DEAD-box Helicase 27 Promotes Hepatocellular Carcinoma Progression Through ERK Signaling

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Abstract

Introduction: DEAD-box helicase 27 (DDX27) belongs to DEAD-Box nucleic acid helicase family. The function of DDX27 in hepatocellular carcinoma (HCC) remain enigmatic. In light of this, we tried to investigate the regulatory role and underlying mechanism of DDX27 in HCC. Materials and methods: DDX27 expression levels were detected by qRT-PCR, Western blot and immunohistochemistry assays in HCC tissues and cells. Colony formation, CCK-8, growth curve, wound healing and transwell assays were conducted to investigate the effect of DDX27 on the proliferation and metastasis of HCC cells. RNA-sequencing was performed to detect the effect of DDX27 on downstream signaling pathway. The effect of DDX27 on HCC progression was evaluated using in vivo murine xenograft model. Results: we found an increased expression of DDX27 in HCC tissues with comparison to its para-tumor tissues. The high expression levels of DDX27 were associated with poor prognosis in HCC patients. DDX27 upregulation promoted cell metastasis. Mechanistic studies suggested that DDX27 overexpression induces the major vault protein (MVP) expression and enhances the phosphorylation levels of ERK1/2. Inhibition of ERK pathway impaired the cellular metastatic abilities induced by DDX27. The induction of DDX27 in HCC progression was further confirmed from tumors in mouse model. Conclusion: our results disclose a novel mechanism by which DDX27 enhances ERK signaling during HCC progression. DDX27 might be used in targeted therapy for HCC patients.

Keywords
cell proliferation, metastasis, hepatocellular carcinoma, DDX27, ERK

Abbreviations
DDX27, DEAD-box helicase 27; HCC, hepatocellular carcinoma; MVP, major vault protein; ERK, extracellular regulated protein kinases; CRC, colorectal cancer; TNF, tumor necrosis factor; NF-kB, Nuclear factor-k-gene binding; NPM, nucleophosmin; GC, gastric cancer; qRT-PCR, quantitative polymerase chain reaction; DMEM, Dulbecco’s modified eagle medium; FBS, fetal bovine serum; cDNA, complementary single-strand DNA; siRNA, small interfering RNA; CCK-8, cell counting Kit-8; GSEA, gene set enrichment analysis; TCGA, the cancer genome atlas; SPSS, statistical package for the social sciences; OS, overall survival; TNM, tumor node metastasis; BCLC, Barcelona clinic liver cancer; AFP, alpha fetoprotein; VEGF, vascular endothelial growth

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most leading cause of cancer-related deaths.1 Although HCC treatment has been evolved considerably by now, prognosis of HCC patients remains poor, especially in advanced-stage and late-stage HCC patients. Most HCC patients cannot be diagnosed at early stage as the difficulty in diagnosis of mild damages of liver function.2 For advanced-stage HCC HCC patients, only available systemic treatments for prolonging survival are the tyrosine kinase inhibitors, such as lenvatinib, sorafenib.3 Sorafenib, being one of the first-line drugs for advanced HCC, still has limited survival benefits because of its drug resistance. Therefore, elucidation of mechanism on HCC progression is benefit for identification new therapeutic targets for HCC.4,5

DDX27 contains a conserved “ DEAD-Box” motif, which is the characteristics of DEAD-Box nucleic acid helicase family.6,7 DDX27 is involved in growth and regeneration of skeletal muscle through regulating ribosome biogenesis in zebrafish.8 Within cancer, studies have shown that DDX27 regulates many biological processes associated with tumorigenesis and metastasis. DDX27 is upregulated in colorectal and gastric cancer.9-11 Increased DDX27 mRNA and protein expression levels predict low survival probability in colorectal cancer (CRC) patients. DDX27 overexpression promotes cell proliferation, migration, and invasion, and raises mouse xenografted tumor growth in CRC cells.12 DDX27 enhances activity of TNF-alpha-mediated NF-kB signaling. DDX27 is associated with Nucleophosmin (NPM1), therefore enhancing interaction between NPM1 and NF-kB-p65 to induce NF-kB’s DNA binding activity. Downregulation of NMP1 impaired DDX27-activating NF-kB signaling in CRC cells.13 DDX27 is also highly amplified in gastric cancer (GC). DDX27 was upregulated in GC tissues in about 27.8% GC patients, whereas para-tumor tissues showed weak to no immunoreactivity for DDX27. Association is found between increased DDX27 expression and poor prognosis in GC patients. Furthermore, downregulation of DDX27 decreased epirubicin or cisplatin resistance in GC cells.13 However, the role of DDX27 in HCC has not been reported.

Here, we determined expression levels of DDX27 in HCC cell lines and HCC tissues. We analyzed the association between DDX27 and the clinical characteristics, and the DDX27 function in progression of HCC. Uregulated expression levels of DDX27 were associated with poor prognosis on HCC. Intriguingly, the overexpression of DDX27 increased the expression of MVP. Moreover, overexpression of DDX27 enhanced cell proliferation and metastasis by regulating ERK1/2 phosphorylation levels. This study reveals that DDX27 modulating ERK pathway, suggesting DDX27 as a possible therapeutic target for HCC.

Materials and Methods

Clinical Tissues

Tumor and para-tumor tissues were collected from patients undergone HCC resection in Affiliated Cancer Hospital of Zhengzhou University (Henan, China). HCC diagnosis was confirmed by pathology. Twenty-six paired of fresh HCC tumor and para-tumor tissues (cohort #1) were examine by reverse transcription and quantitative polymerase chain reaction (qRT-PCR) and Western blotting. Another 127 paired of HCC tumor and para-tumor tissues (cohort #2) were collected for construction of HCC tissue microarray and further microscopic statistical analysis (Olympus-I X 71, Japan). All patients signed the informed consent. Study is performed according to ethics approval approved by the Ethics Committee of Henan Cancer Hospital (No.2018CT065).

Cell Lines

HepG2 and PLC were purchased from the American Type Culture Collection Biobank. The Cells were cultured in dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin.

Immunohistochemistry

Three micrometer paraffine-embedded sections of mice liver samples were processed for immunohistochemical analysis with primary antibody against DDX27 (Sigma-Aldrich, cat.HPA047087) and MVP (Proteintech, cat.16478-1-AP). In the tissues microarrays staining with DDX27 antibody, scores of the staining intensity were defined as 0 (negative staining), 1 (weak staining), 2 (moderate staining), and 3 (high staining). The cancer samples were divided into two groups: low DDX27 expression with staining score as 0 to 2 and high DDX27 expression with staining score 3.

qRT-PCR

Cell pellets were harvested. Total RNA was extracted using Trizol (Invitrogen) according to manufacturer’s protocol. Complementary single-strand DNA (cDNA) was transcribed using FastQuant RT kit (With gDNA, TIANGEN, Beijing, China). The expression levels of DDX27 and MVP were determined by qPCR using SYBR qPCR Kit (TIANGEN, Beijing, China). The primer sequences are listed in Table S1.
Western Blot Analysis

Cell lysates were extracted, quantified, and primary antibody was incubated overnight. After incubation with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz), the blots were subsequently developed by enhanced chemiluminescence (Millipore).

Plasmid Preparation, siRNA and shRNA Knockdown, and Transfection Assays

The DDX27-expressing plasmid and its control vector plasmid were purchased from SinoBiological (Beijing, China). The DDX27-targeting siRNAs and their non-targeting control were purchased from Ribobio (Guangzhou, China, Table S2). The sequences of siRNAs were listed in Table S2. Transfection reagent lipofectamine 2000 (Invitrogen, USA) was used to transient transfect the plasmids and siRNAs into cells. Recombinant genes coding shRNA against DDX27 and non-targeting shRNA (Table S2) were constructed to pLKO.1-TRC cloning vector (Addgene, Cambridge, MA, USA). Lentiviruses were produced and cells were transfected following the protocol online (http://www.addgene.org/plko). Stably transfected HepG2 cells were selected with 3 μg/ml puromycin starting at 48 h after transfection.14

Cell Culture Experiments

The cells were cultured in six-well plates. According to Kratzat’s method. 1 × 10^5 cells were plated in a growth curve assay. At every time lag of 2 days, cells were harvested and counted, and re-plated at the same quantity. 1 × 10^3 cells were subcultured in a colony formation assay. Culture media were refreshed every three days. The colonies were counted manually after stained with 0.01% crystal violet and photographed by Canon EOS200D camera. To do a wound healing assay, 5 × 10^5 cells were plated. When cells reach 80%-90% confluence, cells were scratched in the middle of the well with a pipette tip and changed the culture media to media containing 1% BSA. The migration distance was recorded under microscopy in a time course and photographed at 0 h and 48 h. For the Transwell-chamber assays, 2 × 10^5 of pre-starved cells were plated in the upper chamber with 8 μm pores (Millipore, Billerica, MA, USA), and cultured in the DMEM only. The
lower chamber was added with 600 μl of DMEM with 10% FBS. The polycarbonate membranes were coated with Matrigel matrix (Corning, Lowell, USA) in the invasion assay, while not coated in the migration assay. The migratory or invading cells on the below membranes were fixed with 4% paraformaldehyde, stained with 0.01% crystal violet, and photographed under microscopy. To do the CCK-8 assay, 1 × 10³ cells were cultured into 96-well plate. Culture media were refreshed every three days.

RNA-Sequencing
RNA-sequencing was performed in three paired of DDX27-knockdown HepG2 cells and the control cells using BGISEQ-500 (BGI Company, Shenzhen, China). Hg19 Gene ontology functional analysis was used as reference genome. Gene set enrichment analysis (GSEA) was conducted by BGI Company’s website.

Animal Experiments
Briefly, 1 × 10⁷ cells were injected subcutaneously into right or left thigh region of 4-week-old male BALB/c-nude mice (Chinese Academy of Sciences, Beijing, China). Tumor volume (V) was measured every 2- or 3-days post-injection and calculated as the length × width²/2. Tumors were removed and weighted after the mice were euthanized. The animal study is performed according to ethics approval approved by the Ethics Committee of Henan Cancer Hospital (No. 2018CT065). The reporting of this study conforms to ARRIVE
The animal experiments followed the Guide for the Care and Use of Laboratory Animals, eighth Edition.

Statistical Analysis

The difference between two groups were analyzed using Student’s t-test. The difference between growth curves were analyzed using repeated measures analysis of variance. Kaplan-Meier analysis was performed for survival rate. The bioinformatic analyses of HCC clinical data from The Cancer Genome Atlas (TCGA) was conducted using a cBioPortal online software (http://www.cbioportal.org/index.do). Significance was defined at the 5% level. All statistical test were analyzed by SPSS 16.0 (IL).

Results

**DDX27 was Upregulated in HCC Tissues**

The expression levels of DDX27 were first determined in cohort #1 HCC samples. As shown in Figure 1A and 1B, HCC tissues exhibited higher DDX27 mRNA expression as compared to para-tumor tissues, which were also observed at protein expression levels (Figure 1C). We then examined the expression of DDX27 in various HCC cell lines. DDX27 was expressed in all detected HCC cell lines (Figure 1D and E). In addition, analyses of TCGA data revealed markedly increased DDX27 expression in Stage II-IV HCC tumors as compared to Stage I HCC tumors (Figure 1F). Moreover, we also observed a significant correlation of high DDX27 expression with shorter overall survival (OS) in HCC patients using TCGA dataset (Figure 1G).

**High Expression Levels of DDX27 are Associated With Poor Prognosis in HCC Patients**

In order to explore the relationship of DDX27 expression with HCC prognosis, DDX27 expression levels were determined by immunohistochemistry staining using a HCC tissue array of 126 HCC patients (cohort #2). The different staining intensity of DDX27 immunostaining was observed in Figure 2A respectively. Consistently with results of cohort #1, strong DDX27 expression was mainly found in tumor tissues compared to corresponding para-tumor tissues (Figure 2B and 2C). High level of DDX27 expression in cancer tissues was found in 48.4% (61/126) in our cohort (Table 1). Upregulated DDX27 expression was positively associated with multiple clinical characteristics, including microvascular invasion, surgery time, and patient age. Moreover, higher levels of DDX27 in tumors were markedly associated with poorer OS (Figure 2D).

### Table 1. Clinicopathological Information of the HCC Tissue Samples for Immunohistochemistry.

| DDX27 expression (%) | p-value |
|----------------------|---------|
| Low                  | 65      | 58 (55.8) 46 (44.2) 0.041 |
| High                 | 61      | 70 (57.1) 30 (42.9) 0.163 |

In univariate analyses revealed that high DDX27 expression, BCLC stage (B+C), and high AFP level were showed as the independent prognostic features for the OS (Figure 2E, left panel). All these 10 clinical pathological features were further analyzed by multivariate Cox regression model. Only high DDX27 expression, BCLC stage (B+C), and high AFP level were showed as the independent prognostic features for the OS (Figure 2E, left panel). Furthermore, high DDX27 expression was also associated with shorter disease-free survival (DFS) (Figure 2F). High DDX27 expression level, TNM stage (III&IV), BCLC stage (B+C), satellite metastasis, microvascular invasion, macrovascular invasion, larger tumor size, intraoperative blood transfusion, long surgery time, and high AFP level were correlated with DFS using univariate analyses (Figure 2G, left panel).
stage (B + C), and high AFP level were also the independent prognostic features for the DFS (Figure 2G, right).

**DDX27 Promotes Cell Proliferation in HCC Cells**

To characterize DDX27 function involved in tumor progression, we first performed a series of cell proliferation arrays. DDX27 expression level was upregulated with DDX27-expressing plasmid or silenced with DDX27 siRNAs, respectively. The efficiency of altering DDX27 expression levels was determined at mRNA levels by qPCR and at protein levels by Western blotting (Figure 3A and 3B). The increased DDX27 significantly promoted colony formation and cell viability (Figure 3C and E). Whereas, downregulation of DDX27 decreased clonogenicity and cell proliferation and (Figure 3F–H). The results suggest that DDX27 promote cell proliferation in HCC cells.

**DDX27 Increases Cell Metastatic Ability of HCC Cells**

Next, we determine the effect of DDX27 on cell metastatic ability. In the Transwell-chamber assays, upregulation of DDX27 significantly enhanced migration and invasive abilities in HepG2 and PLC cells compared to their vector control cells (Figure 4A). Conversely, DDX27 knockdown markedly impaired cell migration and invasion (Figure 4B). Moreover, enhanced migration capability was also displayed in DDX27-overexpressing HepG2 cells by wound-healing assay (Figure 4C). Whereas, DDX27 downregulation reduced cell migration (Figure 4D).

**DDX27 Enhanced Activation of ERK1/2 in HCC Cells**

To elucidate potential mechanisms of DDX27 on the tumor progression, an RNA transcriptome-sequencing study was performed using HepG2 cells transfected with DDX27 siRNA or control siRNA (Figure 5A). Totally 388 mRNAs were identified to be the differentially expressed genes. Pathway analysis indicated that these mRNAs were enriched in cancers, metabolic pathways, ubiquitin mediated proteolysis, focal adhesion, and some signaling pathways (Figure 5B). The enriched signaling pathways were including TNF, VEGF, MAPK, p53,
AMPK, and PI3K-Akt pathways. Those signaling pathways were all reported to be related with cancer metastasis.\textsuperscript{17} For example, TNF-α is an inflammation mediator and promotes tumor growth in vivo.\textsuperscript{18} TNF-α can also be used to predict sensitivity to Sorafenib in HCC patients.\textsuperscript{17} P53 could enhance cell cycle arrest and apoptosis, or repress tumor cell ferroptosis.\textsuperscript{19–22} PI3K and Akt pathway induce cancer cell survival.\textsuperscript{23} To evaluate the results of KEGG database analysis affected by DDX27, we assess three pathways, MAPK, p38MAPK, and ERK. Their enrichments were validated by gene set enrichment analysis (GSEA, Figure 5C). These bioinformational analysis provides that DDX27 has potential regulatory mechanisms in tumor metastasis.

Considering the reported association between ERK1/2 and major vault protein (MVP) in HCC,\textsuperscript{24,25} we were interested in MVP, which was one of the differentially expressed mRNAs with the knockdown of DDX27 in RNA-Seq (Figure 5A). The decreased expression level of MVP with the silencing of DDX27 was captured in the RNA-Seq array, and further confirmed by qPCR analysis (Figure 5D). Moreover, MVP protein expression levels was markedly increased in DDX27-overexpressing cells than those in control cells (Figure 5E). Therefore, DDX27 might regulate the expression of MVP during HCC progression. We next confirmed the relationship between the DDX27 expression and ERK1/2 activation by Western blotting. As expected, DDX27 overexpression increased ERK1/2 phosphorylation with comparison to its vector cells (Figure 5E). Then, when cells were treated with a specific ERK phosphorylation inhibitor, SCH772984.\textsuperscript{26,27} The difference in cell proliferation between the DDX27-overexpressing HepG2 cells and its control cells was decreased by SCH772984 treatment by the CCK8 and growth curve array (Figure 5F and G). The enhanced migration and invasion capabilities with DDX27 overexpressing in HepG2 cells were also impaired by SCH772984 by the Transwell-chamber assays (Figure 5H). Taken together, DDX27 might increase MVP expression and induce activation of ERK1/2 to promote HCC progression.

**DDX27 Promotes HCC Cell Proliferation In Vivo**

We further assess the effect of DDX27 on HCC progression using in vivo murine xenograft model. Western blotting and qPCR assays were used to check the continuous down-regulation of DDX27 expression (Figure S1). The DDX27-knockdown HepG2 cells and their control cells were subcutaneously transplanted into nude mice thigh. Silencing of DDX27 significantly decreased tumor growth (Figure 6A–
Moreover, the mice body weights in the shDDX27 mice were significantly higher as compared with the HepG2-pScr mice (Figure 6D). Downregulation of DDX27 in tumors was confirmed by immunohistochemical staining (Figure 6E). The decreased expression level of MVP was also confirmed in tumors from HepG2-shDDX27 mice compared to those of HepG2-pScr mice (Figure 6E). Collectively, these data suggest that DDX27 promotes cell proliferation through upregulating the expression of MVP.

Discussion

The DEAD-Box family of RNA helicases have forty-three members that catalyze variously cellular physiological activities
through reforming RNA or RNA/protein complexes, or RNA annealing. Several DDX family members are reported to be highly multifunctional with causative roles in tumorigenesis and metastasis. For examples, DDX1 promotes testicular tumorigenesis by increase of cyclin-D2, CD9, and NANOG expression. DDX3 enhances cell motility and invasion through induction of epithelial-mesenchymal-like transition, in breast cancer. As for DDX27, it interacts with nucleophosmin and activates NF-κB signaling to promote colorectal cancer (CRC) development. DDX27 contributes to tumorigenesis of gastric cancer by induction of clonogenicity in gastric cancer cells. Nevertheless, DDX27 function in HCC is unknown. In current study, we showed that DDX27 is a pivotal oncogenic gene in HCC, and an independently risk factor for HCC patient survival. DDX27 enhanced metastatic phenotypes in HCC cells. DDX27 also increased MVP expression at mRNA and protein levels. Consistent with in vitro results, DDX27 increased MVP expression in xenografted tumors. Furthermore, DDX27 enhanced metastatic abilities in HCC cells through activation of ERK signaling. Collectively, DDX27 could be a molecular characteristic to predict poor prognosis in HCC. However, this study has limitations. The patients’ sample size was relatively small and from a single hospital and the mechanism research is not deep enough. Whether DDX27 activates ERK pathway through other pathways still needs further research.

ERK is one of the numbers in MAPK family. It is well-known that ERK1/2 pathway regulates key cellular events, including proliferation, differentiation, and survival. Constitutive increased phosphorylation levels of ERK1/2 is closely associated with tumorigenesis and metastasis in most types of cancers, including HCC. In the present story, we determined DDX27 as an HCC oncogene, contributing to stimulating the ERK signaling pathway. DDX27 confers ERK-mediated growth advantage and cell mobility to HCC cells, which can be abolished by ERK specific inhibitor, SCH772984. Thus, the oncogenic effect of DDX27 will be impaired when the ERK pathway is inhibited. Few studies has shown the relationship between DEAD-Box protein family and ERK pathway. DDX3 function can be altering by phosphorylation status of a DEAD-box protein modular. Gle1 is phosphorylated by ERK and c-Jun N-terminal kinase during neurodegeneration. Thus, our study revealed a new mechanism of DEAD-Box protein to promote cancer progression.

Recently, ERK dimers is detected to dominantly localize in the cytoplasm, where ERK interacts with scaffold proteins that provide as ERK dimerization platforms. Though ERK
dimerization is depended on ERK phosphorylation, impairment of ERK dimerization, but not ERK phosphorylation, inhibits the spreading of tumorigenic signals by Ras and BRAF onco
genes.40 Previous study has identified MVP as a partner of ERK1/2.41 Overexpression of MVP is related with chemotherapy failure and radiation resistance of cancer cells.42 In HCC cells, MVP is involved in Src homolog and collagen homolog 3 (Shc3)-induced EMT competences to be resistant to EGFR inhibition.26 Adding to this, we now suggest the increased metastatic capabilities in HCC cells, are probably also by the upregulated expression of MVP with DDX27 over-expression, thereby to facilitate its interaction with ERK, and to further increase ERK extranuclear signaling. Therefore, DDX27 potentiated a prolonged ERK response by increasing mRNA and protein expression of MVP. Moreover, silencing of DDX27 decreased epirubicin or cisplatin resistance in vitro in gastric cancer cells.17 Taken together, the potential significance of DDX27 warrants further investigation of its clinical implications in cancer.

Conclusion
Hence, the increased DDX27 expression in HCC tissues and cell lines, together with its oncogenic function advises the role of DDX27 in triggering HCC progression. Moreover, we identified that DDX27 enhances ERK pathway. In addition, the overexpression of DDX27 is significantly associated with clinical pathological characteristics and shortened survival in HCC patients. In summary, our data support that DDX27/ERK signaling axis is worthy for future research and targeted therapy in HCC treatment.

Authors’ Note
Wang Xiaqian, Zhang Bing, Li Yangwei and Zhi Yafei contributed equally to the work. The study was approved by the Ethics Committee of Henan Cancer Hospital Affiliated to Zhengzhou University (committee approval number: No.2018CT065), and written informed consent was obtained from all patients.

Declaration of Confl cting Interests
The author declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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**Supplemental Material**
Supplemental material for this article is available online.

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