# Validation of ZMYND8 as a New Treatment Target in Hepatocellular Carcinoma

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## Abstract

Background: *ZMYND8 (zinc finger MYND (Myeloid, Nervy and DEAF-1)-type containing 8)* has been known to play an important role in tumor regulation in various types of cancer. However, the results of *ZMYND8* expression and their clinical significance in hepatocellular carcinoma (HCC) have not yet been published. In the present study, we investigate the expression of ZMYND8 protein and mRNA in HCC and elucidate its prognostic significance.

Methods: ZMYND8 protein and mRNA expression in 283 and 234 HCCs were investigated using immunohistochemistry and quantitative real-time polymerase chain reactions. The relationships between ZMYND8 expression with clinicopathologic features and prognosis of HCC patients were evaluated. Furthermore, we performed the invasion, migration, apoptosis, soft agar formation assay and sphere formation assay in HCC cell lines, and evaluated tumorigenicity in a nude mouse model, after *ZMYND8* knockdown.

Results: Overexpression of ZMYND8 protein and mRNA was observed in 20.5% and 26.9% of HCC cases, respectively. High ZMYND8 expression showed significant correlations with microvascular invasion, high Edmondson grade, advanced American Joint Committee on Cancer (AJCC), and increased alpha-fetoprotein level. *ZMYND8* mRNA overexpression was an independent prognostic factor for predicting early recurrence as well as short recurrence-free survival (RFS). Downregulation of *ZMYND8* reduced migration and invasion of HCC cells, and promoted apoptosis of HCC cells in an in vitro model. In a xenograft nude mouse model, knockdown of *ZMYND8* significantly reduced tumor growth.

Conclusions: *ZMYND8* mRNA overexpression could be a prognostic marker of shorter RFS in HCC patients after curative resection. ZMYND8 might play an important role in the proliferation and progression of HCC and could be a promising candidate for targeted therapy.


## Introduction

Hepatocellular carcinoma (HCC) is the most common liver malignancy and remains the fourth leading cause of cancer-related death worldwide (Bray et al. 2018). Although surgical resection is the treatment of choice in HCC, prognosis after hepatectomy is unsatisfactory because of the high incidence of tumor recurrence (Sherman 2008). Thus, it is important to predict and evaluate the risk of recurrence, and prevention with appropriate therapy is crucial to improve patient outcome (Portolani et al. 2006). Recently, several targeted therapeutics other than sorafenib have been discovered for advanced HCC, including tyrosine kinase inhibitors such as regorafenib and levatinib (Bruix et al. 2017; Kudo et al. 2018). Additionally, programmed cell death protein-1 immune checkpoint inhibitors such as nivolumab and pembrolizumab have recently been approved by the FDA, providing additional options for physicians and patients. However, application of these targeted therapies remains limited (El-Khoueiry et al. 2017; Zhu et al. 2018). In the era of precision cancer therapy, identification of reliable molecular markers and target agents is urgently needed to improve the clinical outcomes of HCC patients (Qin and Tang 2004).

ZMYND8 (zinc finger MYND (Myeloid, Nervy and DEAF-1)-type containing 8), which was first introduced as an activated protein-kinase-C-binding protein (Fossey et al. 2000), has recently been shown to play an important role as a chromatin factor in cancer biology (Gong and Miller 2018). ZMYND8 has been reported as a tumor suppressor that works in conjunction with the H3K4me3- specific KDM5 family, such as KDM5C, in breast cancer and prostate cancer (Li et al. 2016; Shen et al. 2016). However, upregulation of ZMYND8 by hypoxia inducible factor (HIF) or overexpression of ZMYND8 via positive feedback of the estrogen receptor (ER) pathway was also correlated with poor prognosis of patients in breast cancer (Chen et al. 2018b; Yu et al. 2017). Immunohistochemical expression of ZMYND8 showed favorable prognostic effects in nasopharyngeal cancers, but adverse effects in colorectal cancers (Chen et al. 2020; Chen et al. 2019). As above, it is not conclusive for the role of *ZMYND8* in cancer progression, and there have been no previous reports evaluating the role of *ZMYND8* in HCC.

In this study, we investigated the expression of ZMYND8 in HCC according to protein and mRNA level and evaluated its association with clinicopathologic parameters and prognostic effects. Further, we performed in vitro and in vivo experiments to evaluate the role of ZMYND8 expression in the progression of HCC.

## Materials And Methods

## Patients and specimens

This study included 283 patients who had Child-Pugh A liver function and underwent curative hepatectomy as a first treatment for primary HCC between July 2000 and May 2006 at Samsung Medical Center, Seoul, Korea. The definition of curative resection was a complete resection of all tumor nodules with no involvement of microscopic resection margins and no visible tumor on computed tomography at 1 month after resection. This study was approved by the Institutional Review Board of Samsung Medical Center with waiver of informed consent (IRB No. 2016-11-112, approved date: 9. 12. 2016).

Patients' electronic medical records were reviewed to obtain the clinical parameters including age, sex, time of surgery, serum α-fetoprotein (AFP) and albumin level. Histopathologic characteristics of HCCs, as well as tumor differentiation, microvascular invasion, major branch of portal vein invasion, multicentric occurrence, intrahepatic metastasis and background non-tumor liver pathology, were determined by reviewing hematoxylin and eosin-stained slides. HCC differentiation was determined using the criteria of Edmondson-Steiner grading system(Edmondson and Steiner 1954). The mitotic index, which was evaluated in a previous study, was used. Briefly, the number of mitotic cells of the tumor was added up in 10 high-power fields (HPFs) of representative whole-section slides stained with hematoxylin and eosin. High mitotic index was defined when 5 or more mitosis events were observed in 10 HPFs (Ha et al. 2016). Multicentric occurrence and intrahepatic metastasis were determined following the criteria of the Liver Cancer Study Group of Japan (Liver Cancer Study Group of Japan 2003). The recurrence of HCC within the first two years is mostly results from intrahepatic metastasis, while late recurrence mainly due to multicentric occurrence (Shimada et al. 2001). Two-years cutoff were used to classify tumor recurrence as either early or late (Imamura et al. 2003). Patients were staged according to the American Joint Committee on Cancer (AJCC) staging manual 7th edition (Edge et al. 2010) and Barcelona Clinic Liver Cancer (BCLC) staging system (Llovet et al. 1999).

Computed tomography and serum AFP were performed every 3 months in all patients after surgery. Magnetic resonance imaging was performed when tumor recurrence was suspected. The follow-up period ranged from 14 to 151.4 months (median, 119.8 months). Recurrence-free survival (RFS) was determined by the difference between the dates of surgery and intra- or extra-hepatic recurrence. Disease-specific survival (DSS) was determined as the difference between the date of surgery and the date of death associated with hepatocellular carcinoma, which was defined as the following clinical outcomes (Hoshida et al. 2008): 1. tumor occupies more than 80% of the liver; 2. tumor in the proximal part of the second branch; 3. tumor occlusion resulting in jaundice; 4. hepatic metastasis; and 5. variceal bleeding due to tumor in the proximal part of the first branch.

# Immunohistochemistry

Immunohistochemistry for ZMYND8 was performed on tissue microarray blocks consisting of two 2 mm cores of HCC tissues from each case. After antigen retrieval using ER2 buffer (pH 8.0) at 100 °C for 20 minutes, The samples were incubated with primary antibody for ZMYND8 (1:200, HPA 020949, Sigma-Aldrich Inc., St.Louis, MO, USA) for 15 minutes, using a Bond-max automated immunostainer (Leica Biosystem, Melbourne, Australia). Chromogenic reactions for antigen-antibody complex were conducted for 10 min and visualized using Bond™ Polymer refine detection, DS9800 (Vision Biosystems, Melbourne, Australia). Normal brain tissue was used as a positive control. For evaluation of immunohistochemistry (IHC) of ZMYND8, the 12-point Remmele scoring system (score: 0–12) was used as described (Cho et al. 2019; Remmele and Stegner 1987). Briefly, the intensity of staining was scored on a 0 to 3 scale, and the proportion of positive tumor cells was scored from 0 to 4. The intensity and the proportion values were then multiplied, with a maximum of 12.

# mRNA expression of ZMYND8 and gene set enrichment assay

We used microarray gene expression profiling data from a previously reported study by our group in the same cohort as with this study (Lim et al. 2013). The data were deposited in Gene Expression Omnibus (GSE 36376, http://www.ncbi.nlm.nih.gov/geo/). This dataset consists of 240 HCC tissue and 193 adjacent non-tumor liver tissues. Among them, data from 234 HCC samples and 178 non-tumor liver samples were used for this study. The normalized values of *ZMYND8* (probe ID: ILMN_2386179) expression with base 2 logarithm were extracted. In order to perform a systematic and comprehensive analysis of the characteristics of *ZMYND8* related gene sets in HCC, differentially expressed genes (DEGs) between high and low expression groups of *ZMYND8* were extracted using the Benjamini & Hochberg correction, and significant DEGs were identified as those with False Discovery Rate (FDR) q value < 0.001. Testing for gene set enrichment of DEGs was performed using the MSigDB (http://www.broad.mit.edu/gsea/msigdb/index.jsp) (Mootha et al. 2003; Subramanian et al. 2005).

# The Cancer Genome Atlas (TCGA) data analysis

To evaluate the prognostic significance of *ZMYND8* mRNA expression, TCGA RNA-seq data were investigated using the Gene Expression Profiling Interactive Analysis (GEPIA) dataset (https://gepia.cancer-pku.cn/) (Tang et al. 2017). High expression group was defined as 4th quartile in Transcripts Per Million (TPM).

## Cell culture

Human liver cancer cell lines (Huh7, SNU449 and PLC/PRF5) were purchased from the Korean Cell Line Bank at December 2017, with authentication by short tandem repeat profiling. SNU449 cells were cultured in RPMI1640 (GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (GIBCO BRL), 100 mg/mL streptomycin, and 100 IU/mL penicillin at 37°C, 5% CO2 condition. Huh7 and PLC/PRF5 were cultured in high glucose DMEM (Life Technologies, Grand Island, NY, USA) in the presence of 10% heat-inactivated fetal bovine serum (Life Technologies), 50 μg/mL streptomycin, and 100 mg/mL penicillin G (Life Technologies) at 37°C with 5% CO2 under humidified atmosphere.

## Lentivirus transduction

Human embryonic kidney 293T cells were used to produce lentivirus. Vector packaging was obtained by transfection of 293T. The cancer cells were transduced with lentivirus expressing human pLKO.1 ShNS.puro control vector, pLKO.1 ShZMYND8-1.puro and pLKO.1 shZMYND8-2.puro vectors.

Short hairpin RNA(shRNA) sequences were encoded in a DNA fragment and inserted into the pLKO.1 vector to construct the following ZMYND8 knockdown plasmids : ShZMYND8-1.puro and ShZMYND8-2.puro. Publicly available MISSION shRNA library provided by Sigma-Aldrich was used to design specific shRNAs. The ShRNA sequence is as follows:

ShZMYND8-1.puro CCGGCCTGGGTTCCAATAAATAATTCTCGAGAATTATTTATTGGAACCCAGGTTTTTG

ShZMYND8-2.puro CCGGTTGCCAATACTTCCTCAGTTTCTCGAGAAACTGAGGAAGTATTGGCAATTTTTG

After viral transduction, selection with 1ug/ml puromycin was performed in transduced cells. The plasmids were verified via sequencing, and their efficacies were assessed by western blotting analysis and qRT-PCR.

## RNA extraction and quantitative real-time RT-PCR

Total RNA was isolated using RNease kits (#74104, Qiagen), and cDNA was synthetized with a high-capacity cDNA reverse transcriptation kit (#4368813, Applied Biosystems). Quantitative Real-time RT-PCR was conducted using the ABI 7900 HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA) as previously described (Yeo et al. 2018). The primer sequences of ZMYND8 were as follows : The forward primer was GGGTTTATCACGCTAAGTGTCTG, and the reverse primer was GGCTTTACTCTGGGTCTCGATG.

## Western blotting analysis

Cells were lysed by a RIPA lysis buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton x-100, 1% Na-Doc, 0.1% SDS and protease inhibitor cocktail (Roche), and BCA protein assay kit (Thermo) was used for quantification of cell extracts. Immunoblotting was performed with ZMYND8 (Origene, TA590385, 1:1000, Rockville, MD, USA) and α-tubulin (Santa Cruz, TU-02, 1:3000, Dallas, TX) and the blots were detected with ECL reagent (#1705061, Bio-Rad).

## Invasion/migration assays

In cell invasion assay, 6.5 mm Costar Transwell chambers with 8.0 μm pore size (Corning, NY, USA) were used. Appropriate Matrigel solution (1 mg/ml; Becton Dickinson, Franklin Lakes, NJ, USA) was added to the transwell filters and solidified in a 37°C incubator. Cells (1x105) were planted onto the Matrigel coating. The cells on the lower surface of the Matrigel-coated filter were stained with hematoxylin and eosin after methanol fixation. The migration assay was performed in the same manner as the invasion assay except that Matrigel coating was not used.

## Soft agar colony formation assay

Cells were seeded at a density of $2x10^4$ cells in 24-well culture plate, coated with 500 μl of solidified bottom agar mixture (DMEM, 10% FBS, 0.8% agar) and 500μl of top agar-medium mixture (DMEM, 10%FBS, 0.4% agar) and were incubated at 37°C. After 1 month, colonies were dyed with 0.05% Crystal Violet and the numbers were counted.

## Sphere forming assay

Cells were grown in modified DMEM/F-12 containing B27 (Invitrogen, Carlsbad, CA, USA), 10 ng/mL EGF, and bFGF (Invitrogen, Carlsbad, CA, USA) in low-attachment 6 well plate (Corning Inc., Corning, NY, USA) at a density of $1x10^4$ cells/well. The medium was replaced every 2 days and the number of spheres were counted after about 14 days of incubation.

# Apoptosis assay

Annexin V apoptosis detection kits (Bio-Vision, SF) was used to quantify cells apoptosis according to the manufacturer's instruction. Cells were stained with FITC-conjugated annexin-V in the dark at room temperature and were suspended in a 1X binding buffer. Cells were measured by a flow cytometer (BD Biosciences).

# Xenograft

Four-week old female Balb/c nude mice (Orient Bio, Korea) were inoculated subcutaneously into the right flank with Huh7 cells ($5x10^6$), which were transfected with lentiviral vector expressing non-specific shRNA (shNS) or ZMYND8 shRNA (shZMYND8) and suspended in 100 μl Matrigel/Serum-free medium (1:1 mixture). Tumor volumes were evaluated every week by a digital caliper and the volumes were calculated using the formula: length x $width^2$ x 1/2. All animal experiments approved by the Institutional Animal Care and Use Committee of Laboratory Animal Research Center at the Samsung Biomedical Research Institute

# Statistical analysis

We used the X-tile statistics software (Yale University, New Haven, CT, USA) (Camp et al. 2004) to determine the optimal cut-off value of ZMYND8 protein and mRNA expression with the most significant difference in RFS. The relationships between ZMYND8 expression and clinicopathologic parameters were analyzed using chi-square test or Fisher's exact test. The Mann-Whitney U test was used for comparing differences of ZMYND8 mRNA expression between tumor and normal tissues, and mitotic index according to ZMYND8 expression status. The correlation between IHC and mRNA expression of ZMYND8 were evaluated using the spearman analysis. The Kaplan-Meier method was performed to construct survival curves. Differences in survival were evaluated using the log-rank method or Breslow test. The Cox regression analysis was performed to assess the factors which were independently associated with survival. P-values less than 0.05 (two-sided) were considered statistically significant. The SPSS software was used for statistical analysis (SPSS Inc., Chicago, IL, USA).

# Results

### Patient characteristics

The clinical characteristics of 283 HCC patients in the present study are summarized in Table 1. The mean age was 52.3 years (range, 17 to 76 years), 235 patients were male and 48 female. Two hundred and fifteen patients (75.8%) were infected with hepatitis B virus and 26 (9.2%) with hepatitis C virus. No viral marker was detected in 38 patients (13.4%). About 83% of patients had AJCC T stage 1 or 2 disease. The mean tumor size was 4.8 cm, and 95 (33.6%) tumors were greater than 5.0cm in size. Microvascular invasion, major portal invasion, intrahepatic metastasis, and multicentric occurrence were observed in 54.1%, 4.0%, 22.6%, and 6.0% of patients, respectively. Tumor recurrence developed in 188 (66.4%) patients, 138 (48.8%) in early recurrence and 50 (17.7%) in late recurrence. Approximately 50% of patients had background liver cirrhosis.

Table 1
The association between ZMYND8 expression and clinicopathologic parameters.

| | | ZMYND8 expression | | | | *ZMYND8* mRNA expression | | |
|---|---|---|---|---|---|---|---|---|
| | No. | Low | High | | No. | Low | High | |
| | n = 283 | n = 225 (%) | n = 58 (%) | p value | n = 234 | n = 171 (%) | n = 63 (%) | p value |
| Age, year | | | | | | | | |
| ≤ 55 | 164 | 126 (56.0) | 38 (65.5) | 0.190 | 136 | 100 (58.5) | 36 (57.1) | 0.854 |
| > 55 | 119 | 99 (44.0) | 20 (34.5) | | 98 | 71 (41.5) | 27 (42.9) | |
| Gender | | | | | | | | |
| Female | 48 | 38 (16.9) | 10 (17.2) | 0.949 | 38 | 31 (18.1) | 7 (11.1) | 0.197 |
| Male | 235 | 187 (83.1) | 48 (82.8) | | 196 | 140 (81.9) | 56 (88.9) | |
| Tumor size, cm | | | | | | | | |
| ≤ 5.0 | 188 | 155 (68.9) | 33 (56.9) | 0.085 | 155 | 124 (72.5) | 31 (49.2) | 0.001 |
| > 5.0 | 95 | 70 (31.1) | 25 (43.1) | | 79 | 47 (27.5) | 32 (50.8) | |
| Edmondson grade | | | | | | | | |
| I | 32 | 28 (12.4) | 4 (6.9) | < 0.001 | 24 | 21 (12.3) | 3 (4.8) | 0.018 |
| II | 227 | 187 (83.1) | 40 (69.0) | | 190 | 140 (81.9) | 50 (79.4) | |
| III | 24 | 10 (4.4) | 14 (24.1) | | 20 | 10 (5.8) | 10 (15.9) | |
| Microvascular invasion | | | | | | | | |
| (-) | 130 | 118 (52.4) | 12 (20.7) | < 0.001 | 105 | 89 (52.0) | 16 (25.4) | < 0.001 |
| (+) | 153 | 107 (47.6) | 46 (79.3) | | 129 | 82 (48.0) | 47 (74.6) | |
| Major portal vein invasion | | | | | | | | |
| (-) | 272 | 218 (96.9) | 54 (93.1) | 0.245 | 225 | 166 (97.1) | 59 (93.7) | 0.256[b)] |
| (+) | 11 | 7 (3.1) | 4 (6.9) | | 9 | 5 (2.9) | 4 (6.3) | |
| Intrahepatic metastasis | | | | | | | | |
| (-) | 219 | 182 (80.9) | 37 (63.8) | 0.006 | 181 | 136 (79.5) | 45 (71.4) | 0.189 |
| (+) | 64 | 43 (19.1) | 21 (36.2) | | 53 | 35 (20.6) | 18 (28.6) | |
| Multicentric occurrence | | | | | | | | |
| (-) | 266 | 209 (92.9) | 57 (98.3) | 0.211 | 222 | 161 (94.2) | 61 (96.8) | 0.522[b)] |
| (+) | 17 | 16 (7.1) | 1 (1.7) | | 12 | 10 (5.8) | 2 (3.2) | |
| Mitotic index | | | | | | | | |
| Low (≤ 4/10 HPF) | 155 | 148 (65.8) | 7 (12.1) | < 0.001 | 128 | 105 (61.4) | 23 (36.5) | 0.001 |
| High (≥ 5/10 HPF) | 128 | 77 (34.2) | 51 (87.9) | | 106 | 66 (38.6) | 40 (63.5) | |
| Tumor necrosis | | | | | | | | |
| (-) | 215 | 188 (83.6) | 27 (46.6) | < 0.001 | 176 | 141 (82.5) | 35 (55.6) | < 0.001 |
| (+) | 68 | 37 (16.4) | 31 (53.4) | | 58 | 30 (17.5) | 28 (44.4) | |
| AJCC T-stage[b)] | | | | | | | | |

| | | ZMYND8 expression | | | *ZMYND8* mRNA expression | | | |
|---|---|---|---|---|---|---|---|---|
| 1 | 122 | 110 (48.9) | 12 (20.7) | < 0.001 | 100 | 85 (49.7) | 15 (23.8) | 0.002[b)] |
| 2 | 113 | 83 (36.9) | 30 (51.7) | | 96 | 60 (35.1) | 36 (57.1) | |
| 3 | 42 | 28 (12.4) | 14 (24.1) | | 33 | 22 (12.9) | 11 (17.5) | |
| 4 | 6 | 4 (1.8) | 2 (3.4) | | 5 | 4 (2.3) | 1 (1.6) | |
| BCLC stage | | | | | | | | |
| 0-A | 163 | 134 (59.6) | 29 (50.0) | 0.347 | 135 | 110 (64.3) | 25 (39.7) | 0.003 |
| B | 107 | 82 (36.4) | 25 (43.1) | | 89 | 55 (32.2) | 34 (54.0) | |
| C | 13 | 9 (4.0) | 4 (6.9) | | 10 | 6 (3.5) | 4 (6.3) | |
| Albumin level, g/dL | | | | | | | | |
| > 3.5 | 28 | 206 (91.6) | 49 (84.5) | 0.108 | 22 | 157 (91.8) | 55 (87.3) | 0.294 |
| ≤ 3.5 | 255 | 19 (8.4) | 9 (15.5) | | 212 | 14 (8.2) | 8 (12.7) | |
| AFP level, ng/mL[a)] | | | | | | | | |
| ≤ 200 | 173 | 148 (68.5) | 25 (43.9) | 0.001 | 145 | 115 (69.7) | 30 (48.4) | 0.003 |
| > 200 | 100 | 68 (31.5) | 32 (56.1) | | 82 | 50 (30.3) | 32 (51.6) | |
| Etiology | | | | | | | | |
| Non-viral | 38 | 29 (12.9) | 9 (15.5) | 0.526 | 32 | 23 (13.5) | 9 (14.3) | 0.985[b)] |
| HBV | 215 | 173 (76.9) | 42 (72.4) | | 179 | 131 (76.6) | 48 (76.2) | |
| HCV | 26 | 20 (8.9) | 6 (10.3) | | 20 | 15 (8.8) | 5 (7.9) | |
| HBV & HCV | 4 | 3 (1.3) | 1 (1.7) | | 3 | 2 (1.2) | 1 (1.6) | |
| Liver cirrhosis | | | | | | | | |
| (-) | 140 | 115 (51.1) | 25 (43.1) | 0.277 | 120 | 87 (50.9) | 33 (52.4) | 0.838 |
| (+) | 143 | 110 (48.9) | 33 (56.9) | | 114 | 84 (49.1) | 30 (47.6) | |
| Early recurrence | | | | | | | | |
| (≤ 2 years) | | | | | | | | |
| (-)[c)] | 145 | 120 (53.3) | 25 (43.1) | 0.165 | 124 | 99 (58.2) | 25 (39.1) | 0.013 |
| (+) | 138 | 105 (46.7) | 33 (56.9) | | 110 | 72 (42.1) | 38 (60.3) | |
| Late recurrence | | | | | | | | |
| (> 2 years) | | | | | | | | |
| (-)[c)] | 95 | 78 (65.0) | 17 (68.0) | 0.774 | 83 | 66 (66.7) | 17 (68.0) | 0.899 |
| (+) | 50 | 42 (35.0) | 8 (32.0) | | 41 | 33 (33.3) | 8 (32.0) | |

AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer;

AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus.

[a)] AFP evaluation was not applicable in 10 cases., [b)] by Fisher's exact test, otherwise by chi-square test, [c)] No early or late recurrence

**ZMYND8 protein expression in HCC**

In HCC, immunoreactivity for ZMYND8 was observed in the nucleus of tumor cells (Fig. 1). No immunoreactivity was detected in background non-tumor hepatocytes. The mean IHC expression Remmele score of ZMYND8 was 1.86 (median, 0, range, 0–12). ZMYND8 expression was regarded as high when the IHC score was greater than 4.0, which was determined as the best cutoff value associated with recurrence free survival (RFS) via the X-tile package. ZMYND8 protein overexpression was observed in 58 of the 283 HCC patients (20.5%). The associations between ZMYND8 protein expression and clinicopathologic characteristics are summarized in Table 1. High ZMYND8 expression was significantly associated with higher Edmondson grade ($p < 0.001$), high mitotic index ($p < 0.001$), tumor necrosis ($p < 0.001$), microvascular invasion ($p < 0.001$), intrahepatic metastasis ($p = 0.006$), advanced AJCC T stage ($p < 0.001$), and increased serum AFP level ($p = 0.001$). The mean mitotic index was significantly higher in the high ZMYND8 expression group than the low expression group (mean ± standard deviation, 17.93 ± 14.42 vs. 5.14 ± 7.93, $p < 0.001$).

### *ZMYND8* mRNA expression in HCC

The quantification of *ZMYND*8 mRNA expression was available in 234 HCC samples. The mean mRNA expression of *ZMYND8* was 7.00 (median, 6.97, range 6.27–8.22). Expression of *ZMYND8* was higher in HCC tissue than in background non-tumor tissue (mean ± standard deviation, 7.00 ± 0.32 vs 6.68 ± 0.16, $p < 0.001$, Fig. 2A). The spearman's correlation analysis revealed a statistically significant positive correlation between ZMYND8 protein and *ZMYND8* mRNA expression level ($r = 0.354$, $p < 0.001$, Fig. 2B). The best cutoff value of *ZMYND8* mRNA level with the highest level of statistical significance related to RFS was 7.12 via the X-tile package. High *ZMYND*8 mRNA expression was observed in 63 of the 234 HCC patients (26.9%). Table 1 summarizes the clinicopathologic parameters with *ZMYND8* mRNA level. High *ZMYND8* mRNA expression showed significant association with larger tumor size ($p = 0.001$), higher Edmondson grade ($p = 0.018$), high mitotic index ($p = 0.001$), tumor necrosis ($p < 0.001$), microvascular invasion ($p < 0.001$), advanced AJCC T stage ($p = 0.002$), advanced BCLC stage ($p = 0.003$), increased AFP level ($p = 0.003$), and early recurrence ($p = 0.013$). The mean mitotic index was significantly higher in the high *ZMYND8* expression group than the low expression group (mean ± standard deviation, 12.65 ± 14.33 vs. 6.09 ± 8.79, $p < 0.001$).

### Association between ZMYND8 expression and prognosis of HCC patients

The high ZMYND8 protein expression group showed a stronger trend toward shorter RFS ($p = 0.053$) and a significantly shorter disease specific survival (DSS) ($p = 0.02$) by the log rank test, as compared to the low ZMYND8 expression group according to a survival analysis with a 79.5 month mean follow-up period (Fig. 3A and 3B). By applying the Breslow test, which attributes greater weight to earlier events, patients with high ZMYND8 protein expression showed significantly shorter RFS ($p = 0.002$). Patients with high *ZMYND8* mRNA expression showed a shorter RFS ($p = 0.003$) and DSS ($p = 0.037$) than those with low expression (Fig. 3C and 3D).

On univariable analysis, larger tumor size, higher Edmondson grade, tumor necrosis, microvascular invasion, major portal vein invasion, intrahepatic metastasis, higher AJCC T-stage, higher BCLC stage, lower albumin level, and high serum AFP level showed unfavorable influences on both RFS and DSS. Viral etiology unfavorably influenced RFS. A high mitotic index showed unfavorable effects on DSS. High ZMYND8 protein expression showed unfavorable effects on DSS ($p = 0.021$, hazard ratio 1.695) and high *ZMYND8* mRNA expression showed unfavorable effects on both RFS ($p = 0.003$, hazard ratio 1.697) and DSS ($p = 0.039$, hazard ratio 1.640) (Table 2).

Table 2
Univariable analysis for recurrence free survival and disease-specific survival

| | Recurrence free survival | | Disease specific survival | |
|---|---|---|---|---|
| | HR (95% CI) | p value | HR (95% CI) | p value |
| Age, year | | | | |
| > 55 vs ≤ 55 | 0.968 (0.723–1.297) | 0.829 | 0.936 (0.625–1.400) | 0.747 |
| Gender | | | | |
| Male vs Female | 0.979 (0.675–1.419) | 0.910 | 0.910 (0.696–1.189) | 0.488 |
| Tumor size, cm | | | | |
| > 5.0 vs ≤ 5.0 | 1.757 (1.309–2.358) | < 0.001 | 2.930 (1.969–4.360) | < 0.001 |
| Edmondson grade | | | | |
| III vs I, II | 2.242 (1.421–3.539) | 0.001 | 2.670 (1.514–4.710) | 0.001 |
| Microvascular invasion | | | | |
| (+) vs (-) | 2.210 (1.644 0 2.971) | < 0.001 | 3.225 (2.057–5.056) | < 0.001 |
| Major portal vein invasion | | | | |
| (+) vs (-) | 3.420 (1.802–6.493) | < 0.001 | 5.024 (2.518–10.024) | < 0.001 |
| Intrahepatic metastasis | | | | |
| (+) vs (-) | 4.980 (3.593–6.903) | < 0.001 | 5.838 (3.890–8.760) | < 0.001 |
| Multicentric occurrence | | | | |
| (+) vs (-) | 1.183 (0.625–2.241) | 0.605 | 0.669 (0.246–1.821) | 0.432 |
| Mitotic index | | | | |
| High vs Low | 1.293 (0.971–1.722) | 0.079 | 2.126 (1.422–3.177) | < 0.001 |
| Tumor necrosis | | | | |
| (+) vs (-) | 2.610 (1.899–3.585) | < 0.001 | 4.345 (2.909–6.492) | < 0.001 |
| AJCC T-stage | | | | |
| 2,3,4 vs 1 | 2.256 (1.672–3.044) | < 0.001 | 3.265 (2.058–5.181) | < 0.001 |
| BCLC stage | | | | |
| B,C vs 0, A | 2.141 (1.605–2.855) | < 0.001 | 3.970 (2.606–6.046) | < 0.001 |
| Albumin level, g/dL | | | | |
| ≤ 3.5 vs > 3.5 | 1.859 (1.190–2.905) | 0.006 | 2.367 (1.362–4.115) | 0.002 |
| AFP level, ng/mL[a)] | | | | |
| > 200 vs ≤ 200 | 1.721 (1.283–2.310) | < 0.001 | 1.772 (1.181–2.658) | 0.006 |
| Etiology | | | | |
| Viral vs Non-viral | 2.032 (1.233–3.348) | 0.005 | 1.526 (0.793–2.935) | 0.206 |
| Liver cirrhosis | | | | |
| (+) vs (-) | 1.314 (0.986–1.752) | 0.062 | 1.031 (0.694–1.531) | 0.882 |

HR, Hazard Ratio; CI, Confidence Interval; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; IHC, Immunohistochemistry

| | Recurrence free survival | | Disease specific survival | |
|---|---|---|---|---|
| ZMYND8 expression (IHC) | | | | |
| High vs Low | 1.406 (0.994–1.988) | 0.054 | 1.695 (1.082–2.654) | 0.021 |
| ZMYND8 expression (mRNA) | | | | |
| High vs Low | 1.697 (1.198–2.405) | 0.003 | 1.640 (1.026–2.620) | 0.039 |

HR, Hazard Ratio; CI, Confidence Interval; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; IHC, Immunohistochemistry

We classified ZMYND8 expression as mRNA and protein and performed multivariable analysis two times with each variable. Multivariable analysis with ZMYND8 protein expression showed that intrahepatic metastasis and tumor necrosis were independent predictors of both shorter RFS and shorter DSS, and low AFP level was an independent predictor for shorter DSS. ZMYND8 protein overexpression was not an independent prognostic factor for RFS or DSS (Table 3). On multivariable analysis with *ZMYND8* mRNA expression, intrahepatic metastasis and tumor necrosis were found to be an independent predictor of both shorter RFS and DSS. *ZMYND8* mRNA expression was an independent predictor for RFS ($p = 0.020$, hazard ratio 1.572), not for DSS ($p = 0.864$) (Table 4).

Table 3
Multivariable analysis for recurrence free survival and disease-specific survival including ZMYND8 protein expression

| | Recurrence free survival | | Disease specific survival | |
|---|---|---|---|---|
| | HR (95% CI) | p value | HR (95% CI) | p value |
| Tumor size, cm | | | | |
| > 5.0 vs ≤ 5.0 | 0.934 (0.657–1.330) | 0.707 | 1.504 (0.0921–2.458) | 0.103 |
| Edmondson grade | | | | |
| III vs I, II | 1.359 (0.813–2.271) | 0.241 | 0.979 (0.508–1.887) | 0.949 |
| Microvascular invasion | | | | |
| (+) vs (-) | 1.056 (0.687–1.624) | 0.804 | 0.715 (0.369–1.385) | 0.320 |
| Major portal vein invasion | | | | |
| (+) vs (-) | 0.796 (0.382–1.657) | 0.542 | 1.180 (0.545–2.555) | 0.674 |
| Intrahepatic metastasis | | | | |
| (+) vs (-) | 4.492 (2.896–6.967) | < 0.001 | 4.962 (2.840–8.670) | < 0.001 |
| Mitotic index | | | | |
| High vs Low | 0.727 (0.507–1.042) | 0.083 | 1.449 (0.879–2.389) | 0.145 |
| Tumor necrosis | | | | |
| (+) vs (-) | 2.302 (1.502–3.527) | < 0.001 | 3.609 (2.122–6.138) | < 0.001 |
| Albumin level, g/dL | | | | |
| ≤ 3.5 vs > 3.5 | 1.363 (0.817–2.277) | 0.236 | 1.372 (0.720–2.616) | 0.336 |
| AFP level, ng/mL[a)] | | | | |
| > 200 vs ≤ 200 | 1.393 (1.008–1.925) | 0.044 | 1.148 (0.718–1.836) | 0.565 |
| Etiology | | | | |
| Viral vs Non-viral | 1.515 (0.894–2.565) | 0.122 | 0.962 (0.471–1.962) | 0.915 |
| ZMYND8 expression (IHC) | | | | |
| High vs Low | 0.898 (0.585–1.377) | 0.621 | 0.735 (0.419–1.288) | 0.282 |
| HR, Hazard Ratio; CI, Confidence Interval; IHC, Immunohistochemistry | | | | |

Table 4
Multivariable analysis for recurrence free survival and disease-specific survival including ZMYND8 mRNA expression

| | Recurrence free survival | | Disease specific survival | |
|---|---|---|---|---|
| | HR (95% CI) | p value | HR (95% CI) | p value |
| Tumor size, cm | | | | |
| > 5.0 vs ≤ 5.0 | 0.793 (0.539–1.167) | 0.240 | 1.470 (0.854–2.530) | 0.165 |
| Edmondson grade | | | | |
| III vs I, II | 1.469 (0.821–2.630) | 0.195 | 0.808 (0.387–1.689) | 0.571 |
| Microvascular invasion | | | | |
| (+) vs (-) | 1.032 (0.634–1.681) | 0.898 | 0.656 (0.308–1.398) | 0.275 |
| Major portal vein invasion | | | | |
| (+) vs (-) | 0.679 (0.299–1.542) | 0.355 | 1.583 (0.682–3.676) | 0.285 |
| Intrahepatic metastasis | | | | |
| (+) vs (-) | 5.854 (3.572–9.594) | < 0.001 | 4.758 (2.576–8.788) | < 0.001 |
| Mitotic index | | | | |
| High vs Low | 0.673 (0.452–1.002) | 0.051 | 1.470 (0.844–2.561) | 0.174 |
| Tumor necrosis | | | | |
| (+) vs (-) | 2.064 (1.326–3.215) | 0.001 | 3.473 (1.957–6.164) | < 0.001 |
| Albumin level, g/dL | | | | |
| ≤ 3.5 vs > 3.5 | 1.207 (0.691–2.109) | 0.509 | 1.208 (0.599–2.437) | 0.597 |
| AFP level, ng/mL[a)] | | | | |
| > 200 vs ≤ 200 | 1.257 (0.871–1.813) | 0.222 | 1.029 (0.615–1.724) | 0.913 |
| Etiology | | | | |
| Viral vs Non-viral | 1.699 (0.931–3.103) | 0.084 | 1.020 (0.458–2.272) | 0.961 |
| *ZMYND8* expression (mRNA) | | | | |
| High vs Low | 1.572 (1.074–2.300) | 0.020 | 1.047 (0.622–1.761) | 0.864 |
| HR, Hazard Ratio; CI, Confidence Interval | | | | |

### Prognostic effect of ZMYND8 mRNA expression in TCGA data

We found the unfavorable prognostic effect of *ZMYND8* expression in an independent HCC cohort consisting of TCGA RNAseq samples. Patients with high expression of *ZMYND8* mRNA showed shorter RFS ($p = 0.008$) and OS ($p = 0.003$) than those with low expression ($p < 0.001$) (Fig. 3E and 3F).

### Gene set enrichment analysis in ZMYND8-high HCC

List of DEGs between high and low expression group of *ZMYND8* was summarized in supplementary Table 1, and list of significantly enriched gene sets according to *ZMYND8* expression status was summarized in Table 5. Gene signatures related to cell proliferation were significantly enriched, including gene sets of E2F targets, G2M checkpoint, MYC targets, mitotic spindle and apoptosis. In addition, the expression of *ZMYND8* was strongly associated with the enrichment of gene sets involved in hypoxia, oxidative phosphorylation and angiogenesis, as well as apical junction and epithelial-mesenchymal transition. Gene set of DNA repair was also significantly enriched.

Table 5
List of enriched gene sets according to ZMYND8 expression status in hepatocellular carcinomas.

| Gene Set Name | # Genes in Gene Set (K) | Description | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|---|
| HALLMARK_E2F_TARGETS | 200 | Genes encoding cell cycle related targets of E2F transcription factors. | 58 | 0.29 | 1.44E-43 | 7.19E-42 |
| HALLMARK_G2M_CHECKPOINT | 200 | Genes involved in the G2/M checkpoint, as in progression through the cell division cycle. | 47 | 0.235 | 5.62E-31 | 1.41E-29 |
| HALLMARK_COAGULATION | 138 | Genes encoding components of blood coagulation system; also up-regulated in platelets. | 37 | 0.2681 | 6.92E-27 | 1.15E-25 |
| HALLMARK_XENOBIOTIC_METABOLISM | 200 | Genes encoding proteins involved in processing of drugs and other xenobiotics. | 43 | 0.215 | 9.30E-27 | 1.16E-25 |
| HALLMARK_MYC_TARGETS_V1 | 200 | A subgroup of genes regulated by MYC - version 1 (v1). | 41 | 0.205 | 9.99E-25 | 9.99E-24 |
| HALLMARK_MYC_TARGETS_V2 | 58 | A subgroup of genes regulated by MYC - version 2 (v2). | 18 | 0.3103 | 4.93E-15 | 4.11E-14 |
| HALLMARK_FATTY_ACID_METABOLISM | 158 | Genes encoding proteins involved in metabolism of fatty acids. | 25 | 0.1582 | 6.37E-13 | 4.55E-12 |
| HALLMARK_MTORC1_SIGNALING | 200 | Genes up-regulated through activation of mTORC1 complex. | 26 | 0.13 | 2.22E-11 | 1.39E-10 |
| HALLMARK_BILE_ACID_METABOLISM | 112 | Genes involve in metabolism of bile acids and salts. | 19 | 0.1696 | 1.07E-10 | 5.94E-10 |
| HALLMARK_MITOTIC_SPINDLE | 199 | Genes important for mitotic spindle assembly. | 23 | 0.1156 | 3.23E-09 | 1.61E-08 |
| HALLMARK_PEROXISOME | 104 | Genes encoding components of peroxisome. | 16 | 0.1538 | 1.35E-08 | 6.16E-08 |

| Gene Set Name | # Genes in Gene Set (K) | Description | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|---|
| HALLMARK_COMPLEMENT | 200 | Genes encoding components of the complement system, which is part of the innate immune system. | 21 | 0.105 | 8.26E-08 | 3.44E-07 |
| HALLMARK_ADIPOGENESIS | 200 | Genes up-regulated during adipocyte differentiation (adipogenesis). | 20 | 0.1 | 3.69E-07 | 1.42E-06 |
| HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION | 200 | Genes defining epithelial-mesenchymal transition, as in wound healing, fibrosis and metastasis. | 18 | 0.09 | 6.24E-06 | 1.84E-05 |
| HALLMARK_GLYCOLYSIS | 200 | Genes encoding proteins involved in glycolysis and gluconeogenesis. | 18 | 0.09 | 6.24E-06 | 1.84E-05 |
| HALLMARK_KRAS_SIGNALING_UP | 200 | Genes up-regulated by KRAS activation. | 18 | 0.09 | 6.24E-06 | 1.84E-05 |
| HALLMARK_OXIDATIVE_PHOSPHORYLATION | 200 | Genes encoding proteins involved in oxidative phosphorylation. | 18 | 0.09 | 6.24E-06 | 1.84E-05 |
| HALLMARK_ANDROGEN_RESPONSE | 100 | Genes defining response to androgens. | 12 | 0.12 | 1.25E-05 | 3.46E-05 |
| HALLMARK_APOPTOSIS | 161 | Genes mediating programmed cell death (apoptosis) by activation of caspases. | 15 | 0.0932 | 2.41E-05 | 6.34E-05 |
| HALLMARK_UV_RESPONSE_UP | 158 | Genes up-regulated in response to ultraviolet (UV) radiation. | 14 | 0.0886 | 7.71E-05 | 1.90E-04 |
| HALLMARK_APICAL_JUNCTION | 200 | Genes encoding components of apical junction complex. | 16 | 0.08 | 8.36E-05 | 1.90E-04 |
| HALLMARK_ESTROGEN_RESPONSE_LATE | 200 | Genes defining late response to estrogen. | 16 | 0.08 | 8.36E-05 | 1.90E-04 |
| HALLMARK_HYPOXIA | 200 | Genes up-regulated in response to low oxygen levels (hypoxia). | 15 | 0.075 | 2.79E-04 | 6.06E-04 |

| Gene Set Name | # Genes in Gene Set (K) | Description | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|---|
| HALLMARK_ANGIOGENESIS | 36 | Genes up-regulated during formation of blood vessels (angiogenesis). | 6 | 0.1667 | 3.17E-04 | 6.61E-04 |
| HALLMARK_DNA_REPAIR | 150 | Genes involved in DNA repair. | 12 | 0.08 | 6.22E-04 | 1.24E-03 |
| HALLMARK_UNFOLDED_PROTEIN_RESPONSE | 113 | Genes up-regulated during unfolded protein response, a cellular stress response related to the endoplasmic reticulum. | 10 | 0.0885 | 7.97E-04 | 1.53E-03 |
| HALLMARK_HEDGEHOG_SIGNALING | 36 | Genes up-regulated by activation of hedgehog signaling. | 5 | 0.1389 | 2.35E-03 | 4.31E-03 |
| HALLMARK_IL2_STAT5_SIGNALING | 199 | Genes up-regulated by STAT5 in response to IL2 stimulation. | 13 | 0.0653 | 2.41E-03 | 4.31E-03 |
| HALLMARK_MYOGENESIS | 200 + B3B31:C31 | Genes involved in development of skeletal muscle (myogenesis). | 13 | 0.065 | 2.52E-03 | 4.35E-03 |
| Table is sorted in the order of q value, and only the list of q values less than 0.005 was displayed. | | | | | | |

### ZMYND8 expression affects invasiveness and tumorigenesis of HCCs

We performed in vitro and in vivo assays to evaluate the clinical observations of *ZMYND8* mentioned above. After knockdown of *ZMYND8* with treatment of Sh-ZMYND8 in the Huh7, SNU449, PLC/PRF5 cell lines (Fig. 4A-B), sphere formation, soft agar colony formation migration and invasion of cancer cells were dramatically decreased (Fig. 4C-D). The rate of early and late apoptosis increased after Sh-ZMYND8 treatment (Fig. 4E). Downregulation of *ZMYND8* inhibited the growth of HCC xenografts in Balb/c nude mice (Fig. 4F).

## Discussion

In the present study, we first demonstrated that *ZMYND8* mRNA expression was up-regulated in HCC tissues compared to background non-tumor liver tissue, and high ZMYND8 expression by both mRNA and protein was associated with aggressive clinicopathologic parameters such as high Edmondson grade, microvascular invasion, intrahepatic metastasis, advanced AJCC T stage, and high AFP level, as well as frequent tumor necrosis and high mitotic index. Patients with high expression of ZMYND8 showed shorted RFS and DSS in a large cohort of HCC patients with long-term follow-up. Especially, *ZMYND8* mRNA expression was an independent predictor for shorter RFS. The prognostic effect of *ZMYND8* expression was also confirmed in an independent cohort of TCGA dataset. These clinical observations were confirmed by in vitro and in vivo experiments. Knockdown of *ZMYND8* induced significant decrease in migration, invasion, and tumorigenicity on in vitro model, and suppressed the tumor growth of HCC xenografts in vivo model. These results suggest that ZMYND8 plays a critical role in HCC progression.

ZMYND8 was initially identified as a receptor for activated C-kinase (RACK) protein that binds to an activated protein-kinase-C beta I (PKCβ1) (Fossey et al. 2000), and has been shown to play as a central chromatin factor in DNA damage response and cancer (Gong and

Miller 2018). There have been several studies regarding ZMYND8 as a tumor suppressor. ZMYND8 collaborates with H3K4me3- specific KDM5 family, such as KDM5C or KDM5D, and regulates transcription (Li et al. 2016; Shen et al. 2016). Shen et al. showed that loss of ZMYND8 or KDM5C induced enhanced cell migration and invasion, as well as tumor growth in a breast ZR-75-30 mouse xenograft model, by increasing the level of H3K4me3, subsequent enhancer RNA levels, and expression of neighboring genes such as oncogenic family of S100A proteins (Shen et al. 2016). Li et al. demonstrated that ZMYND8 and KDM5D are necessary for repressing metastasis-linked gene, and loss of these genes lead to increased cell migration and invasiveness of a prostate DU145 mouse xenograft model (Li et al. 2016). Basu et al. demonstrated *ZMYND8* as a target gene of all-*trans* retinoic acid (ATRA), involved in ATRA-mediated inhibition of cancer proliferation, and regulated epithelial to mesenchymal transition to maintain the epithelial phenotypes by selective enrichment on *CLDN1/CDH1* (Basu et al. 2017a; Basu et al. 2017b). Low expression of ZMYND8 protein by IHC have been reported as an independent prognostic factor for worse survival in nasopharyngeal carcinoma (Chen et al. 2019).

However, there have been studies reporting *ZMYND8* as an oncogenes. Kuroyanagi et al. found *ZMYND8* as one of the most significantly changed genes in the tumor angiogenesis-positive group than in the negative group, using a prostate DU145-xenografted zebrafish model, and showed that ZMYND8 promotes tumor angiogenesis and cancer cell proliferation induced by hypoxia (Kuroyanagi et al. 2014). Yu et al. showed that overexpression of ZMYND8 involved in a positive feedback circuit of the estrogen receptor pathway was more prevalent in luminal B breast cancers and associated with poor survival (Yu et al. 2017). High expression of ZMYND8 protein by IHC has been reported as an independent prognostic factor for shorter survival in colorectal cancers (Chen et al. 2020). In a recent comprehensive study by Chen et al., up-regulation of *ZMYND8* was correlated with poor survival in breast cancer. Knockdown of *ZMYND8* decreased tumor growth and lung metastasis in a hypoxia induced factor (HIF) dependent manner, by epigenetic mechanism. Specifically, Acetylation of ZMYND8 at lysine 1007 and 1034 by HIF coactivator p300 induced RNA polymerase II phosphorylation and subsequent transcriptional elongation of the HIF target genes (Chen et al. 2018b). These results highlighting oncogenic function of ZMYND8 are consistent with those of our study performed in HCC. It seems that ZMYND8 has the dual role as a tumor suppressor or promoter, and that role depends on the specific types of genes it interacts with and the types of tumors.

HCC is one of the most hypoxic tumors, and activation of hypoxia signaling pathway, including HIF and its transcriptional coactivators, mediates increased tumor cell proliferation, invasion, and metastasis. (Chen and Lou 2017). Under a hypoxia state, ZMYND8 physically interacts with HIF-1a and HIF-2a, and activates HIF transcriptional activity in breast cancer (Chen et al. 2018a; Chen et al. 2018b). Additionally, ZMYND8 is acetylated by the histone acetyltransferase p300 and promotes the HIF target gene expression (Chen et al. 2018a). Interestingly, high expression of both HIF and p300 are associated with aggressive features and poor prognosis of HCC (Li et al. 2011; Xiang et al. 2012). In the present study, high expression of ZMYND8 was significantly associated with a high mitotic index and tumor necrosis, reflecting the hypoxic state of HCC. Also, *ZMYND8* expression was significantly associated with gene set pathways associated with cell cycle, mitosis, hypoxia, and angiogenesis, supporting the results of previous studies. Recent studies have been targeting the molecules of the hypoxia pathway in HCC. Ma et al. demonstrated that 2-Methoxyestradiol, a HIF inhibitor, reduced the expression of both HIF-1a and HIF-2a as well as their downstream molecules, and consequently suppressed tumor proliferation and angiogenesis of HCC cells (Ma et al. 2014). Moreover, Inajaki et al. reported that the proliferation and invasion of HCC cells significantly reduced after p300 inhibitor treatment (Inagaki et al. 2016). Thus, we could infer that ZMYND8, which is one of the main transcription regulator complexes combined with HIF and p300 can also become potential target for novel HCC therapy.

The limitation of our study is that we could not elucidate the specific molecular mechanism of *ZMYND8* and its functional relationship with other genes. Despite the limitation, we provide valuable information about the close association between *ZMYND8* expression and clinicopathologic features in a large cohort of HCC for the first time, with relevant experimental data. Also, we could indirectly suggest that *ZMYND8* may has a crucial role on aggressive tumor behavior in HCC by interacting with genes involved in pathway such as cell cycle, mitosis, hypoxia, angiogenesis and epithelial-mesenchymal transition through gene set enrichment assay. Interestingly, DNA mismatch repair pathway was also significantly enriched. Gong et al. reported *ZMYND8* as a key chromatin modulator of the DNA damage response and DNA repair (Gong et al. 2015; Gong et al. 2017), and Wang et al. showed that upregulation of *ZMYND8* promoted tumor growth by reducing DNA damage and thereby evading cytotoxic T lymphocyte surveillance in breast cancer (Wang et al. 2020). The association between *ZMYND8* expression and genes involved in DNA mismatch repair pathway is a possible regulator of immune response and provide the insight for immunotherapy. Further study is necessary to unveil the specific mechanisms of *ZMYND8* involved in the progression of HCC.

## Conclusions

We demonstrated that high expression of ZMYND8 is associated with advanced stage and poor patient survival in HCC for the first time, and knockdown of *ZMYND8* decreased invasion, migration, and proliferation in an in vitro model, as well as tumorigenesis in an in vivo xenograft model. ZMYND8 may be used as a prognostic biomarker and is a potential candidate for therapeutic targets in HCC.

## Abbreviations

HCC: Hepatocellular carcinoma; ZMYND8: zinc finger MYND (Myeloid, Nervy and DEAF-1)-type containing 8; HIF: Hypoxia inducible factor; ER: Estrogen receptor; AFP: α-fetoprotein; HPFs: High power fields; AJCC: American Joint Committee on Cancer; BCLC: Barcelona Clinic Liver Cancer; RFS: Recurrence-free survival; DSS: Disease-specific survival; IHC: Immunohistochemistry; DEG: Differentially expressed genes; FDR: False Discovery Rate; TCGA: The Cancer Genome Atlas; GEPIA: Gene Expression Profiling Interactive Analysis; shRNA: Short hairpin RNA; RACK: Receptor for activated C-kinase; PKCβ1: Protein-kinase-C beta I; ATRA: all-*trans* retinoic acid

## Declarations

### Ethics approval and consent to participate

The Institutional Review Board of Samsung Medical Center approved this study and waived informed consent for this study.

### Consent for publication

All authors agreed to publish the article.

### Availability of data and materials

All datasets used and analyzed during this study are available from the corresponding author on reasonable request.

### Competing interest

All contributing authors have no financial support relevant to this article and no competing interests to declare.


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### Author's contributions

SC performed data analysis, generated tables and figures, and drafted the manuscript. KWL performed in vitro and in vivo assays, analyzed the data and drafted the manuscript. HHK analyzed data. SP, SYY, JWJ, MSC supported data acquisition. SHK and CKP contributed to knowledge. SYH conceptualized and designed the study, collected and analyzed the data, drafted and revised the manuscript. All authors have read and agreed to the published version of the manuscript.


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## Figures

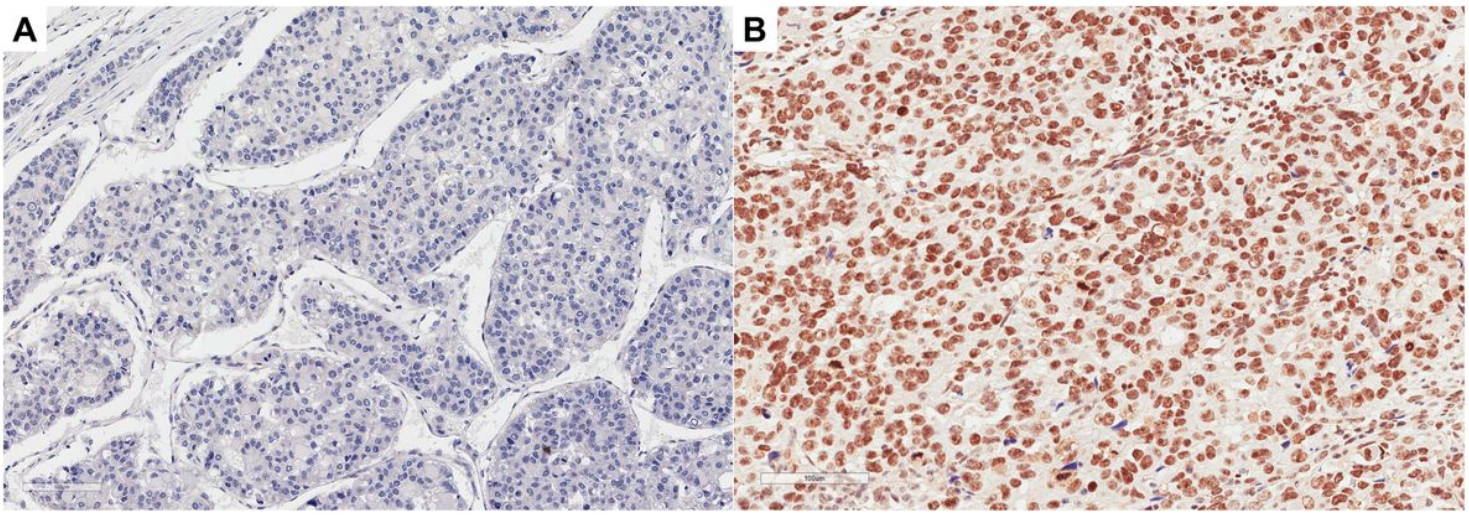
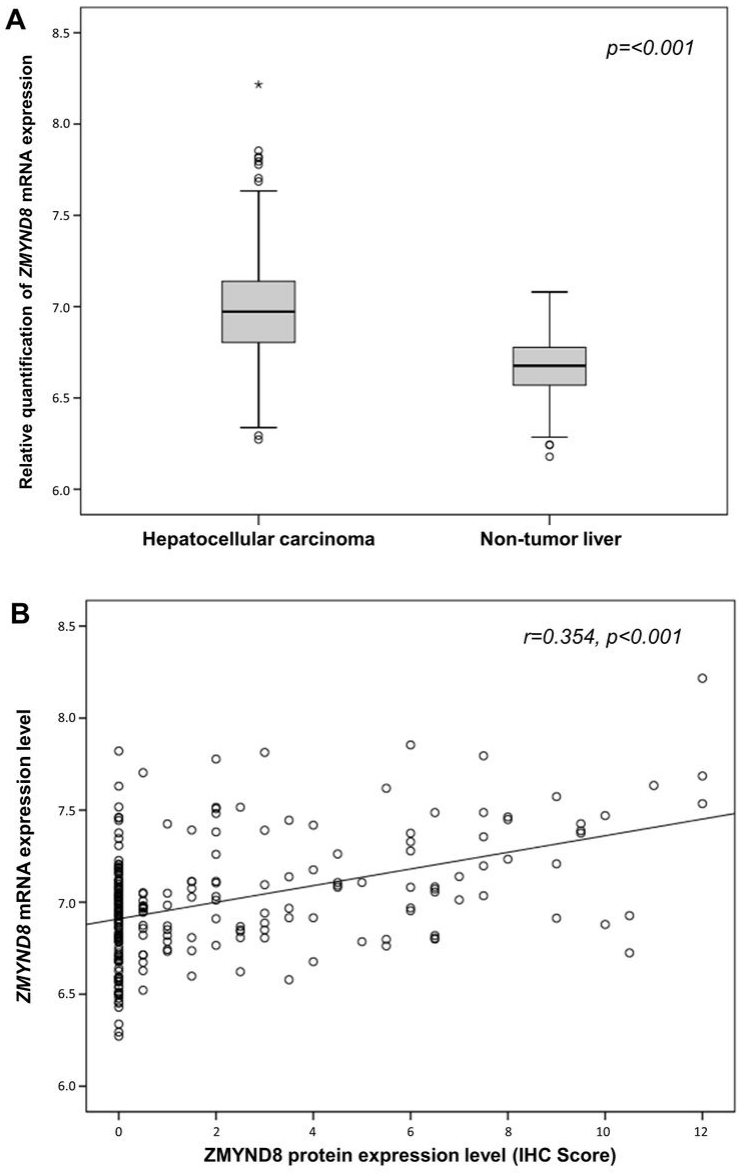


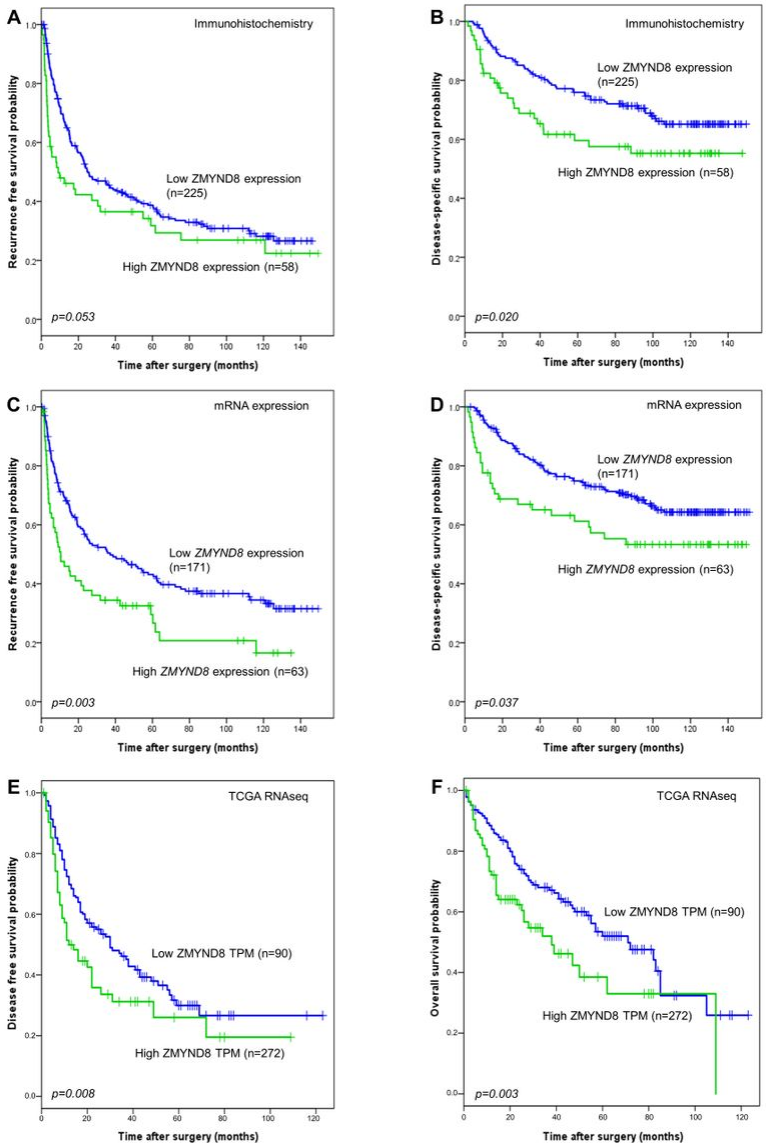

**Figure 1**

Representation of ZMYND8 expression in hepatocellular carcinoma by immunohistochemistry. (A) Tumor cells show negative staining for ZMYND8. (B) Tumor cells show nuclear staining for ZMYND8.




## Figure 2

The relative quantification of ZMYND8 mRNA expression in hepatocellular carcinoma and background non-tumor liver (A), and the relationship between ZMYND8 protein and ZMYND8 mRNA expression (B). IHC, immunohistochemistry




## Figure 3

Kaplan-Meier survival curves according to ZMYND8 expression. (A-B) Survival curves according to ZMYND8 protein expression by immunohistochemistry (C-D) Survival curves according to ZMYND8 mRNA expression by microarray gene expression data (E-F) Survival curves according to ZMYND8 mRNA expression by TCGA RNAseq data. TCGA, The Cancer Genome Atlas

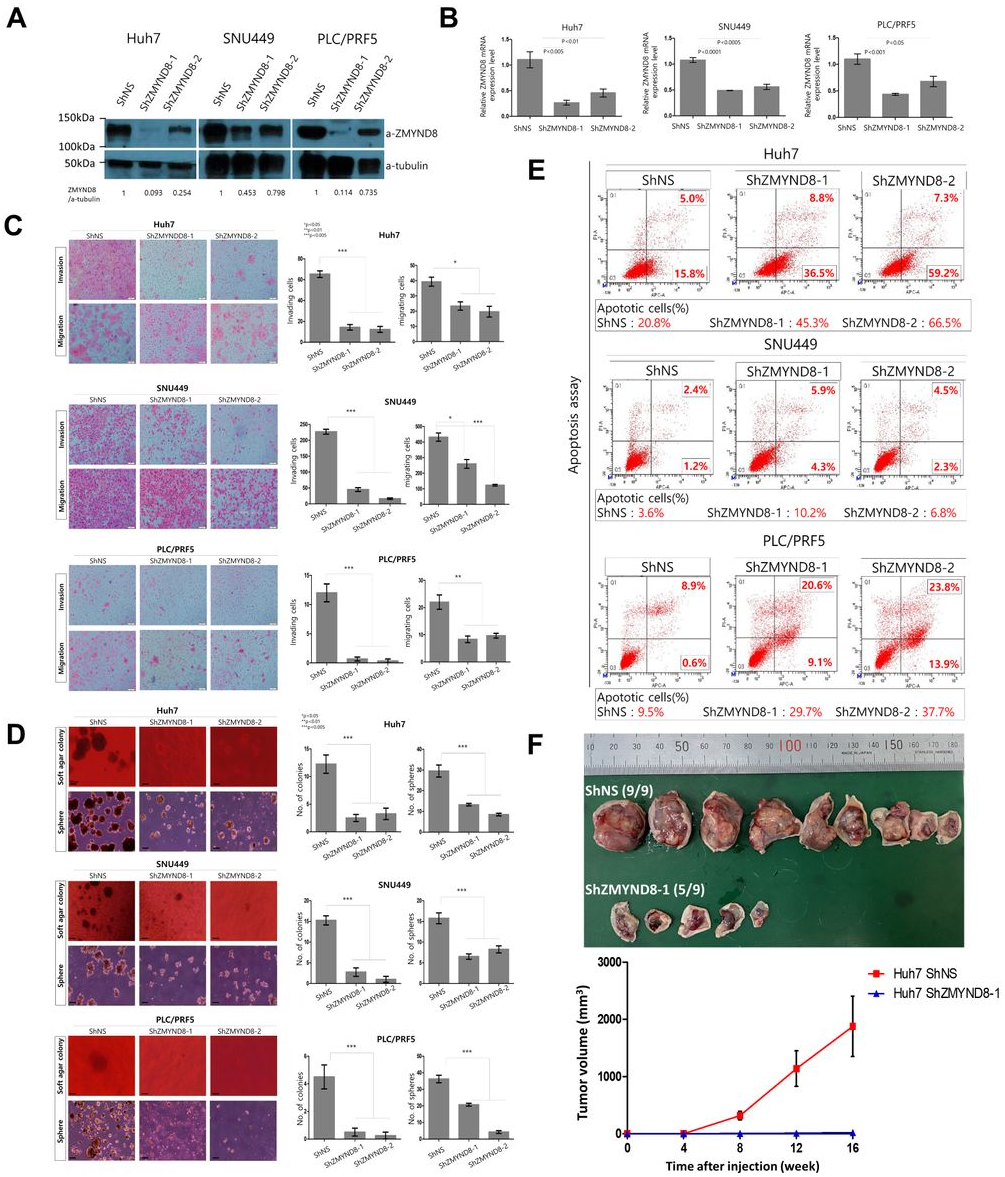



**Figure 4**

Downregulation of ZMYND8 expression by ShRNA was confirmed at protein (A) and mRNA level (B). Knockdown of ZMYND8 resulted in significantly reduced migration, invasion (C), sphere formation, colony formation (D), and increased apoptosis of HCC cells (E). Inhibition of tumor growth was observed in a ZMYND8 knockdown HCC Xenograft in nude mice (F).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.pdf