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# MFAP2 Promotes the Proliferation of Cancer Cells and Is Associated With a Poor Prognosis in Hepatocellular Carcinoma

**Xiang Zhu, MD[1,2], Ye Cheng, MD[2], Fan Wu, MD[2], Haoyao Sun, MD[3], Wubin Zheng, MD[2], Wei Jiang, MD[2], Junfeng Shi, PhD[4], Shijie Ma, MD[5], and Hongyong Cao, MD[2]**

## Abstract

**Backgrounds:** Microfibril-associated protein 2 (MFAP2) is an extracellular matrix protein that regulates the function of microfibrils by interacting with fibrillin. MFAP2 has been reported to play an important role in metabolic diseases and has been shown to be significantly overexpressed in head and neck squamous cell carcinoma and Hepatocellular carcinoma (HCC). However, the molecular function and prognostic value of MFAP2 have never been reported in HCC or other tumors. **Methods:** In the present study, expression characteristics of MFAP2 in HCC, its influence on the development of HCC, as well as its function and potential mechanism in HCC were verified by Quantitative reverse transcription-polymerase chain reaction, bioinformatics data mining and in vitro cell experiments. **Results:** MFAP2 was prominently high-expressed in HCC and associated with cancer stages. HCC patients with higher MFAP2 expression displayed lower overall survival (OS) and disease-specific survival(DSS), while there was no significant difference in recurrence-free survival (RFS). In vitro experiments showed that downregulation of MFAP2 inhibited proliferation, migration level of HCC cells. Transcription factors, DNA methyltransferases, immune factors may interact with MFAP2 mRNA to promote tumor progression in HCC. **Conclusion:** These findings suggest that MFAP2 may play a key role in the development of HCC. Therefore, MFAP2 may be a valuable prognostic marker and an effective anticancer target in HCC.

## Keywords

MFAP2, hepatocellular carcinoma, prognosis, proliferation

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## Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies in the world, has become the fifth most common malignancy and the second leading cause of tumor-related death.[1] Though the clinical treatment of HCC has been significantly improved in recent years thanks to advances in surgical techniques, radiotherapy, interventional therapy and endocrine therapy, the long-term survival rate of HCC patients remains low.[2] Therefore, it is crucial to find reliable biomarkers and therapeutic targets for curing HCC clinically.

Microfibrillar-associated protein 2 (MFAP2), also known as microfibril-associated glycoprotein 1 (MAGP1), is a component of extracellular elastic microfibrils, which interacts with fibrillin to influence the function of microfibers.[3,4] MFAP2 is the most widely distributed protein of MAGPs and a component protein of microfibrils in most vertebrates. A prominent

[1] Department of General Surgery, The East District of Suzhou Municipal Hospital, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou, Jiangsu, People's Republic of China
[2] Department of General Surgery, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, People's Republic of China
[3] Department of Radio-Oncology, Suzhou Municipal Hospital, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou, Jiangsu, People's Republic of China
[4] Department of Oncology, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, People's Republic of China
[5] Department of Gastroenterology, The Affiliated Huaian No.1 People's Hospital of Nanjing Medical University, Huaian, Nanjing, People's Republic of China

**Corresponding Authors:**
Hongyong Cao, Department of General Surgery, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, China.
Email: caohongy6167@163.com
Shijie Ma, Department of Gastroenterology, The Affiliated Huaian No.1 People's Hospital of Nanjing Medical University, Huaian, Jiangsu, China.
Email: msj_1234@163.com

feature of this protein is its ability to interact with TGF-β family growth factors, Notch and Notch ligands, and a variety of elastic fibrin.[5] It has been confirmed that mutations in MFAP2 were linked to hemostasis and thrombosis, thoracic aneurysms, metabolic disease, and osteopenia in humans.[3-5] Thus far, there have been few studies on the role of MFAP2 in tumors. Recent reports indicated that MFAP2 was highly expressed in human HCC and head and neck squamous cell carcinoma tissues.[6-8] As far as we know, the role of MFAP2 in HCC has still not been reported up to now. Therefore, we investigated the expression, possible molecular mechanisms, and clinical significance of MFAP2 in HCC in the present study.

## Materials and Methods

### Patients and Clinical Tissue Samples

A total of 47 HCC tissues and corresponding adjacent non-tumorous tissue samples were collected from HCC patients who adopting surgery treatment during January 2016 and December 2018 in Nanjing First Hospital. All patients are carriers of the HBV virus. The patients received neither radiotherapy nor chemotherapy before the operation. Written informed consent was obtained from each patient before they participated in this study. All experiments using animal and human samples were reviewed and approved by the Ethics Committee of Nanjing Medical University (No.2019669).

### Cell Culture and Transfection

HCC cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% Penicillin-Streptomycin(Gibco, USA) at 37°C in a humidified incubator containing 5% CO2. Non-targeting control siRNA (si-NC) and small interfering RNA against MFAP2 (si-MFAP2) were synthesized by Hongxin Biotechnology Company (Nanjing, China). Transfections were performed with the solution produced by applied biological materials company (Canada) and the Opti-MEM (Gibco, USA). Cells were collected 48 h after transfection for further study. The target sequence of si-MFAP2 was as follows: si-MFAP2-1: 5′-GCAGCAAGUCCAACAGGAATT-3′; si-MFAP2-2: 5′-GUGUGUACGUCAUUAACAATT-3′; si-MFAP2-3: 5′-CGGGACAAGUUCUCCAAAUTT-3′. si-MFAP2-2 was used for further experiments.

### RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using Trizol solution (Thermo, USA) according to the manufacturer's instructions. QRT-PCR were performed to detect the expressions of genes using the TaKaRa®qPCR SYBR Green Master Mix kit (DaLian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for internal control. The outcomes were evaluated via the 2^-ΔΔCt method. The primer pairs for MFAP2 were as follows: 5′-CGCCGTGTGTACGTCATTAAC-3′ (Forward) and 5′-CCATCACGCCACATTTGGA-3′ (Reverse).

### Cell Proliferation Experiments

Cell proliferation was measured by a Cell Counting kit-8 (CCK-8) assay. Firstly, YY-8103 and HuH-7 cells were transfected with either si-MFAP2 or si-NC and incubated at 37°C. Then the CCK-8 solution (Biosharp, China) was added into each well and incubated for 2 h. The absorbance was measured at 0, 24, 48 and 72 h time points at a wavelength of 450 nm. All experiments were conducted in triplicate at least 3 times.

### Transwell Assay

YY-8103 and HuH-7 cells were cultured overnight in serum-free RPMI-1640 medium and then suspended in serum-free RPMI-1640 medium. Cells ($4 \times 10^4$/well) were seeded into the upper chamber of transwell inserts (pore size, 8 μm; Costar; Corning Incorporated, Corning, NY, USA) in 24-well plates. The lower chambers were filled with 500 μL RPMI-1640 supplemented with 10% FBS as a chemoattractant. The cells were then treated with or without si-MFAP2 (5 ng/mL) and incubated for 24 h. After incubation for 24 h, the cells migrated into the lower chambers were fixed with 4% paraformaldehyde for 30 min, and stained with 1% crystal violet (Biosharp, China) for 10 min, and the remaining unmigrated cells were removed from the top layer with cotton swabs. The cells migrated into the bottom chamber were counted and photographed under the inverted microscope. Five mid-power fields were randomly selected to calculate the mean number of cells. All experiments were carried out in triplicates.

### MFAP2 Expression Level and Survival Analysis

To detect MFAP2 expression in different tumor tissues and corresponding para-cancer tissues, we used the TCGA portal (www.tcgaportal.org) and FIREBROWSE (http://firebrowse.org/). The Human Protein Atlas (https://www.proteinatlas.org/), which compiles many reports and forms of the tissues, cells and pathology atlas, as well as gene information in tissues and cells was utilized for obtaining MFAP2 mRNA expression in human tissues and location in cells. UALCAN (http://ualcan.path.uab.edu/) is a comprehensive and interactive web resource for analyzing cancer OMICS data. In this study, it was used for subgroup analysis of MFAP2 expression in HCC. TCGA portal (www.tcgaportal.org) was also used to examine relationship between MFAP2 expression and survival probability of HCC patients. We then used Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p=background) to compare correlations between MFAP2 expression and OS, DSS and RFS. The Kaplan-Meier survival plot was used to compare the 2 groups of patients, and hazard ratios with 95% confidence intervals and log rank p-values were calculated.

### Gene Correlations Analysis

GEPIA2 (http://gepia2.pku.cn/#index) is an open-access dataset that uses standard processing methods to analyze RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from TCGA and GTEx projects. GEPIA2 provides customizable features such as tumor/normal differential expression, similar gene testing, patient survival curves, profiling based on cancer types or pathological stages, analysis of correlation and dimension reduction. It was utilized through the whole study to assess the correlations between all significant genes.

### TFs Identification

The Cistrome Data Browser Toolkit (http://dbtoolkit.cistrome.org) allows users to detect transcription factors (TFs) that may regulate the genes of interest to determine binding factors, histone modifications, and chromatin accessibility within the maximum 2 Mb of the genome of interest. The samples with the most similarity among chip-seq, dnpase-seq, and atac-seq were determined based on the overlap of user-supplied sets of genome spacers.[9] In this study, we used the Cistrome DB Toolkit to predict which TFs were most likely to regulate MFAP2 expression in HCC.

### DNA Methylation Modification Analysis

MEXPRESS (https://mexpress.be/) is a data visualization tool for the visualization of TCGA expression, DNA methylation, and clinical data, as well as their relationships.[10] Here, we used MEXPRESS to study the methylation status of MFAP2 gene and the relationship between MFAP2 mRNA expression and different clinical characteristics of HCC patients.

### MFAP2 mRNA Mutation Analysis

The TCGA portal (www.tcgaportal.org) is an online portal that allows multiple tumors to be aligned in parallel, as well as detailed analysis of individual tumors, it was used for the analysis of MFAP2 mRNA mutations.

### Protein-Protein Interaction and Functional Enrichment Analysis

Metascape (http://metascape.org/) is a powerful annotation analysis tool for gene function, which can help users do an analysis of batch genes and proteins and realize the cognition of gene or protein function.[11] This web-based portal combines functional enrichment, interactive group analysis, gene annotation, and membership search by leveraging data from more than 40 separate knowledge bases. Here, heatmap and network of enrichment terms associated with MFAP2 were acquired from Metascape. STRING (https://string-db.org/cgi/input.pl) is a database of known and predicted protein-protein interactions, including direct (physical) and indirect (functional) associations; these interactions result from computational predictions, knowledge transfer between organisms, and interactions gathered from other (primary) databases.[12] We used STRING to generate a network of interactions between MFAP2 and other key proteins.

### Immune-Related Analysis

TISIDB (http://cis.hku.hk/TISIDB/index.php) is a central portal for displaying the interactions between tumor and immune system, which integrates a variety of heterogeneous data types.[13] Here, it was used to analyze the Spearman correlations between the expression of MFAP2, immunoregulatory factors, and tumor-infiltrating lymphocytes (TILs).

### Statistical Analysis

Data are showed as mean $\pm$ standard deviation (SD). All statistical analysis of cell line experiments were evaluated by GraphPad Prism 8 with the Student's t test or one-way analysis of variance (ANOVA). The correlations of gene expressions were evaluated using Spearman's correlation. P-values $<0.05$ were considered as statistically significant. Corresponding significance levels were presented in the figures.

## Results

### MFAP2 mRNA Is Over-Expressed in HCC Samples

Using qRT-PCR, the MFAP2 mRNA expression levels were defined for 47 paired primary cancerous and adjacent noncancerous tissues from HCC patients and the results showed the expression of MFAP2 mRNA in HCC tissues were significantly higher than that of adjacent noncancerous tissues (Figure 1A). Furthermore, we used the ROC curve to investigate the diagnostic value of MFAP2 mRNA in distinguishing HCC tissues from adjacent nontumorous tissues. When the expression level of MFAP2 mRNA was analyzed for this purpose, the area under the ROC curve (AUC) was 0.7730 (Figure 1B). As shown in Table 1, MFAP2 mRNA level was not associated with age, gender, differentiation in patients with HCC. However, up-expression of MFAP2 mRNA level was positively associated with TNM stage ($P = 0.028$) and tumor size ($P = 0.036$).

The expression of MFAP2 mRNA in normal and tumor tissues was firstly detected using TCGA portal and FIREBROWSE. The results showed that the expression level of MFAP2 in tumor tissues was much higher than that in the corresponding normal tissues (Figure 1C). Then we compared the expression of MFAP2 mRNA in HCC and normal tissues purposely, and caught the fact that MFAP2 mRNA expression in HCC tissues was significantly higher than that in normal tissues (Figure 1D). Next, a more detailed and specific analysis of MFAP2 mRNA expression in HCC was performed by using UALCAN. The results of subgroup analysis based on nodal metastasis status, individual cancer stages, and tumor grade indicated that the levels of MFAP2 mRNA in HCC patients

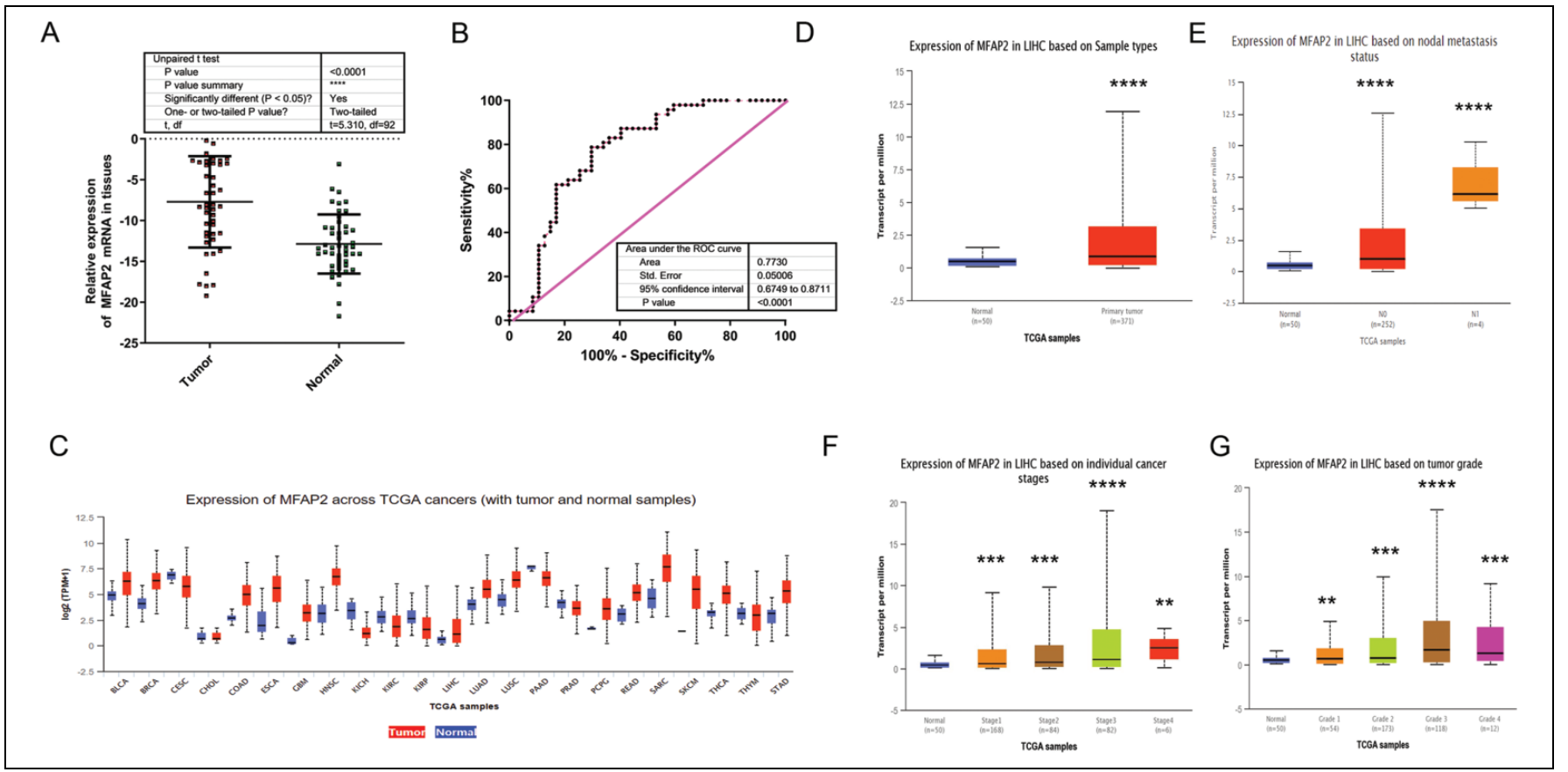


**Figure 1.** MFAP2 is over-expressed in HCC samples. A, MFAP2 mRNA expression in HCC tissues and normal tissues. B, The ROC curve has been used to evaluate MFAP2 potential diagnostic value. C, MFAP2 mRNA expression in human cancers. D, MFAP2 mRNA expression in HCC samples and normal samples. E-G, Differences in MFAP2 mRNA expression depending on nodal metastasis status, individual cancer stages, and tumor grade (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

**Table 1.** The Relationship Between MFAP2 Expression Levels in HCC Tissues and Clinicopathological Factors of Patients.

| Variable | Case | Low expression | High expression | P-value |
|---|---|---|---|---|
| Age (year) | | | | 0.252 |
| ≥ 60 | 32 | 5 | 27 | |
| < 60 | 15 | 5 | 10 | |
| Gender | | | | 0.414 |
| Female | 12 | 1 | 11 | |
| Male | 35 | 9 | 26 | |
| Diameter | | | | 0.036* |
| ≥ 5 (cm) | 24 | 2 | 22 | |
| < 5 (cm) | 23 | 8 | 15 | |
| Differentiation | | | | 0.377 |
| Low | 9 | 3 | 6 | |
| middle/ high | 38 | 7 | 31 | |
| TNM Stage | | | | 0.028* |
| IA-IIB | 21 | 8 | 13 | |
| IIIA-IV | 26 | 2 | 24 | |

*P < 0.05.

were significantly higher than that in matched group (Figure 1E-G).

## *MFAP2 Is Associated With a Poor Prognosis in HCC Patients*

In terms of the up-regulation of MFAP2 in 62 HCC patients, Kaplan–Meier survival analysis was performed to further evaluate the correlation between MFAP2 expression and prognosis of patients with HCC. The results revealed that patients with higher levels of MFAP2 expression had significantly shorter survival times than those with lower levels of MFAP2 expression (Figure 2A). As shown in Figure 2B-C, online prediction revealed that patients with higher MFAP2 expression had shorter Survival Probability, OS, and DSS than patients with lower MFAP2 expression. While RFS was not associated with the expression of MFAP2 in tumor patients (Figure 2D).The relationship between HCC patients with positive and negative HBV infections and OS was further analyzed, and it was found that HCC patients with positive HBV infections were associated with prognosis but the negatives HBV infection were not statistically significant (Figure 2E-F).

## *MFAP2 Knockdown Suppresses Proliferation and Migration of HCC Cells*

As shown in our results, we confirmed that MFAP2 mRNA expression was significantly increased in several HCC cell lines compared to the normal HL-7702 cell lines (Figure 3A). si-MFAP2-2 and si-NC were transfected into YY-8103 and HuH-7 cells accordingly, and successful knockdown was confirmed by qRT-PCR, MFAP2 expression was significantly reduced using the si-MFAP2-2 (Figure 3B-C). To investigate the role of MFAP2 on tumor physiological characteristics, CCK-8 assays were performed in YY-8103 and HuH-7 cell lines. CCK-8 assays showed that MFAP2 gene knockdown significantly inhibited the proliferation of both HCC cell lines compared to control group (Figure 3D-E). It indicated the inhibiting role of MFAP2 knockdown on HCC cells aggression. Moreover, the suppression of MFAP2 by si-MFAP2-2 exhibited a lower relative migration rate compared with control ones seen from transwell assay in the confluent monolayer of the cultured HCC cell lines (Figure 3F-G). These findings demonstrated that inhibition of MFAP2 can slow the progression of hepatocellular carcinoma in vitro including proliferation and migration.

## *The Expression of MFAP2 mRNA Is Obviously Related to RAD21 and DNMTs*

In order to probe which factors might regulate MFAP2 expression in a supposed molecular regulatory network, we identified transcription factors (TFs) that might regulate transcription of MFAP2 using the Cistrome Data Browser Toolkit. The top 20 regulatory TFs in all tumors are showed in the acquired plot (Figure 4A). Next, we focused on validating MFAP2 regulators in HCC related cell lines such as hepatocellular carcinoma, SMMC-7721, HuH-7, and HepG2. Finally, it is found that RAD21 had the greatest regulatory potential (RP) in those cells (Figure 4B). The following analyses testified that the expression of RAD21 mRNA was obviously correlated with MFAP2 in the GEPIA2 database (Figure 4C).

There is still no clear understanding of the potential role of DNA methylation modifications as prognostic markers in HCC, though more and more research indicated that aberrant DNA methylation plays an important role in the development of HCC.[14] In addition, we know that abnormal methylation modification (mainly selective hypermethylation) at the CpG island prevents TFs from binding to DNA, thereby inhibiting transcription.[14-16] Therefore, we used the MEXPRESS to detect DNA methylation modifications of the MFAP2 gene in HCC (Figure 4D). Meanwhile, we also found that MFAP2 was positively correlated with the expression levels of DNA methyltransferases (DNMTs) in HCC (Figure 4E). These findings revealed that transcription factors and DNA methylation modifications might participate in tumorigenesis of HCC by regulating MFAP2 expression.

## *TP53 Mutation Interacts With MFAP2 to Involve in Tumorigenesis of HCC*

TP53, also known as BCC7, or P53, which encodes a tumor suppressor protein that contains transcriptional activation, DNA binding, and oligomerization domains. Mutations in this gene have been linked to several types of human cancers, including hepatocellular carcinoma.[17] So we came up with the result that there was a clear correlation between MFAP2 and TP53, as the expression of MFAP2 decreased, the mutation frequency of TP53 also decreased, when investigating the relationship between MFAP2 expression and frequencies of mutations in the tumor-related genes in HCC (Figure 4F). Next, the outcome of gene correlation analysis using GEPIA2 showed

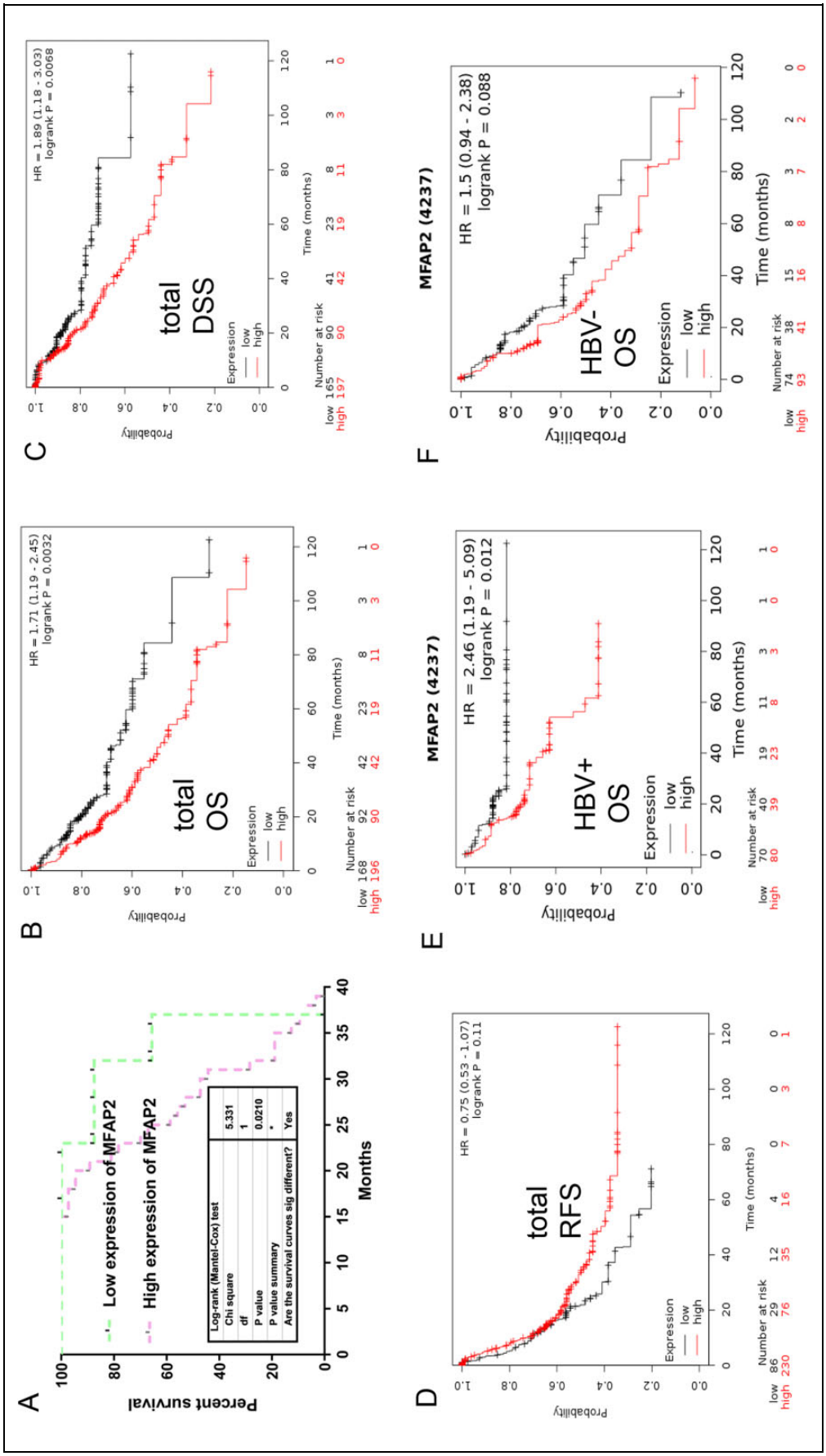


**Figure 2.** MFAP2 is associated with a poor prognosis in HCC patients. A, High MFAP2 expression was correlated with poor survival probability in HCC patients. B-C, High MFAP2 expression was correlated with poor OS and DSS in HCC patients. D, RFS was not associated with MFAP2 expression in HCC patients. E-F, The relationship between HCC patients with positive and negative viral infections and OS (OS, overall survival; DSS, disease-specific survival; RFS, recurrence-free survival).

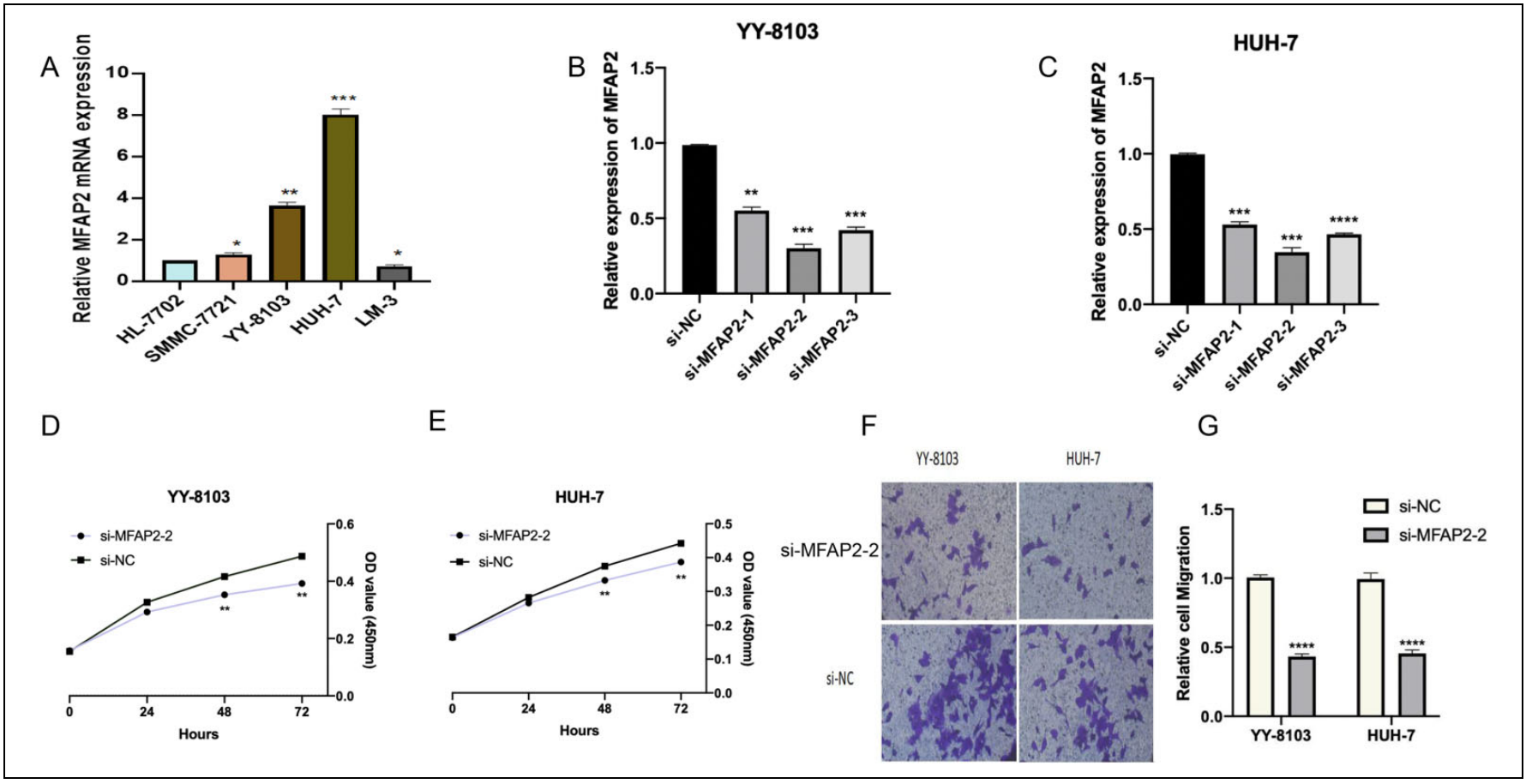


**Figure 3.** Knockdown of MFAP2 suppresses cell proliferation and migration. A, MFAP2 expression in HCC cells. B-C, Knockdown in YY-8103 and HuH-7 cells with si-MFAP2 or si-NC. D-E, CCK-8 assays showed that down-regulation of MFAP2 inhibited proliferation of YY-8103 and HuH-7 cells. F-G, Suppression of MFAP2 by si-MFAP2 exhibited a lower relative migration rate compared with control group in YY-8103 and HuH-7 cells (si-NC, siRNA negative control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

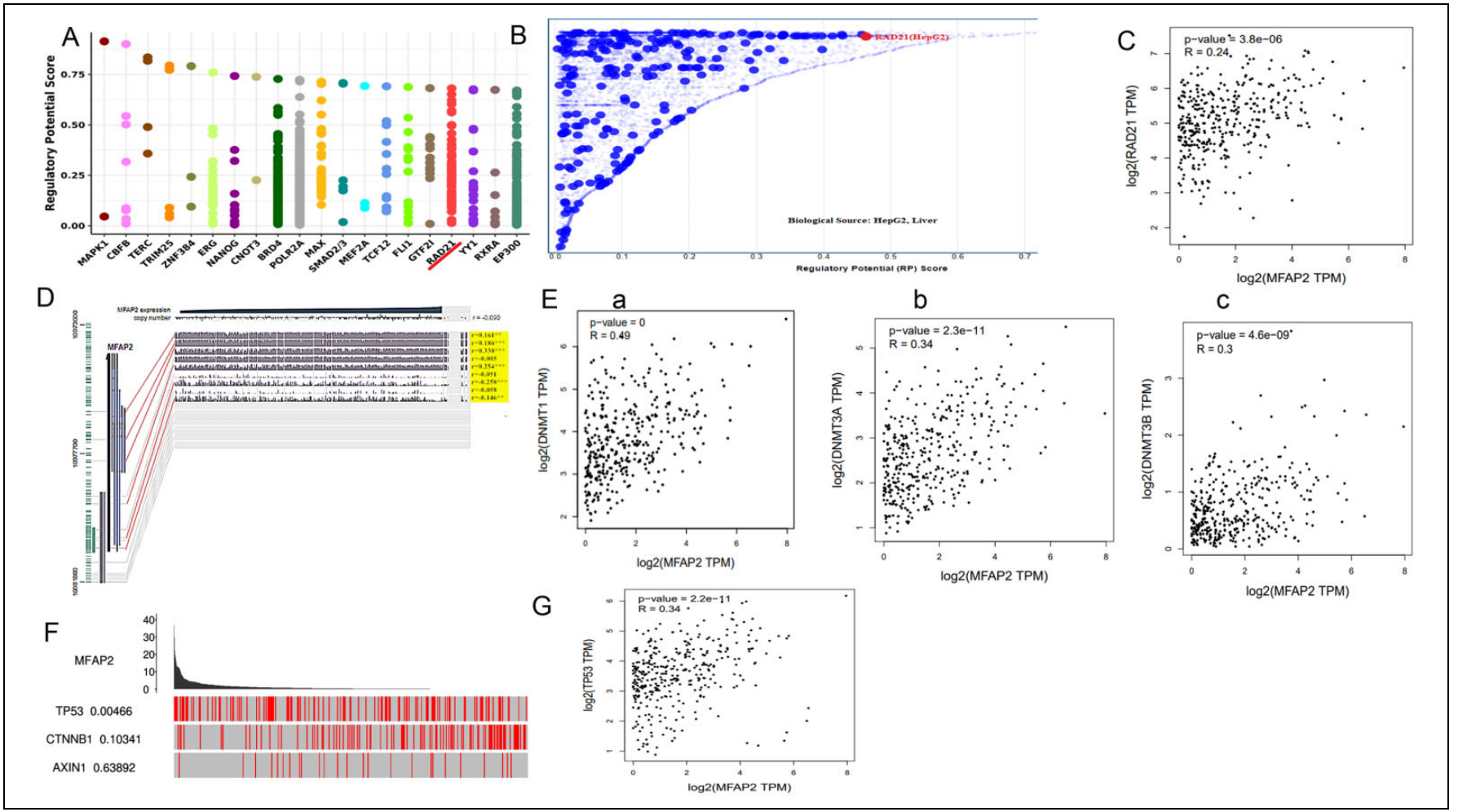


**Figure 4.** Relations between TFs, tumor-related genes and MFAP2 in HCC. A, Top 20 TFs that potentially regulate MFAP2. B, TFs with high regulatory potential in HCC cell lines (10 k distance to TSS). C, Correlation between RAD21 and MFAP2 mRNA expression. D, MFAP2 DNA methylation modification in HCC. E, Correlation between MFAP2 mRNA expression and DNMTs expression. F, Correlation between MFAP2 mRNA expression and mutation frequency of tumor-related genes. G, Correlation between MFAP2 and TP53 mRNA expression (TSS, transcription start site).

that TP53 expression was obviously correlated with expression of MFAP2 (Figure 4G).

### Protein Interactions and Enrichment Analysis of MFAP2

The STRING database currently was used to obtain the interaction network between MFAP2 and other important proteins, and the results indicated that some proteins such as ELN, EFEMP2, FBLN1, and so on, which could bind directly to MFAP2 (Figure 5A). Enrichment analysis of co-expression genes conducted using Metascape displayed that MFAP2 is mainly involved in the processes of ATP formation, T cell activation, and hematopoietic cell lineage (Figure 5B-C). And the results also clearly showed that it was associated with the development of tumors of the digestive system (Figure 5D).

### MFAP2 Expression Is Associated With Immune Factors

The interaction between tumor and immune system plays a vital role in the occurrence, development and treatment of tumor. Cytolytic immune cells (T/NK cells) are initially used to recognize and kill cancer cells, while tumor tissues can shape the surrounding microenvironment and promote immune escape. Particularly, tumors can disrupt infiltration and function of T/NK cells, and antigen presentation by soluble and cellular surface mediators (such as PD-L1) and immunosuppressive cells, such as regulatory T cells (Tregs) and marrow derived suppressive cells (MDSCs). Hence, elucidating the interaction between tumor and immune cells will help predict the immunotherapy response and develop new immunotherapy targets. We investigated the relationship between the expression of MFAP2 and immune factors in HCC and got the fact that some immunostimulators, immunoinhibitors, and TILs for which expression was significantly correlated with MFAP2 expression by filtering: $p < 0.05$ and $|\pm \text{rho}| \geq 0.1$ were showed in Figure 6A-C ($p \geq 0.05$ or $|\pm \text{rho}| < 0.1$ was marked as black). Some Immunosuppressive membrane proteins whose expressions, such as TIGIT, CTLA4, have significant correlation with MFAP2 expression (Figure 6D-E). We also found a significant and meaningful relationship between the expression of MFAP2 and Regulatory T cells (Tregs) (Figure 6F). In particular, there was a significantly negative correlation between the expression of immunostimulant IL6R and MFAP2 (Figure 6G).

## Discussion

Many factors have been reported to be associated with the development of HCC, including chronic hepatitis virus infection, genetic mutations, cell damage, alcoholic liver disease and aflatoxin poisoning.[18] Despite extensive experimental studies, the molecular mechanisms of HCC have not been fully understood to date. Most undetected early HCC patients are not suitable for curative treatment, which may be one of the reasons for poor prognosis.[19] Therefore, potential highly effective diagnostic and therapeutic markers are urgently needed.[18,19] Bioinformatics analysis plays a critical role in cancer research, and it promotes the understanding of carcinogenesis by combining genome-level data with systematic bioinformatics approaches. In the present study, we investigated the expression of MFAP2 in hepatocellular carcinoma (HCC) and many other human cancer types and found that MFAP2 was up-regulated in a variety of tumors. We specially found that MFAP2 was obviously over-expressed in HCC when compared with normal tissues and was correlated with survival probability, OS, and DSS. In addition, to evaluate whether MFAP2-silencing contributes to inhibition of tumor cells, we conducted in vitro studies which demonstrated that MFAP2 knockdown inhibited the proliferation and migration of hepatocellular carcinoma cells in a time-dependent manner that may be a potential biomarker for HCC.

Multiple reports have shown similar skeletal phenotypes in both fibrillin-1 mutant mice and MAGP-1 (MFAP2) deficient mice. The overlapping functions of MFAP2 and fibrillin-1 regulate osteoclast number and bone resorption.[20] Researches suggest that MFAP2 is not necessary for elastic fiber assembly in mice, but is important for other processes of tissue homeostasis or differentiation.[3-5] Therefore, it may be that these fibrillin mutations alter the ability of fibrillin to bind to MFAP2, leading to the emergence and aggravation of the disease. Previous studies have demonstrated that versican, a large extracellular matrix proteoglycan, was found associated to the microfibrils through its interaction with fibrillin-1, which played an important role in tumor invasion and metastasis.[21] The results from Segade et al. suggested that MFAP2 was involved in ECM function and modulating the expression of genes that function in cell adhesion, migration and control of ECM deposition in human osteosarcoma.[5] Our present results indicate that inhibition of MFAP2 can delay the proliferation and migration of HCC in vitro. Taken together, MFAP2 might promote the development and progression of HCC through its interaction with the mutant fibrillin-1. Future studies should investigate whether MFAP2 is associated with fibrillin-1 in HCC cells.

RAD21 (double-strand-break repair protein Rad21 homolog, also known as SCC1) is a component of the cohesive proteins that are essential for chromosome separation and DNA repair.[22] Recently, RAD21 has been found to be associated with the development and prognosis of malignant tumors. As a target gene of multiple genes or a regulatory gene of other genes, it is also widely involved in human physiological and pathological processes.[23,17] Ahn et al. found that RAD21 was involved in transcriptional regulation of migration/invasion related genes induced by mutant p53-r248 in human ovarian cancer cells.[24] In our present study, we found that the expression of RAD21 mRNA was obviously correlated with MFAP2 based on the GEPIA2 database and the mutation frequency of P53 was significantly correlated with the expression of MFAP2. The expression level of RAD21 in HCC tissues was higher than that in adjacent non-tumor tissues, and its expression level was associated with OS in HCC patients. Previous study identified RAD21 as a new binding partner of mutant P53 in ovarian cancer, the specific molecular mechanism by which

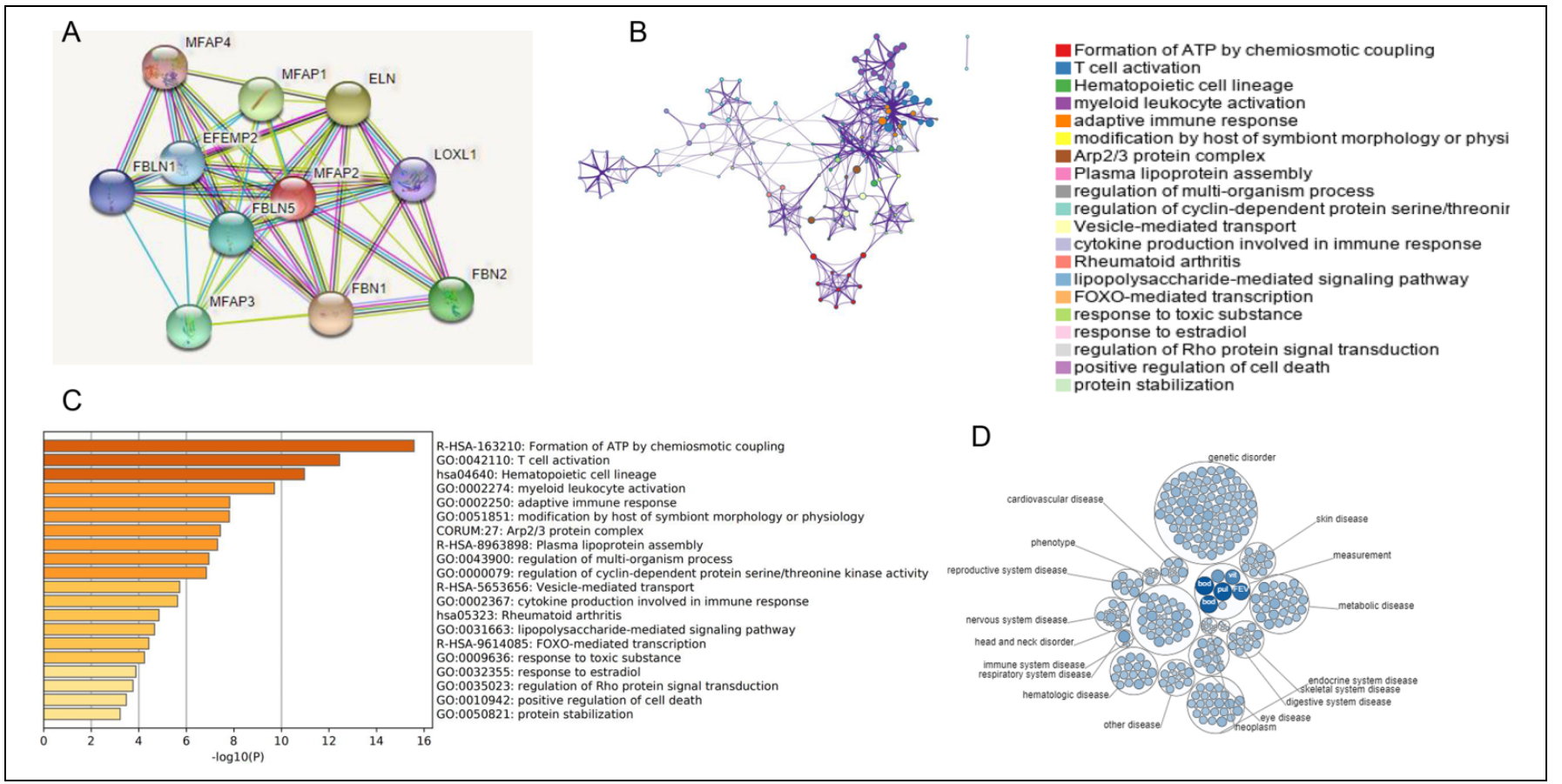


**Figure 5.** Protein interactions and enrichment analysis of MFAP2. A, Interaction network between MFAP2 and other proteins. B, Enriched terms network with cluster ID coloring; nodes that share the same cluster ID are often close to each other. C, KEGG analysis of gene co-expressed with MFAP2. D, Enrichment analysis showed that MFAP2 was associated with the development of tumors of the digestive system.

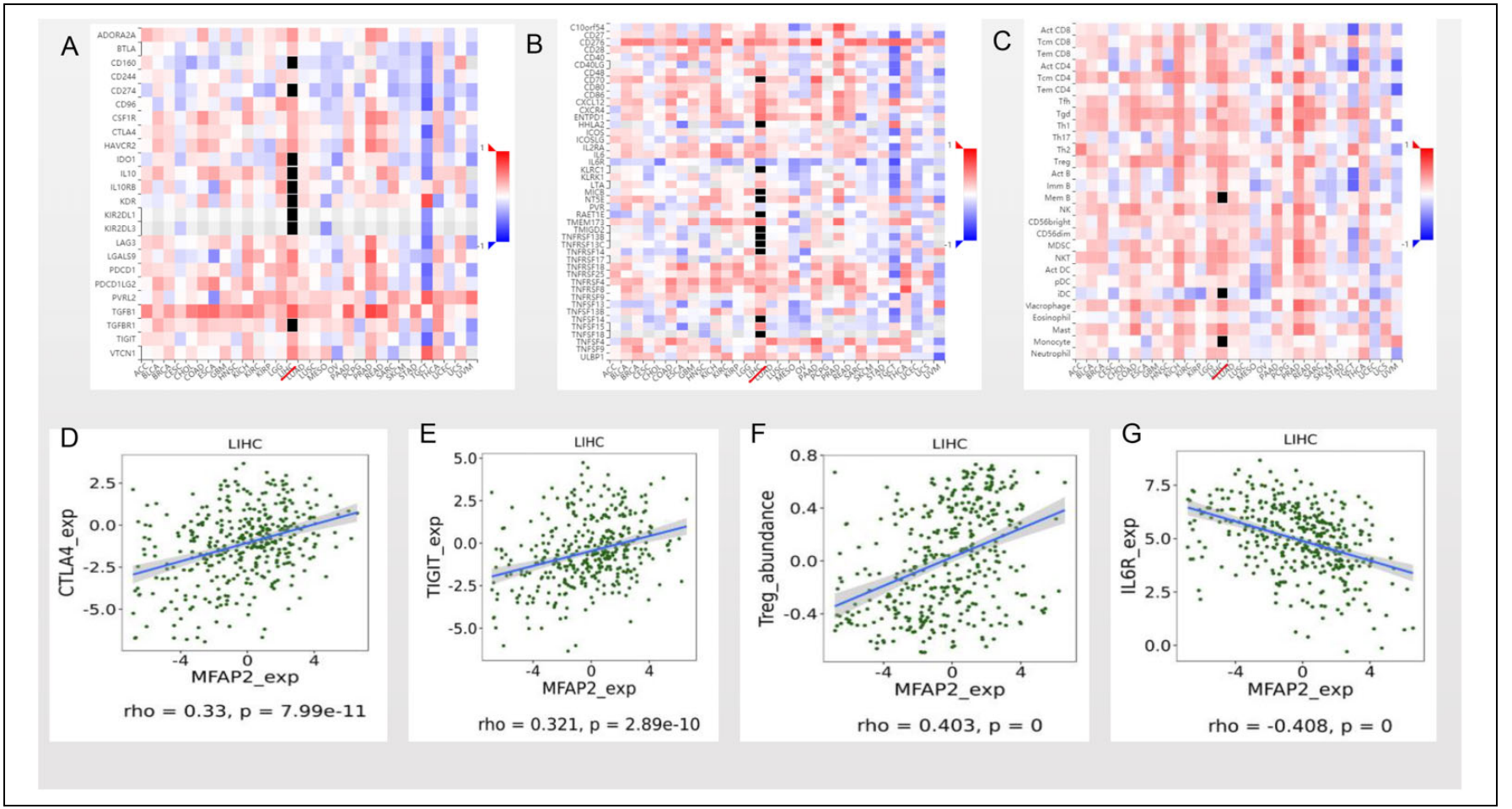


**Figure 6.** Relations between MFAP2 expression and cancer-related immune factors. A, Spearman correlation between MFAP2 expression and immunoinhibitors in HCC. Common inhibitory factors include CTLA4, LAH3, PDCD1. The prediction results show that MFAP2 is positively correlated with these inhibitory factors, suggesting that MFAP2 may be involved in tumor immune escape. B, Spearman correlation between MFAP2 expression and immunostimulators in HCC. The prediction results show that MFAP2 is positively correlated with immunostimulators including IL6, ICOS. C, Spearman correlation between MFAP2 expression and TILs in HCC. D-E, Immunosuppressive membrane proteins CTLA4, TIGIT have significant correlation with MFAP2 expression. F, MFAP2 was significantly linked with Tregs. G, Expression of IL6R was negatively correlated with MFAP2.

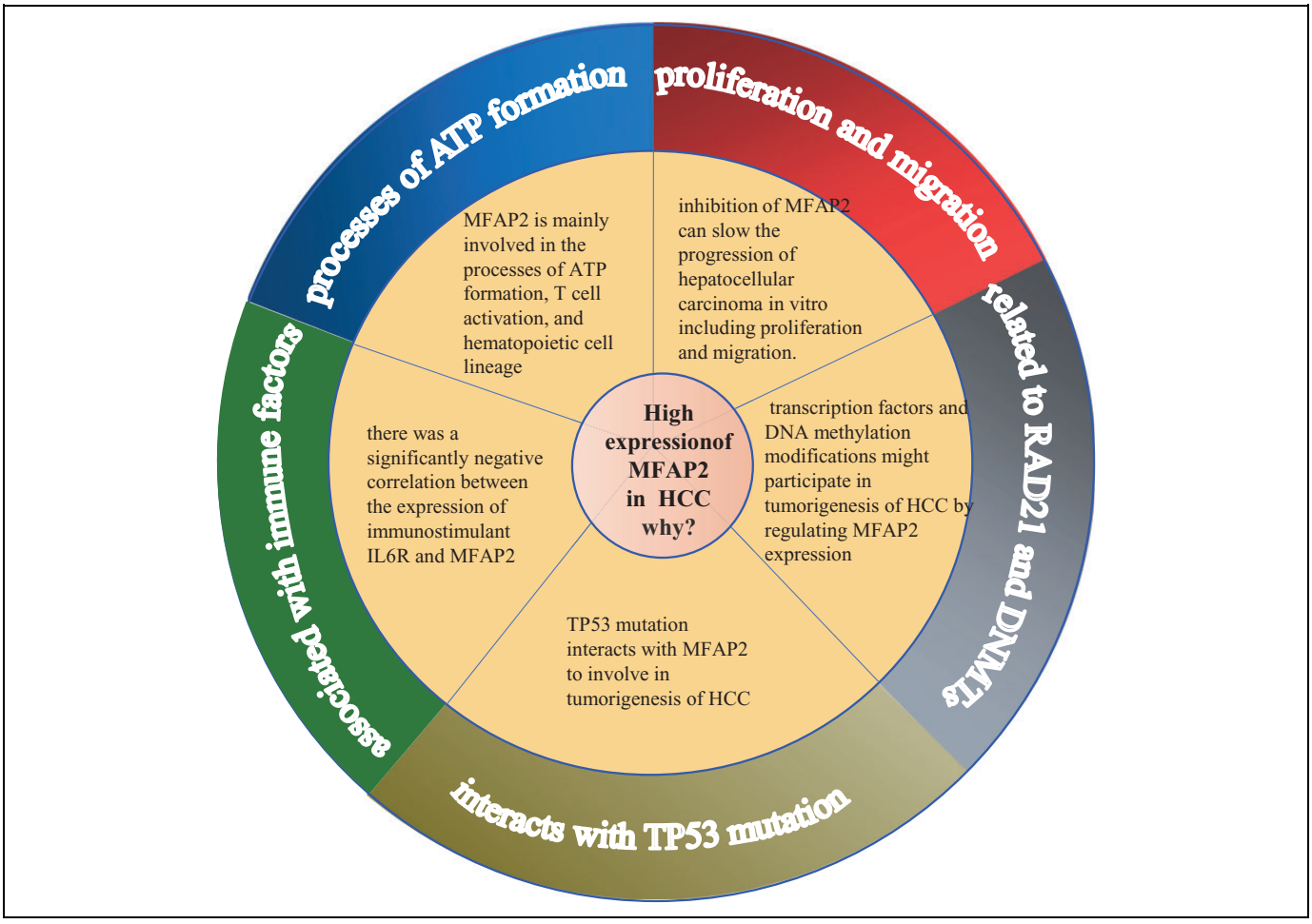


**Figure 7.** A summary mechanism of MFAP2 in HCC. MFAP2 is involved in tumor progression and prognosis. Transcription factors, DNA methyltransferases, immune factors may interact with MFAP2 mRNA to promote tumor progression in HCC.

RAD21 regulates the expression of MFAP2 and the relationship to mutant p53 in HCC remains to be investigated.[23,17,25]

DNA methylation regulates cell differentiation and is involved in tumorigenesis, and is mediated by a family of DNA methyltransferase enzymes (DNMTs). Abnormal gene methylation is considered to be one of the main mechanisms that trigger HCC and may be a useful biomarker for predicting HCC risk.[14,15] DNMTs are involved in epigenetic regulation of the genome and are promising new targets for the treatment of cancer and other diseases.[16] Liu's and Lai's studies demonstrated respectively that DNMT1 and DNMT3b were upregulated in the sorafenib-resistant human hepatocellular carcinoma cells.[26,27] So inhibition of DNMTs by their inhibitors such as RNA interference or nanaomycin A (a selective DNMT3b inhibitor) significantly increased the sensitivity of sorafenib in a dose-dependent manner. The DNMT inhibitor acacytidine down-regulates the activity of DNMT1 and DNMT3b, leading to increased drug sensitivity in tumor cells in hormone refractory prostate cancers.[28] Epigallocatechin gallate (EHCCG), the most active compound in green tea, induces cell cycle arrest and apoptosis of cancer cells by inhibiting class 1 HDACs and DNMTs in colon cancer.[29] Several methods for inhibiting DNMT activity have been reported, including DNMTs depletion mediated by small interfering RNAs or covalent enzyme capture using suicide-nucleoside substrates such as azacytidine and decitabine.[30] In recent years, the rational development of small molecule non-nucleoside inhibitors such as RG108 has become a research hotspot. DNA methylation can lead to transcriptional inactivation by directly inhibiting transcription factor binding, blocking the DNA sequence it recognizes, histone deacetylases (HDACs), or recruiting methylated binding proteins that interact with transcription factors directly.[31] Significant DNA methylation modification was found in the MFAP2 gene, and its expression was positively correlated with DNMTs expression. Our results suggested that alterations in DNA methylation modifications of MFAP2 gene were likely to be involved in the occurrence and development of HCC. Therefore, DNMTs inhibitors mentioned above, such as RG108 or EHCCG, combined with TF inhibitors may be an effective treatment for HCC patients. In order to elucidate the molecular mechanisms underlining their roles, further research is necessary.

The host immune response has been emphasized as a genetic biomarker for the disease with the production of multiple immune cells in HCC. Immune checkpoints involve membrane expression of different molecules, fine-tuning the immune responses.[32] Tregs are prominent among the immunosuppressive candidate cells in and around the tumor niche. Langhans et al. confirmed that peripheral Tregs upregulate checkpoint inhibitors and are involved in systemic immune dysfunction and anti-tumor activity through several inhibitory pathways, which may contribute to tumor development in HCC.[33] CTLA4 is a structural homologue of CD28 and expressed on activated T cells. Recently, some studies have focused on the relationship between CTLA4 SNPs and susceptibility to HCC, and Wang's research demonstrated that CTLA4 could affect susceptibility to HCC via changing the immune status of the individuals.[34] TISIDB results indicated that MFAP2 expression is closely related to Tregs and some immunological checkpoints which have recently received more and more attention, such as CTLA4, TIGIT in HCC. Therefore, inhibitors that target these immune checkpoints might be particularly effective in the treatment of HCC patients, especially when used in combination with MFAP2 inhibitors. Finally, IL6R was negatively correlated with MFAP2; if the expression of MFAP2 is an effective indicator for judging the prognosis of HCC patients, IL6R might also be used as a useful biomarker for immunotherapy to reflect the immune microenvironment status of HCC patients.

In summary, our data indicated that MFAP2 was significantly upregulated in HCC and was associated with tumor progression and prognosis, the higher MFAP2 expression, the poorer prognosis and survival. MFAP2 might promote the proliferation, migration and invasion in HCC cells. The study of DNMTs and TF inhibitors that may down-regulate MFAP2 has important clinical significance. MFAP2 might also be a promising prognostic biomarker and serve as a potential immunotherapy target in HCC patients (Figure 7).

## Conclusion

MFAP2 may play a key role in the development of HCC. Therefore, MFAP2 may be a valuable prognostic marker and an effective anticancer target in HCC.

### Authors' Note

Xiang Zhu, Ye Cheng, and Fan Wu contributed equally. All experiments using animal and human samples were reviewed and approved by the Ethics Committee of Nanjing Medical University (No.2019669). Written informed consent for experimental use of the specimens was acquired from each participant, and the research was carried out according to the relevant guidelines and regulations. There are 3 first authors in this manuscript and they have equally contributed to this project. XZ was responsible for drafting the manuscript. YC was responsible for designing and performing the experiments. FW was responsible for the manuscript language editing and data analysis. Furthermore, we have 2 corresponding authors in this manuscript. JFS and HYS has contributed to data interpretation, editing and critical revision of the manuscript. SJM and HYC have contributed to study design and critical revision of the manuscript. All authors read and approved the final manuscript. The medical ethics committee of Nanjing Medical University approved the study.

### Declaration of Conflicting Interests

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### ORCID iD

Xiang Zhu https://orcid.org/0000-0002-2773-0412

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