MEP1A Contributes to Tumor Progression and Predicts Poor Clinical Outcome in Human Hepatocellular Carcinoma

Han-Yue OuYang,1-3 Jing Xu,2,3 Jun Luo,4 Ru-Hai Zou,5 Keng Chen,6 Yong Le,1,3 Yong-Fa Zhang,1,3 Wei Wei,1,3 Rong-Ping Guo,1,3 and Ming Shi1–3

Although many staging classifications have been proposed for hepatocellular carcinoma (HCC), determining a patient’s prognosis in clinical practice is a challenge due to the molecular diversity of HCC. We investigated the relationship between MEP1A, a candidate oncogene, and clinical outcomes of HCC patients; furthermore, we explored the role of MEP1A in HCC. In this report, it was demonstrated by quantitative real-time polymerase chain reaction that MEP1A messenger RNA levels were significantly elevated in HCC tumor tissues compared with matched adjacent nonneoplastic tissues and nonmalignant liver disease tissues. Immunohistochemical analyses of tissue samples from two independent groups of 394 HCC patients showed that positive expression of MEP1A in tumor cells was an independent and significant risk factor affecting survival after curative resection in both cohort 1 (hazard ratio = 2.05, 95% confidence interval 1.427-2.946; \( P < 0.001 \)) and cohort 2 (hazard ratio = 1.89, 95% confidence interval 1.260-2.833; \( P = 0.002 \)). Analysis of Barcelona Clinic Liver Cancer stage 0-A subgroup further showed that patients with positive MEP1A expression in tumor cells had poorer surgical prognoses than those with negative MEP1A expression in tumor cells (cohort 1 \( P = 0.001 \), cohort 2 \( P < 0.001 \)). Both in vitro and in vivo assays showed that MEP1A promoted HCC cell proliferation, migration, and invasion. Further analyses found that MEP1A played an important role in regulating cytoskeletal events and induced epithelial-mesenchymal transition in HCC cells. Conclusion: MEP1A is a novel prognostic predictor in HCC and plays an important role in the development and progression of HCC. (HEPATOLOGY 2016;63:1227-1239)

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant neoplasms, with increasing incidence and mortality worldwide.1,2 Despite improved diagnostic and treatment strategies, the overall survival (OS) of patients with HCC remains poor.3,4 In clinical practice, several staging systems have been established for the assessment of prognoses (e.g., the Barcelona Clinic Liver Cancer [BCLC] and tumor-node-metastasis [TNM] staging systems); however, correlations between tumor stage and the actual prognosis have not always been found.5,6 Therefore, the study of tumor biomarkers to identify patients at high risk for poor survival in HCC and the targeting of intensive clinical follow-up or
postsurgical adjuvant therapies in such patients would be ideal.

Meprin α, a metalloprotease coded by the human gene MEP1A, belongs to the metzincin superfamily. Meprin α is capable of cleaving a wide variety of substrates, such as basement membrane proteins, protein kinases, and cytokines. In normal human tissues, meprin α has been detected in the intestines, leukocytes, skin, pancreas, and testes. Although abnormal MEP1A expression has been implicated in several diseases, such as inflammatory bowel disease, nephritis, and Alzheimer’s disease, MEP1A expression in tumors has been observed in only colorectal cancer.

In the present study, the frequent aberrant expression of MEP1A in HCC tissues was analyzed by quantitative real-time polymerase chain reaction (PCR) and immunohistochemical (IHC) staining. We demonstrated that aberrant expression of MEP1A in tumor cells correlated with multiple malignant clinicopathological characteristics and was an independent prognostic factor for the OS and recurrence of HCC in patients. Both in vitro and in vivo assays showed that MEP1A promoted HCC cell proliferation, invasion, and metastasis. Additionally, MEP1A played an important role in regulating cytoskeletal events and induced epithelial-mesenchymal transition (EMT) in HCC cells, leading to enhanced invasiveness of cancer cells.

Materials and Methods

PATIENTS AND TISSUE SPECIMENS

This study was approved by the institutional review boards of the participating hospitals. Written informed consent was obtained from all patients. Formalin-fixed, paraffin-embedded tissues were obtained from 212 patients who underwent curative resection for HCC at the Cancer Center of Sun Yat-sen University (SYSU) between 2002 and 2006. These samples were examined as cohort 1. An HCC tissue microarray containing 182 multicenter samples from patients who underwent resection between 2001 and 2009 (including 73 patients at the First Affiliated Hospital of SYSU, 54 at Sun Yat-sen Memorial Hospital, and 55 at the Third Affiliated Hospital of SYSU) was examined as cohort 2. The tissue microarray was constructed according to a described method. The inclusion criteria used for patient enrollment in the cohorts were an absence of anticancer therapies or distant metastasis before the operation; a lack of concurrent autoimmune disease, human immunodeficiency virus, or syphilis; and the availability of follow-up data. Patients with Child-Pugh class B or C were excluded from our study. The histologic grade of tumor differentiation was assigned according to the Edmondson-Steiner grading system. The BCLC staging system and the seventh edition of the International Union Against Cancer/American Joint Committee on Cancer TNM staging system were used for staging. The clinicopathological characteristics of the patients in cohort 1 and cohort 2 are summarized in Supporting Table S1.

Additionally, 94 pairs of freshly resected HCC and adjacent nonneoplastic liver tissues were collected from patients who had undergone hepatectomies for the curative treatment of HCC at the Cancer Center of SYSU in 2014. Nonmalignant liver disease tissues, including 18 cases of focal nodular hyperplasia and eight cases of hepatocellular adenoma, were obtained from the Department of Pathology at the Cancer Center of SYSU. None of the patients received any neoadjuvant therapies, such as radiotherapy or chemotherapy, before surgery.
Informed consent was obtained from patients regarding the use of their liver specimens for research.

**FOLLOW-UP**

The detailed follow-up procedures have been described. OS was defined as the interval between surgery and death or between surgery and the last observation for surviving patients. The time to recurrence was defined as the interval between surgery and recurrence or between surgery and the last observation for patients without recurrence. The median follow-up was 34.4 months (range 1.9-120 months) for cohort 1 and 44.3 months (range 2.0-100 months) for cohort 2. Of the 394 patients who were examined during the follow-up period, 232 patients (58.9%) died and 286 patients (72.6%) were diagnosed with tumor recurrence.

**STATISTICAL ANALYSIS**

For continuous variables, the data are expressed as the mean ± standard error of the mean. The significance of differences between values was determined using the Student t test. The chi-squared test was applied to examine the correlation between MEP1A expression and clinical pathological parameters. Survival curves for patients were calculated using the Kaplan-Meier method and analyzed using the log-rank test. Prognostic factors were examined by univariate and multivariate analyses using the Cox proportional hazards model. All differences were deemed significant at \( P < 0.05 \). All statistical analyses were performed with SPSS software version 18.0 (SPSS, Chicago, IL).

Detailed information regarding animal models, plasmid constructs and transfection, immunohistochemistry, immunofluorescence, RNA isolation and quantitative real-time PCR, western blot analyses, antibodies and reagents, cell lines and culture conditions, in vitro cell growth assays, cell migration and invasion assays, and wound healing assays is provided in the Supporting Information.

**Results**

**ELEVATED EXPRESSION OF MEP1A IN HUMAN HCC TISSUES BY QUANTITATIVE REAL-TIME PCR**

To compare the expression pattern of MEP1A in HCC tumor tissues and adjacent nonneoplastic tissues, we examined the levels of MEP1A in 94 pairs of matched HCC tumor-derived and non-tumor-derived specimens by quantitative real-time PCR. Eighteen cases of focal nodular hyperplasia and eight cases of hepatocellular adenoma were collected as disease controls. We found that the expression levels of MEP1A were remarkably elevated in HCC tumor tissues compared with matched adjacent non-neoplastic tissues and nonmalignant liver diseases (Fig. 1A). Moreover, the expression levels of MEP1A in tumor tissues were significantly higher in advanced stage patients (TNM stages II and III) than in early stage patients (TNM stage I) (\( P < 0.01 \), Fig. 1A). MEP1A messenger RNA levels were also significantly higher in patients with poorly differentiated HCC than in those with well-differentiated HCC (\( P < 0.001 \), Fig. 1B).

**IHC STAINING OF MEP1A IN HCC TISSUES AND ITS CORRELATION WITH CLINICOPATHOLOGIC FEATURES AND POOR SURVIVAL IN HCC PATIENTS**

As the survival data were not available for the specimens used in the quantitative real-time PCR, we performed IHC staining in specimens from a set of 212 HCC patients (cohort 1) to study the correlation between MEP1A expression and HCC prognosis. The results showed that all endothelial cells of blood vessels were positive for MEP1A expression in both tumor and nontumor tissues (Fig. 2A). However, the MEP1A expression levels in the tumor cell cytoplasm varied widely among different HCC specimens (Fig. 2A,B). Thus, we focus on the aberrant expression of MEP1A in tumor cells. Based on MEP1A expression in the tumor cell cytoplasm, patients were divided into two groups, the MEP1A− group (negative expression in tumor cells; Fig. 2A) and the MEP1A+ group (positive expression in tumor cells; Fig. 2B). We analyzed the relationship between MEP1A expression levels in tumor cells and the clinicopathological characteristics. Interestingly, patients in the MEP1A+ group were significantly associated with aggressive clinicopathologic features (i.e., microvascular invasion, portal vein tumor thrombus, poorly differentiated tumors, and late clinical stage) (Table 1). Another independent external set (cohort 2, \( n = 182 \)) confirmed the results from cohort 1 (Fig. 2C,D; Supporting Table S2). To confirm the correlation between MEP1A expression levels...
in tumor cells and HCC prognoses, we compared the time to recurrence and OS between these two groups. Kaplan-Meier survival analysis revealed that patients in the MEP1A\(^+\) group had shorter time to recurrence and worse OS than those in the MEP1A\(^-\) group in both cohorts (Fig. 3; Supporting Fig. S1). To determine whether the positive expression of MEP1A in tumor cells was an independent prognostic factor for HCC, a multivariate survival analysis was performed for these two independent cohorts. The hazard ratios for OS in the MEP1A\(^+\) group from cohorts 1 and 2 were 2.05 (95% confidence interval 1.427-2.946, \(P < 0.001\)) and 1.89 (95% confidence interval 1.260-2.833, \(P = 0.002\)), respectively (Table 2; Supporting Table S3). These data indicated that the MEP1A expression level in tumor cells was an independent prognostic factor for HCC.

**EFFECT OF MEP1A EXPRESSION ON THE PROGNOSIS OF HCC PATIENTS WITH DIFFERENT BCLC STAGES AND SPECIFIC SUBGROUPS**

All patients were stratified according to the BCLC staging system. Kaplan-Meier plots of patients with different BCLC stages are shown in Fig. 4 and in Supporting Fig. S2. Of the 128 patients in cohort 1 at stage 0-A, 34 were identified as having positive MEP1A expression in tumor cells. Patients with positive MEP1A expression in tumor cells had a poorer surgical prognosis than those with negative MEP1A expression in tumor cells (\(P < 0.001\), Fig. 4A). Further analysis of cohort 2 demonstrated that MEP1A expression in tumor cells predicted poorer survival for patients at stage 0-A (\(P < 0.001\), Fig. 4B). In cohort 1, of the 44 patients at stage B and the 40 patients at stage C, the prognosis of patients with MEP1A expression in tumor cells was poorer than that of patients with negative MEP1A expression in tumor cells (\(P = 0.001\), Fig. 4A). Further analysis of cohort 2 demonstrated that MEP1A expression in tumor cells predicted poorer survival for patients at stage 0-A (\(P < 0.001\), Fig. 4B). In cohort 1, of the 44 patients at stage B and the 40 patients at stage C, the prognosis of patients with MEP1A expression in tumor cells was poorer than that of patients with negative MEP1A expression in tumor cells (\(P = 0.109\), \(P = 0.026\), respectively; Supporting Fig. S2A); however, this finding could not be validated in cohort 2 (\(P = 0.843\), \(P = 0.101\); Supporting Fig. S2B).

We further explored the prognostic value of MEP1A in specific subgroups of HCC patients. We divided patients into two subgroups according to microvascular invasion (MVI). The OS of patients with positive MEP1A expression in tumor cells significantly decreased compared with those with negative MEP1A expression in tumor cells in the non-MVI groups in cohorts 1 and 2 (\(P < 0.001\), \(P < 0.001\); Supporting Fig. S3). However, for patients in the MVI group, no significant associations between MEP1A expression and prognosis were observed in
FIG. 2. IHC characteristics of MEP1A in HCC specimens. All endothelial cells of blood vessels were positive for MEP1A expression in both tumor and adjacent nonneoplastic liver tissues. (A,B) Paraffin-embedded samples from cohort 1. (A) A well-differentiated HCC specimen. Representative staining of negative MEP1A expression in the tumor cell cytoplasm (×200). The panel below shows an enlargement of the indicated area (×400). (B) A poorly differentiated HCC specimen. Representative staining of positive MEP1A expression in the tumor cell cytoplasm. (C,D) Tissue microarray samples from cohort 2. (C) Representative staining of negative MEP1A expression in the tumor cell cytoplasm (left ×40, right ×400). (D) Representative staining of positive MEP1A expression in the tumor cell cytoplasm (left ×40, right ×400).
TABLE 1. Correlation Between MEP1A Expression and Clinicopathologic Characteristics in HCC (Cohort 1, n = 212)

| Clinicopathological Variable       | MEP1A Expression Levels | No. | Negative | Positive | P       |
|------------------------------------|-------------------------|-----|----------|----------|---------|
| Age (year)                         |                         |     |          |          |         |
| ≤50                                |                         | 113 | 75       | 38       | 0.840   |
| >50                                |                         | 99  | 67       | 32       |         |
| Gender                             |                         |     |          |          |         |
| Female                             |                         | 28  | 22       | 6        |         |
| Male                               |                         | 184 | 120      | 64       | 0.162   |
| Hepatitis B surface antigen        |                         |     |          |          |         |
| Negative                           |                         | 20  | 17       | 3        |         |
| Positive                           |                         | 192 | 125      | 67       | 0.072   |
| Serum AFP (ng/mL)                  |                         |     |          |          |         |
| <400                               |                         | 117 | 85       | 32       |         |
| ≥400                               |                         | 95  | 57       | 38       | 0.051   |
| Tumor size (cm)                    |                         |     |          |          |         |
| ≤5                                 |                         | 76  | 58       | 18       | 0.031   |
| >5                                 |                         | 136 | 84       | 52       |         |
| Tumor number                       |                         |     |          |          |         |
| Solitary                           |                         | 144 | 101      | 43       | 0.155   |
| Multiple                           |                         | 68  | 41       | 27       |         |
| Microvascular invasion             |                         |     |          |          |         |
| No                                 |                         | 186 | 133      | 53       | <0.001  |
| Yes                                |                         | 28  | 9        | 17       |         |
| PVTT                               |                         |     |          |          |         |
| No                                 |                         | 172 | 123      | 49       | 0.004   |
| Yes                                |                         | 40  | 19       | 21       |         |
| Liver cirrhosis                    |                         |     |          |          |         |
| No                                 |                         | 120 | 80       | 40       | 0.911   |
| Yes                                |                         | 92  | 62       | 30       |         |
| Differentiation grade              |                         |     |          |          |         |
| I+II                               |                         | 132 | 97       | 35       | 0.010   |
| III+IV                             |                         | 80  | 45       | 35       |         |
| BCLC stage                         |                         |     |          |          |         |
| 0-A                                |                         | 128 | 94       | 34       | 0.014   |
| B-C                                |                         | 84  | 48       | 36       |         |
| TNM stage                          |                         |     |          |          |         |
| I                                  |                         | 120 | 89       | 31       | 0.011   |
| II-IV                              |                         | 92  | 53       | 39       |         |

Abbreviations: AFP, alpha-fetoprotein; PVTT, portal vein tumor thrombus.

We also divided the patients into two subgroups according to tumor size. Patients with a tumor diameter >5 cm had a poorer prognosis than those with a tumor diameter ≤5 cm in both cohort 1 (P < 0.001, Table 2) and cohort 2 (P = 0.001; Supporting Table S3). Furthermore, the effect of MEP1A expression in tumor cells on prognosis in the two subgroups was examined. The results showed that patients with positive MEP1A expression in tumor cells may have significantly shorter OS than those with negative MEP1A expression in tumor cells in either the ≤5 cm or the >5 cm subgroup (Supporting Fig. S4).

MEP1A DISPLAYS AN ONCOCENIC FUNCTION IN HCC

Because MEP1A expression in tumor cells in HCC tissues was positively associated with malignant clinicopathological features, we explored the potential biological function of MEP1A in HCC tumorigenesis. First, we examined the MEP1A expression pattern in HCC cell lines (Hep3B, SK-Hep-1, HuH7, SMMC-7721) and normal liver cells (L02). Notably, all HCC cell lines displayed significantly higher messenger RNA and protein levels of MEP1A than normal liver cell lines (Supporting Fig. S5A,B). To further investigate the role of MEP1A in malignancy, Hep3B and SMMC-7721 cells were stably transfected with an MEP1A expression plasmid (pEZ-Lv201-MEP1A) or a control vector (pEZ-Lv201). The ectopic expression of MEP1A in the cells was confirmed by
quantitative real-time PCR and western blot analyses. Functional assays were used to characterize the tumorigenicity of MEP1A. The results showed that overexpression of MEP1A in HCC cell lines significantly promoted the cell growth rate ($P < 0.05$; Supporting Fig. S5C) and foci formation frequency ($P < 0.01$; Supporting Fig. S5D).

To demonstrate whether MEP1A could enhance tumorigenicity in vivo, Hep3B-MEP1A, Hep3B-Vector, 7721-MEP1A, and 7721-Vector cells were subcutaneously injected into nude mice. After 3 weeks, the mice were sacrificed and the xenograft tumors were harvested and measured. The results showed that the xenograft tumors of the MEP1A overexpression group were significantly larger and heavier ($P < 0.01$) than those of the control group (Supporting Fig. S5E). The corresponding images for the hematoxylin and eosin (H&E) and IHC of MEP1A staining are shown in Supporting Fig. S5F.

**MEP1A OVEREXPRESSION INCREASES CELL MIGRATION AND ENHANCES TUMOR METASTASIS**

In addition to its oncogenic role, positive MEP1A expression in tumor cells was significantly associated with clinicopathological features related to tumor metastasis, such as microvascular invasion, portal vein thrombus, and ascending clinical stage (Table 1; Supporting Table S2). To determine the role of MEP1A in cell migration and invasion, wound healing and transwell assays were performed in vitro. The results showed that

---

**TABLE 2. Univariate and Multivariate Analyses of Prognostic Factors in HCC (Cohort 1, n = 212)**

| Variables                  | OS                  | Time to Recurrence |
|----------------------------|---------------------|--------------------|
|                           | Univariate          | Multivariable      | Univariate          | Multivariable      |
|                           | $P$                 | HR (95% CI)        | $P$                 | HR (95% CI)        |
| Age (year)                |                     |                    |                     |
| $\leq 50$                 | 0.013               | n.a.               | 0.009               | n.a.               |
| $>50$                     |                     |                    |                     |
| Gender                    |                     |                    |                     |
| Female                    | 0.603               | 0.422              | 0.253               | 0.010              |
| Male                      |                     |                    |                     |
| Hepatitis B surface antigen |                   |                    |                     |
| Negative                  | 0.747               | 0.010              | 0.004               | n.a.               |
| Positive                  |                     |                    |                     |
| Serum AFP (ng/mL)         |                     |                    |                     |
| $<400$                    | 0.038               | n.a.               | 0.010               | n.a.               |
| $\geq 400$                |                     |                    |                     |
| Tumor size (cm)           |                     |                    |                     |
| $\leq 5$                  | 0.000               | 1.669 (1.116-2.496)| 0.013               | 1.685 (1.186-2.395)| 0.004               |
| $>5$                      |                     |                    |                     |
| Tumor number              |                     |                    |                     |
| Solitary                  | 0.000               | 1.815 (1.262-2.611)| 0.001               | 1.604 (1.144-2.248)| 0.006               |
| Multiple                  |                     |                    |                     |
| PVTT                      |                     |                    |                     |
| No                        | 0.000               | 5.373 (3.468-8.326)| $<0.001$            | 0.000               | 4.465 (2.990-6.667)| $<0.001$            |
| Yes                       |                     |                    |                     |
| Microvascular invasion    |                     |                    |                     |
| No                        | 0.000               | n.a.               | 0.000               | n.a.               |
| Yes                       |                     |                    |                     |
| Liver cirrhosis           |                     |                    |                     |
| No                        | 0.243               | 0.127              | 0.015               | n.a.               |
| Yes                       |                     |                    |                     |
| Differentiation grade     |                     |                    |                     |
| I+ II                     | 0.015               | n.a.               | 0.013               | n.a.               |
| III+IV                    |                     |                    |                     |
| MEP1A                     |                     |                    |                     |
| Negative                  | 0.000               | 2.050 (1.427-2.946)| $<0.001$            | 0.000               | 1.599 (1.138-2.246)| 0.007               |
| Positive                  |                     |                    |                     |

Abbreviations: AFP, alpha-fetoprotein; CI, confidence interval; HR, hazard ratio; n.a., not applicable; PVTT, portal vein tumor thrombus.
wound healing was more rapid in Hep3B-MEP1A cells and 7721-MEP1A cells than in control cells (Fig. 5A). For the transwell migration assay, in vitro cultured HCC cell lines were allowed to migrate through the 8-µm pores of polycarbonate filters. Compared with the control cells, MEP1A overexpression induced a three-fold to four-fold increase in the migration of HCC cell lines (Fig. 5B). This effect was further examined using Matrigel invasion assays, wherein MEP1A was shown to increase the invasive activity of HCC cell lines (Fig. 5C).

To evaluate the effects of MEP1A on tumor metastasis in vivo, four groups of 12 mice each were injected intravenously in the tail vein with Hep3B-MEP1A, Hep3B-Vector, 7721-MEP1A, or 7721-Vector cells. After 8 weeks, the mice were sacrificed and the metastatic nodules in the lungs were counted. Significantly more metastatic nodules were induced in the lungs of mice injected with Hep3B-MEP1A and 7721-MEP1A cells than those injected with vector cells (Fig. 5D). H&E staining confirmed that the nodules in the lungs were metastatic tumors.

Another HCC model was used to confirm the effect of MEP1A overexpression on tumor formation and metastasis by the orthotopic transplantation of 7721-MEP1A or 7721-Vector cells into BALB/c mice (n = 12 each group). The liver tumors from the group of 7721-MEP1A mice were significantly larger than those from the 7721-Vector group (Fig. 5E). Significantly more metastatic nodules were also induced on the surfaces of the lungs from the 7721-MEP1A group than those from the 7721-Vector group. H&E staining of liver tumors and lung nodules in the 7721-Vector group and the 7721-MEP1A group is shown in Fig. 5F.

KNOCKDOWN OF MEP1A INHIBITS CELL GROWTH AND INVASION

Next, we investigated the effect of MEP1A knockdown on cell proliferation and invasion in Huh7 and SK cells. Scrambled short hairpin RNA (shRNA) was used as a negative control. Quantitative real-time PCR and western blot analyses indicated that the expression levels of MEP1A could be effectively reduced by shRNA (Fig. 6B,C). MEP1A knockdown in the cells significantly inhibited the cell growth rate (P < 0.05; Supporting Fig. S6A) and frequency of foci formation (P < 0.01; Supporting Fig. S6B). To investigate whether MEP1A knockdown could inhibit tumorigenicity in vivo, MEP1A shRNA-transfected and scrambled shRNA-transfected SK cells were subcutaneously injected into nude mice (n = 12 each group). We observed that tumors from the SK-shMEP1A group were significantly smaller than those from the control group (Supporting Fig. S6C). Additional wound healing and transwell assays showed that knockdown of MEP1A in SK and Huh7 cells inhibited cell migration and invasion (Supporting Fig. S6D-F).

MEP1A EXPRESSION INDUCES EMT IN HCC CELLS

EMT is one of the key events in tumor invasion and metastasis defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. This transition is accompanied by loss of cell polarity and E-cadherin expression and by the acquisition of spindle-shaped morphology, high cell motility, and invasiveness. In our study, we observed that mesenchyme-like
morphological features were induced in MEP1A-transfected cells (Fig. 6A). Results by quantitative real-time PCR and western blot showed that overexpression of MEP1A in Hep3B and SMMC-7721 cells markedly enhanced the levels of vimentin, matrix metalloproteinase-2 (MMP2), and MMP9 and concomitantly reduced the expression of E-cadherin. Opposite expression patterns of these genes were observed in MEP1A knockdown cells (Fig. 6B,C). Immunofluorescence staining was also performed to analyze the localization of E-cadherin and the accumulation of vimentin in MEP1A-transfected cells (Fig. 6D). Interestingly, we found that the alterations of E-cadherin expression were accompanied by an opposite parallel regulation of ZEB1 in HCC cells (Fig. 6B,C). We also confirmed this by IHC staining of MEP1A, E-cadherin, vimentin, and ZEB1 in consecutive human HCC tissue sections (Fig. 6E). These results suggest that ZEB1 may play an important role in MEP1A-mediated EMT.

Growing evidence has shown that EMT can be triggered by epidermal growth factor through activation of the extracellular signal-regulated kinase (ERK) pathway. Because meprin α can lead to an increase in epidermal growth factor receptor and ERK1/2 phosphorylation through the release of epidermal growth factor and transforming growth factor-alpha in human colorectal adenocarcinoma cells, we asked whether MEP1A affects the ERK signaling pathway in HCC cells. Thus, the level of phosphorylated ERK1/2 was analyzed in MEP1A overexpression and knockdown cells by western blot. The level of phosphorylated ERK1/2 was increased when MEP1A was up-regulated in Hep3B and SMMC-7721 cells (Fig. 6C). In contrast, an obvious decrease of phosphorylated ERK1/2 was observed in MEP1A knockdown cells (Fig. 6C). These results were confirmed by increased expression of phosphorylated ERK1/2 in xenograft tumors from the MEP1A overexpression group (Supporting Fig. S7A).

Further, we asked whether MEP1A promoted EMT and migration of HCC cells through activation of the ERK/ZEB1 pathway. MEP1A-transfected cells were then treated with U0126, an ERK pathway inhibitor. The results showed that U0126 effectively decreased the levels of phosphorylated ERK and ZEB1 induced by MEP1A in both Hep3B and SMMC-7721 cell lines (Supporting Fig. S7B). Wound healing and transwell assays showed that U0126 could significantly reverse the effect of MEP1A on cell migration (Supporting Fig. S7C–E).

**Discussion**

We identified the frequent aberrant expression of MEP1A in HCC tissues by quantitative real-time PCR and IHC. This expression pattern was associated with malignant clinicopathological characteristics. More importantly, we demonstrated that this MEP1A expression pattern in HCC tissues was associated with poor prognoses and recurrence after curative resection. Furthermore, multivariate analyses revealed that MEP1A expression in tumor cells was an independent and significant risk factor affecting recurrence and survival after curative resection in two independent cohorts.

According to the BCLC staging system, patients in stages 0 and A are believed to be in the early stages of HCC and to have better outcomes after curative resection. However, a few of these early stage patients still have poor prognoses in clinical practice. Our results suggest that MEP1A expression in tumor cells predicted poorer survival for early stage patients (Fig. 4A,B). Thus, the findings of the present study suggest that the measurement of MEP1A expression in tumor cells could identify worse prognoses among early stage HCC patients.

In our study, the OS of patients with positive MEP1A expression in tumor cells significantly decreased compared with that of patients with negative MEP1A expression in tumor cells in the non-MVI groups in the two cohorts (Supporting Fig. S3). This result suggests that MEP1A expression in tumor cells
FIG. 6. MEP1A induces EMT in HCC cells. (A, left) Morphological changes in 7721 and Hep3B cells were examined under a microscope (×200). (right) F-actin was analyzed by phalloidin staining (red). 7721-MEP1A and Hep3B-MEP1A cells showed a greater number of stress fibers than control cells. (B) Relative expression levels of ZEB1, E-cadherin, vimentin, MMP2, and MMP9 were examined by quantitative real-time PCR in MEP1A-transfected, MEP1A knockdown, and their respective control cells. (C) Western blot analyses for E-cadherin, vimentin, MMP2, MMP9, ERK1/2, phosphorylated ERK1/2, and ZEB1 in MEP1A-transfected, MEP1A knockdown, and their respective control cells. (D) Immunofluorescence staining of E-cadherin and vimentin in Hep3B-MEP1A cells and Hep3B-Vector cells. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. (E) Representative images of IHC staining of MEP1A, E-cadherin, vimentin, and ZEB1 in consecutive human HCC tissue sections. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Vec, vector.
could be used to identify patients with worse prognoses in the non-MVI groups, indicating that MEP1A may be a useful indicator for prognostic assessment before the presence of MVI.

Notably, Lottaz et al. demonstrated that meprin z promoted migration and angiogenesis in colorectal cancer cells.(26) Matters et al. reported that actinonin (an inhibitor of meprin z) could decrease the invasiveness of human breast carcinoma cells. However, none of these studies has reported the clinical prognostic value of MEP1A in human cancer. Additionally, all of these studies were conducted in vitro.

In our study, a series of in vitro and in vivo assays were employed to investigate the role of meprin z in regulating HCC cell motility, invasion, and metastasis. The results showed that meprin z substantially promoted cell motility and invasiveness. Furthermore, we demonstrated that meprin z promoted EMT and migration of HCC cells through the activation of the ERK/ZEB1 pathway.

Given that MEP1A is frequently overexpressed in HCC, meprin z may be a potentially viable therapeutic target for treating HCC. Recently, several synthetic compounds were identified as inhibitors of meprin z. The hydroxamate actinonin was shown to be the most potent inhibitor with a 20 nM inhibition constant value for human meprin z.(28) In vitro, actinonin has been shown to have an antiproliferative effect on a variety of human cancer cell lines, including prostate, lung, breast, and ovarian carcinomas, as well as lymphoma and leukemia cell lines; however, the mechanism of this effect has not been investigated.(29,30) Actinonin was also reported to have significant antitumor effects on AKR leukemia in vivo.(29) However, additional research regarding specific meprin z inhibitors and appropriate animal models for HCC are required.

In summary, this study investigated the correlation between MEP1A expression in tumor cells, tumor recurrence, and patient survival in HCC. Our results suggest that MEP1A expression in tumor cells is an ideal biomarker that has a strong association with disease outcomes, particularly for early stage HCC patients. Furthermore, we demonstrated that MEP1A plays an important role in the progression of HCC by promoting cell migration and invasion.

REFERENCES

1) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.

2) Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet 2012;379:1245-1255.

3) Villanueva A, Hoshida Y, Battiston C, Tovar V, Sia D, Alsinet C, et al. Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma. Gastroenterology 2011;140:1501-1512.

4) Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell 2004;5:215-219.

5) Marrero JA, Fontana RJ, Barratt A, Askari F, Conjeevaram HS, Su GL, et al. Prognosis of hepatocellular carcinoma: comparison of 7 staging systems in an American cohort. Hepatology 2005;41:707-716.

6) Roayaie S, Blume IN, Thung SN, Guido M, Fiel MJ, Hiotis S, et al. A system of classifying microvascular invasion to predict outcome after resection in patients with hepatocellular carcinoma. Gastroenterology 2009;137:850-855.

7) Sterchi EE, Stocker W, Bond JS. Meprins, membrane-bound and secreted astacin metalloproteinases. Mol Aspects Med 2008;29:309-328.

8) Kronenberg D, Bruns BC, Mooli C, Vadon-Le Goff S, Sterchi EE, Traupe H, et al. Processing of procollagen III by meprins: new players in extracellular matrix assembly? J Invest Dermatol 2010;130:2727-2735.

9) Broder C, Becker-Pauly C. The metalloproteinases meprin alpha and meprin beta: unique enzymes in inflammation, neurodegeneration, cancer and fibrosis. Biochem J 2013;450:253-264.

10) Bertenshaw GP, Turk BE, Hubbard SJ, Matters GL, Bylander JE, Crisman JM, et al. Marked differences between metalloproteinases meprin A and B in substrate and peptide bond specificity. J Biol Chem 2001;276:13248-13255.

11) Sterchi EE, Naim HY, Lentze MJ, Hauri HP, Fransen JA. N-Benzoyl-L-tyrosyl-p-aminobenzoic acid hydrolase: a metalloendopeptidase of the human intestinal microvillus membrane which degrades biologically active peptides. Arch Biochem Biophys 1988;265:105-118.

12) Beynon RJ, Shannon JD, Bond JS. Purification and characterization of a metallo-endoproteinase from mouse kidney. Biochem J 1981;199:591-598.

13) Bond JS, Matters GL, Banerjee S, Dusheek RE. Meprin metalloprotease expression and regulation in kidney, intestine, urinary tract infections and cancer. FEBS Lett 2005;579:3317-3322.

14) Banerjee S, Oneda B, Yap LM, Jewell DP, Matters GL, Fitzpatrick LR, et al. MEP1A allele for meprin A metalloproteinase is a susceptibility gene for inflammatory bowel disease. Mucosal Immunol 2009;2:220-231.

15) Wang Z, Herzog C, Kaushal GP, Goldenk N, Mayeux PR. Actinonin, a meprin A inhibitor, protects the renal microcirculation during sepsis. Shock 2011;35:141-147.

16) Lottaz D, Maurer CA, Hahn D, Buchler MW, Sterchi EE. Nonpolarized secretion of human meprin alpha in colorectal cancer generates an increased proteolytic potential in the stroma. Cancer Res 1999;59:1127-1133.

17) Xu J, Ding T, He Q, Yu XJ, Wu WC, Jia WH, et al. An in situ molecular signature to predict early recurrence in hepatitis B virus-related hepatocellular carcinoma. J Hepatol 2011;54:313-321.

18) Choi SS, Diehl AM. Epithelial-to-mesenchymal transitions in the liver. Hepatology 2009;50:2007-2013.

19) Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420-1428.

20) Reichel P, Dengler M, van Zijl F, Huber H, Führlinger G, Reichel C, et al. Axl activates autocrine transforming growth factor-α signalling in breast cancer via MET activation. EMBO Mol Med 2013;5:909-922.
factor-beta signaling in hepatocellular carcinoma. Hepatology 2015;61:930-941.

21) Lemieux E, Bergeron S, Durand V, Asselin C, Saucier C, Rivard N. Constitutively active MEK1 is sufficient to induce epithelial-to-mesenchymal transition in intestinal epithelial cells and to promote tumor invasion and metastasis. Int J Cancer 2009;125:1575-1586.

22) Shin S, Dimitri CA, Yoon SO, Dowdle W, Blenis J. ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events. Mol Cell 2010;38:114-127.

23) Ichikawa K, Kubota Y, Nakamura T, Weng JS, Tomida T, Saito H, et al. MCRIP1, an ERK substrate, mediates ERK-induced gene silencing during epithelial-mesenchymal transition by regulating the corepressor CtBP. Mol Cell 2015;58:35-46.

24) Minder P, Bayha E, Becker-Pauly C, Sterchi EE. Meprinalpha transactivates the epidermal growth factor receptor (EGFR) via ligand shedding, thereby enhancing colorectal cancer cell proliferation and migration. J Biol Chem 2012;287:35201-35211.

25) Bergin DA, Greene CM, Sterchi EE, Kenna C, Geraghty P, Belaaouaj A, et al. Activation of the epidermal growth factor receptor (EGFR) by a novel metalloprotease pathway. J Biol Chem 2008;283:31736-31744.

26) Lottaz D, Maurer CA, Noel A, Blacher S, Huguenin M, Nierergelt A, et al. Enhanced activity of meprin-alpha, a pro-

migratory and pro-angiogenic protease, in colorectal cancer. PLoS One 2011;6:e26450.

27) Matters GL, Manni A, Bond JS. Inhibitors of polyamine biosynthesis decrease the expression of the metalloproteases meprin alpha and MMP-7 in hormone-independent human breast cancer cells. Clin Exp Metastasis 2005;22:331-339.

28) Kruse MN, Becker C, Lottaz D, Kohler D, Yiallouros I, Krell HW, et al. Human meprin alpha and beta homo-oligomers: cleavage of basement membrane proteins and sensitivity to metalloprotease inhibitors. Biochem J 2004;378:383-389.

29) Xu Y, Lai LT, Gabrilove JL, Scheinberg DA. Antitumor activity of actinonin in vitro and in vivo. Clin Cancer Res 1998;4:171-176.

30) Lee MD, She Y, Soksi MJ, Borella CP, Gardner JR, Hayes PA, et al. Human mitochondrial peptide deformylase, a new anticancer target of actinonin-based antibiotics. J Clin Invest 2004;114:1107-1116.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28397/supplinfo.