we confirmed that miR-3194-3p down-expression and BCL9 over-expression, as well as their combination, were obviously correlated with poor prognosis of HCC patients. These results suggest that miR-3194-3p and BCL9 may be promising predictors for the prognosis of HCC. Finally, we elucidated the underlying mechanism for reduced expression of miR-3194-3p in HCC. Previous researches have
shown hypoxia tumor microenvironment play important roles in metastasis of HCC [33]. Moreover, BCL9, which was the target of miR-3194-3p, was also regulated by hypoxia condition. To confirm the reason, we showed that miR-3194-3p level was decreased in hypoxia environment. Importantly, we also found that overexpression of miR-3194-3p abrogated the prompting effects of hypoxia on migration, invasion, and EMT progress. These results suggest that hypoxia-induced miR-3194-3p loss promotes the metastasis and EMT of HCC.

Altogether, our observations suggested that miR-3194-3p inhibits HCC migration, invasion, and EMT via decreasing Wnt/β-catenin signaling through targeting BCL9 and might be a therapeutic target for HCC.

**Conclusion**

In conclusion, we demonstrate miR-3194-3p is down-regulated in HCC tissues and cell lines, and its reduced expression is associated with adverse clinicopathological features. We confirm that miR-3194-3p inhibits migration, invasion, and EMT process of HCC cells by directly targeting BCL9-mediated Wnt/β-catenin pathway. Notably, a combination of down-regulated miR-3194-3p and over-expressed BCL9 is potential prognostic predictors for the survival of HCC patients. Moreover, we confirmed that hypoxia was the underlying reason for miR-3194-3p down-regulation. In summary, the down-regulation of miR-3194-3p was involved in tumor metastasis and maybe a novel prognostic factor and potential therapeutic target for HCC.

**Ethical approval**

Ethics approval and consent to participate. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of The First Affiliated Hospital of Xi’an Jiaotong University.
University and with the 1964 Helsinki declaration and its later amendments. All written informed consent to participate in the study was obtained from HCC patients for samples to be collected from them.

Author contributions
ZL and QL conceived and designed the experiments; BY, YL performed the experiments; LS and TC analyzed the data; YN contributed reagents/materials/analysis tools; BY and YL wrote the paper. All authors read and approved the final manuscript.

Disclosure statement
No potential conflict of interest was reported by the authors.

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Data availability
All data generated or analyzed during this study are included in this article.

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