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Original Article

Abnormal expression of YEATS4 associates with poor prognosis and promotes cell proliferation of hepatic carcinoma cell by regulation the TCEA1/DDX3 axis

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Abstract: YEATS domain containing 4 (YEATS4) is usually amplified and functions as an oncogene in several malignancies, such as colorectum, ovarian, breast and lung. However, the biological role of YEATS4 in hepatocellular carcinoma (HCC) has not yet been discussed. Herein, we found that YEATS4 was significantly upregulated in HCC compared to para-cancerous tissues, and was associated with poor prognosis, large tumor size, poor differentiation and distant metastasis. In addition, YEATS4 promoted HCC cell proliferation and colony formation by binding to and increasing the transcriptional activity of the TCEA1 promoter. Concurrently, upregulation of TCEA1 increased the stability of the DDX3 protein, a member of the DEAD box RNA helicase family, and augmented the proliferative and colony forming ability of HCC cells. Furthermore, YEATS4 accelerated tumor growth *in vivo* in a xenograft HCC model. Taken together, our study provides evidence for the first time on the potential role of the YEATS4/TCEA1/DDX3 axis in regulating HCC progression, and presents YEATS4 as a promising therapeutic target and prognosis maker for HCC.

Keywords: YEATS4, TCEA1, DDX3, hepatic carcinoma, prognosis, proliferation

Introduction

Hepatocellular carcinoma (HCC) is a malignant tumor of the liver, and has a high mortality rate with an annually increasing incidence worldwide [1]. The poor prognosis of HCC patients is largely attributed to the difficulty in early diagnosis, as well as the high metastasis, invasion and recurrence of this cancer [2]. Therefore, it is vital to explore the pathways involved in HCC initiation, and thereby identify early specific biomarkers and potential therapeutic targets for HCC.

YEATS4 was first identified in the nucleolix [3]. High sequence homology to the human MLLT1 and MLLT3 proteins indicated a transcription factor function [4]. Recently, YEATS4 has been shown to be involved in the genesis and progression of various cancers. In the pancreatic and gastric cancers, it acts as an oncogene via the Wnt/β-catenin/TCF signaling pathway [5, 6]. YEATS4 is also upregulated in NSCLC and contributes to cancer cell proliferation and tumor growth by inhibiting p53 expression [7]. Furthermore, it has been linked with increased drug resistance in ovarian cancer [8]. Downregulation of YEATS4 by miR-218 sensitized colorectal cancer cells to L-OHP-induced cell apoptosis by weakening cytoprotective autophagy [9]. However, the precise function of YEATS4 in HCC, and underlying mechanisms and patient prognosis is unclear.

SOX is an important transcriptional elongation factor that can directly bind to RNA polymerase II, and allow it to read through various transcription arrest sites [10, 11]. SOX has 3 distinct isoforms: the ubiquitously expressed TCEA1 that was identified in the network-based protein-protein interactions (PPI) for meningioma relative to healthy controls, the testis-specific SOX2

[12], and SOX3 which controls murine embryonic stem cell (mESC) fate and maintains its pluripotency by regulating the Lefty1-Nodal-Smad2 pathway [13]. TCEA1 is also up-regulated in HCC [14], although its potential role TCEA1 in liver cancer is not completely clear.

DDX3 (DEAD box protein 3) belongs to the DEAD box RNA helicase family that are characterized by the presence of a helicase domain and involved in RNA post-transcriptional procession. They are also members of the DEAD/H-box family [15], which are aberrantly expressed in various solid and hematological malignancies [16-18]. Studies have shown that high-levels of DDX3 could promote proliferation and neoplastic transformation of immortalized human breast cancer epithelial cells [19]. In addition, studies have hinted to DDX3 growth-regulatory functions of DDX3 in hepato-carcinogenesis and progression [20, 21].

In this study, we showed that YEATS4 transcriptionally activated TCEA1 expression TCEA1 by binding to its promoter, TCEA1 and the latter upregulated DDX3 levels by increasing protein stability. These interactions significantly accelerated HCC cells proliferation and growth *in vitro* and *in vivo*, thereby indicating that up-regulation of YEATS4 is associated with poor prognosis in HCC patients.

Materials and methods

Cell culture

Human liver cancer cell lines were purchased from the cell bank of Shanghai Institute of Cell Biology and cultured in medium (HyClone) supplemented with 10% fetal calf serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Millipore).

Immunoblotting

Cells were harvested and protein was extracted using RIPA buffer (Sigma) with 1% v/v protease inhibitor cocktail (Sigma) and 1% v/v phosphatase inhibitor cocktail (Sigma). Equal amounts of protein lysates were separated by SDS-PAGE and were transferred onto PVDF membranes. The filters were probed with the following specific primary antibodies: anti-YEATS4 and anti-DDX3 (Abcam), anti-TCEA1 and anti-PCNA (Proteintech Group), anti-ha, anti-flag and anti-β-actin (Cell Signaling Technology) (Cell Signaling Technology). The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce) and visualized by chemiluminescence. Experiments were repeated in triplicate independently.

Human tissue specimens

Tumor samples were obtained with informed consent from HCC patients at Zhongshan Hospital Xiamen University, between 2007 and 2011. The study was approved by the Xiamen University Medical Ethics Committee. Overall survival was calculated from the date of surgery to the date of death or final follow-up.

Hematoxylin-eosin and immunohistochemistry staining

Tissues were fixed with 10% neutral formalin, embedded in paraffin, and 3-μm-thick sections were prepared by the pathological technologist. For Hematoxylin-Eosin (HE) staining, sections were deparaffinized and hydrated with a gradient alcohol. After soaking in PBS, sections were stained with HE. For IHC, sections were stained using the streptavidin-peroxidase (S-P) method. In brief, sections were deparaffinized, hydrated and soaked in 3% H_2O_2 for 10 minutes at room temperature, and then incubated with Pygo2 polyclonal antibody (1:3000, ab109001, Abcam) at 4°C overnight. Biotinylated secondary antibody and diaminobenzidine (DAB) were purchased from Maixin Biotechnology (Fuzhou, China).

Plasmid construction and lentivirus preparation

For target gene knockdown, control and shRNA sequences (shown in **Table 1**) against YEATS4 were cloned into the pLKO.1-Puro vector. For over-expression, genomic sequence of the YEATS4 and TCEA1 coding region was respectively cloned into the backbone of the plv-Puro vector vector downstream of the CMV promoter. Then 293T cells were transfected with the above mentioned plasmids and packaging vectors by using the Turbofect Transfection Reagent (Thermo). Infected cells were cultured for selection with puromycin (InvivoGen).

RNA extraction and real-time PCR

RNA was extracted from tissues or cell samples using the Trizol reagent (Invitrogen) according

Table 1. YEATS4 abnormal expression correlates with clinic-pathological factors of HCC patients

Clinical-pathology Factors	A abnormal expression	N	X^2	P
Age (yr)				
< 55	20 (80.0%)	25	0.125	0.724
≥ 55	40 (83.3%)	48		
Gender				
Male	35 (83.3%)	42	0.088	0.768
Female	25 (80.6%)	31		
Tumor size				
< 5 cm	21 (70.0%)	30	5.172	0.023*
≥ 5 cm	39 (90.7%)	43		
Metastasis				
Yes	38 (92.7%)	41	7.033	0.008**
No	22 (68.8%)	32		
Differentiation				
Poor	28 (93.3%)	30	5.074	0.034*
Moderate	12 (85.7%)	14		
Well	20 (71.4%)	28		
Liver cirrhosis status				
No	19 (73.1%)	26	2.261	0.200
Yes	41 (87.2%)	47		
Serum HBV level (cps/ml)				
< 1000	26 (83.9%)	31	0.166	0.684
≥ 1000	28 (80.0%)	35		
Serum AFP level (ug/L)				
< 400	27 (79.4%)	34	0.279	0.597
≥ 400	32 (84.2%)	38		

*p < 0.05; **P < 0.01.

to the manufacturer's instructions. Primers were designed and synthesized by BGI-Tech. The sequences of the primer pairs are shown in Table S1. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. Gapdh mRNA was quantified in parallel as the endogenous control.

Luciferase reporter assay

For the luciferase reporter assays, a 3050bp genomic sequence of the 5'-UTR (-3000-+50 bp) non-coding region was cloned into the backbone promoter region of the pGL3.0 vector. After 48 h, the luciferase activity was measured using a luciferase assay system (Promega) according to the manufacturer's instructions.

Cck8 assay

Approximately 2 × 10^3 cells per well were plated on to 96-well plates in triplicate. Cell Counting Kit-8 (CCK-8) assays were performed using the Kit (#YB-K001, Yiyuan Biotechnologies, Guangzhou, China) according to the manufacturer's instructions for the purpose of detecting cell proliferation ability. Optical density (OD) values were measured at a wavelength of 450 nm by a microplate reader. All experiments were performed three times, and the average results were calculated.

EdU assay

The EdU incorporation assay was performed using the EdU assay kit (RiboBio, Guangzhou, China), according to the manufacturer's instructions. Briefly, cells were cultured in triplicate in 24-well plates. The cells were then incubated with 50 nM EdU for an additional 2 h at 37°C. The cells were fixed with 4% formaldehyde for 15 min at room temperature and treated with 0.5% Triton X-100 for 20 min at room temperature to permeabilize them. After three washes with phosphate-buffered saline (PBS), the cells were incubated with the Apollo reaction cocktail (100 l/well) for 30 min. After being washed three times with phosphate buffered saline (PBS), the cells were visualized using a fluorescence microscope (Zeiss, Oberkochen, Germany).

Colony formation assay

A total of 1 × 10^3 cells per well were seeded into 6-well plates and cultured for about 2 weeks in culture media. These cultures were stained with 0.4% crystal violet. Clones beyond 2 mm were counted and the number of clones per well was averaged from three wells. Experiment was repeated at least three times.

In vivo assays for tumor growth

Cells (5 × 10^6) in 0.2 ml of serum-free culture medium were inoculated subcutaneously into the right side of the backs of the nude mice. After a tumor formed, the tumor size was esti-

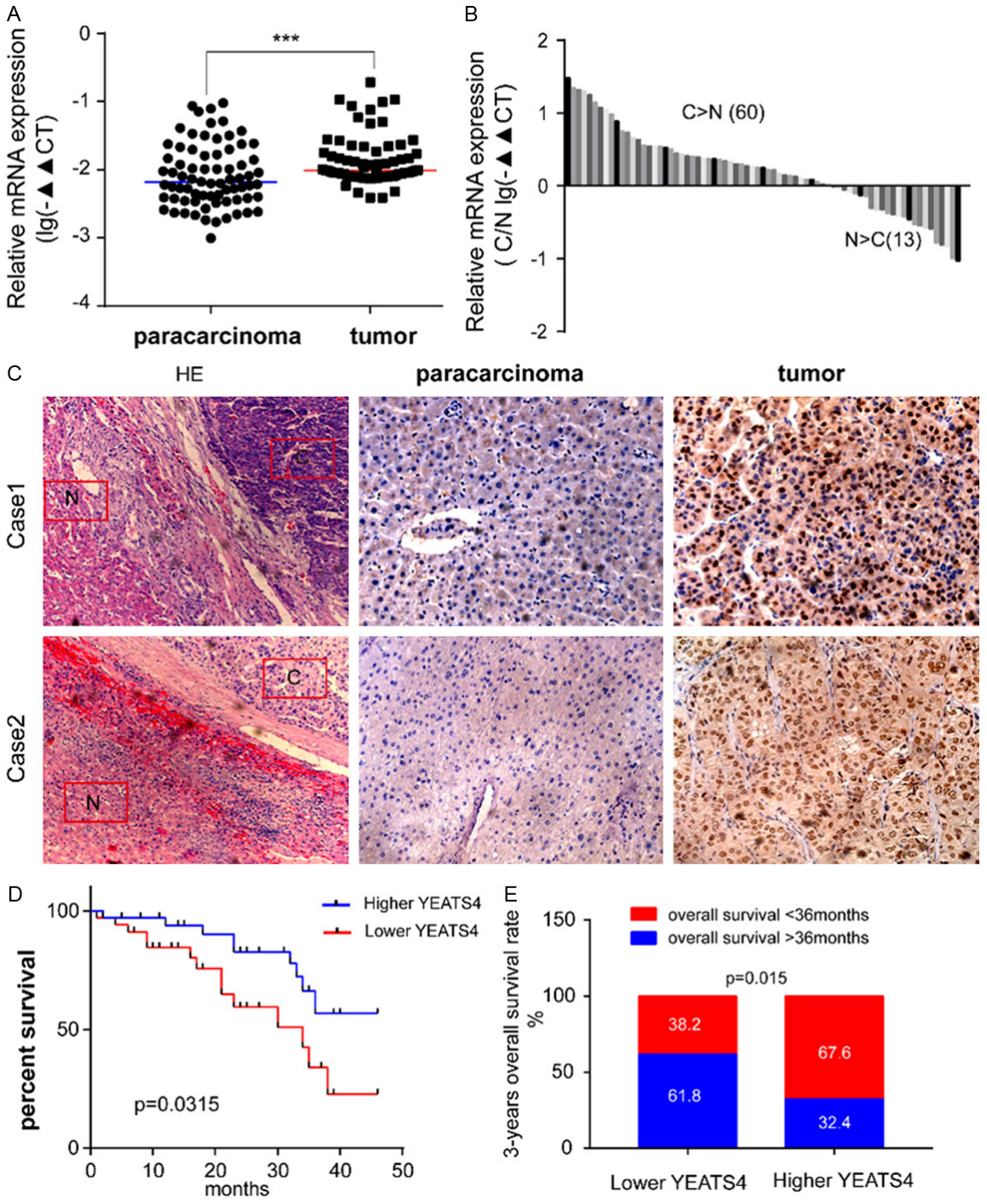

Figure 1. YEATS4 expression is upregulated in HCC tissues and negatively correlated with patient survival. A, B. RT-PCR: YEATS4 mRNA expression in HCC was significantly higher than that in the adjacent normal liver tissues (60 vs 13 samples, P≤0.001). Data were analyzed with 2-related samples Wilcoxon nonparametric test. C. IHC: YEATS4 protein expression in HCC was significantly higher than that in the adjacent normal liver tissues and located hepatic carcinoma cell nuclear. D. Kaplan-Meier analysis of 73 patients with HCC classified according to YEATS4 expression. Patients with abnormal expression of YEATS4 had shorter survival time than patients with normal YEATS4 expression (P = 0.0315). E. The 3-year survival rate among differential expressions of YEATS4 protein. **P≤0.01, ***P≤0.001.

mated according to the formula: volume (mm^3) = $a^2 \times b/2$, where "a" is the major diameter of the tumor and "b" is the minor diameter perpendicular to the major one. Eight weeks later,

Table 2. Cox regression model for prediction of 73 patients with HCC

Variables	Univariate analysis			Multivariate analysis		
	P	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI
Age (yr)	0.346	1.492	0.649-3.428	0.096	2.554	0.847-7.695
Gender	0.397	0.706	0.315-1.580	0.290	1.691	0.639-4.473
Metastasis	0.015	2.908	1.231-6.871	0.041	0.418	0.168-1.041
Tumor size	0.471	0.349	0.598-3.043	0.171	1.928	0.753-4.934
Poor differentiation	0.038	2.674	1.057-6.764	0.122	2.177	0.813-5.828
YEATS4 abnormal expression	0.016	2.855	1.213-6.718	0.038	2.895	1.061-7.896

the animals were sacrificed. The tumors were removed and cryopreserved at -70°C for western blot analysis. Lungs were removed and embedded in paraffin for hematoxylin and eosin (H&E) staining.

Co-IP assay

Cells were lysed in lysis buffer supplemented with protease inhibitors. Immunoprecipitation and western blot analyses were performed as previously described [22]. Briefly, the cell lysate was incubated with the appropriate antibody and protein A-agarose beads for 3 h. The immunoprecipitate was collected, washed three times with lysis buffer, and examined using western blot analysis with different antibodies, as required, after separation by SDS-PAGE. The immunoreactive products were visualized via enhanced chemiluminescence.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using an EZ-ChIP kit (Millipore, Catalog No. 17-10461) according to manufacturer's instructions. The TCEA1 promoter region located -3000 to -50 bp upstream of the transcription start site was amplified, and products were quantified by real-time PCR using both the ChIP-enriched DNA and input DNA as template. Enrichment by ChIP was assessed relative to the input DNA and normalized to the level of gapdh. The PCR primers for TCEA1 were listed in Table S1.

Statistical analyses

All data were expressed as the mean ± SD and analyzed using SPSS version 21.0 (IBM). Statistical analysis was performed with two-related samples using the Wilcoxon nonparametric test for comparing two different groups. Correlation analysis was used to explore the relationship between YEATS4 and TCEA1. Survival curves were calculated by the Kaplan-Meier test. Cox regression analysis was used to evaluate the risk factors associated with postoperative survival. *P* value less than 0.05 was considered statistically significant.

Results

Correlation between YEATS4 expression and clinico-pathological features

To determine the expression level of YEATS4 in HCC tissues, total RNA and protein were extracted from 73 paired HCC and adjacent noncancerous liver tissues. YEATS4 mRNA levels were detected by real-time PCR, and were significantly upregulated in the HCC tissues compared to the paired normal tissues ($n = 73$, $P < 0.001$; **Figure 1A**, **1B**). YEATS4 protein expression as per immunohistochemistry (IHC) was also consistent with its mRNA levels (**Figure 1C**), with strong YEATS4-positive nuclear staining seen in the tumor tissues and undetectable in the normal tissues. The correlation between YEATS4 expression and clinico-pathological factors of HCC was analyzed by chi-square analysis. As shown in **Table 1**, higher YEATS4 expression was significantly correlated with unfavorable features such as large tumor size ($P = 0.023$), poor differentiation ($P = 0.034$) and distant metastasis ($P = 0.008$). Taken together, YEATS4 may contribute to HCC progression by promoting tumor growth and metastasis.

YEATS4 expression levels in HCC is correlated with poor prognosis

To determine whether up-regulation of YEATS4 could influence the clinical outcome in HCC patients after surgical resection, Kaplan-Meier

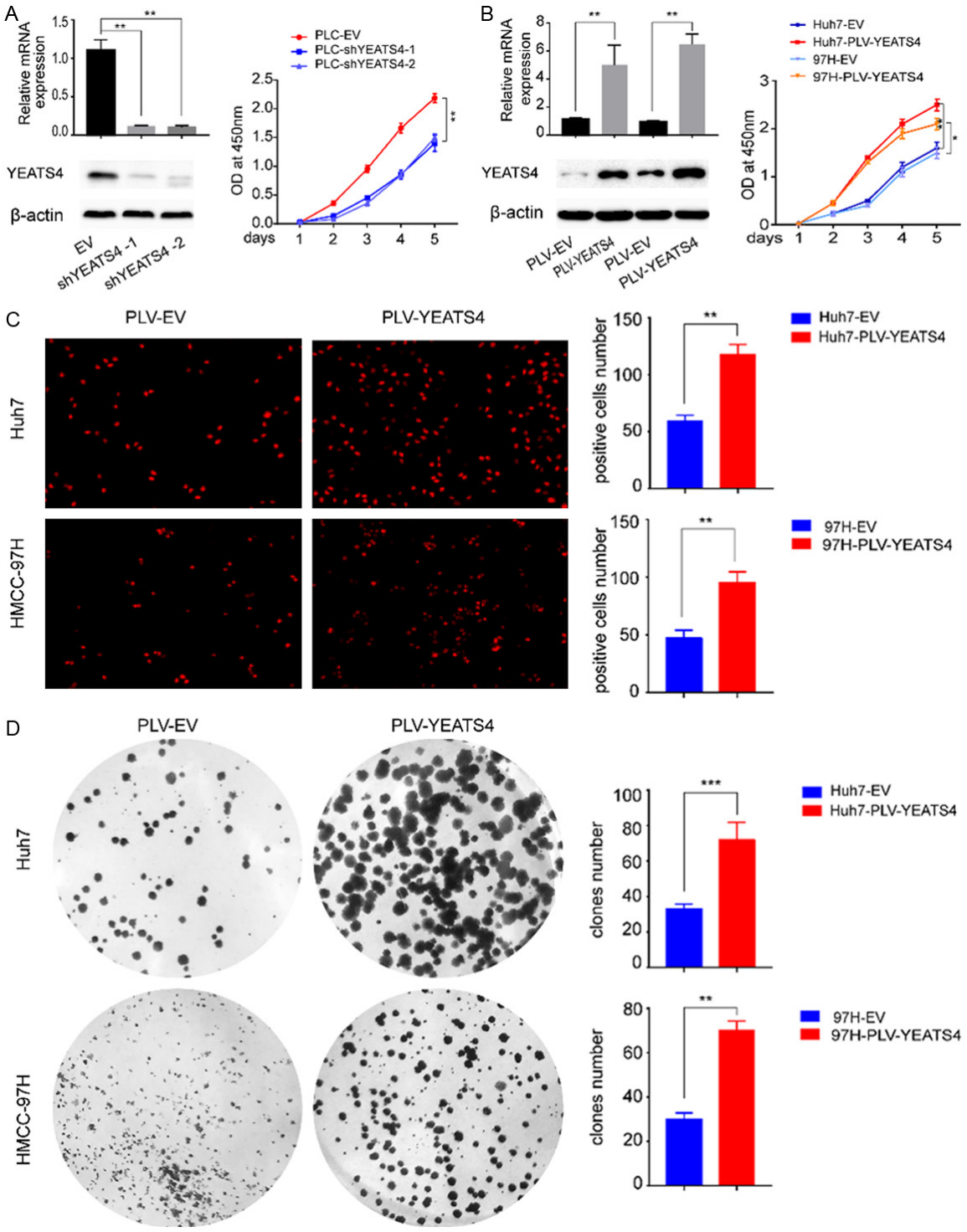

Figure 2. YEATS4 promotes cell proliferation and clone formation in hepatocarcinoma cells in vitro. A. (left) YEATS4 mRNA and protein was dramatic decreased in PLC/PRL/5-shYEATS4 cells. (right) Down-regulation of YEATS4 represses PLC/PRL/5 cell proliferation in CCK-8 assay. B. (left) YEATS4 mRNA and protein was dramatic increased in Huh7 and MHCC-97H-overexpression YEATS4 cells. (right) Up-regulation of promotes Huh7 and MHCC-97H cell proliferation in CCK-8 assay. C. YEATS4-overexpression Huh7 and MHCC-97H cells had a significantly higher positive EdU incorporation rate than Ctrl cells. D. Colony formation assay showed that a marked increase in colony number and size was seen in groups with YEATS4 ectopic expression hepatocarcinoma cells. **P≤0.01, ***P≤0.001.

analysis was performed. We found a negative correlation between YEATS4 levels and clinical prognosis (**Figure 1D**). As shown in **Figure 1E**, based on the 3-years survival rate, patients with abnormally high YEATS4 expression had a shorter overall post-operative survival time compared to those with lower YEATS4 levels (32.4% vs 61.8%, P = 0.015). In addition, the Cox proportional hazards regression model showed that high expression of YEATS4 was an independent risk factor of HCC prognosis (P = 0.016 and P = 0.038, **Table 2**). The prognosis of the patients was in turn significantly associated with the tumor differentiation status and metastasis, whereas no significant association was seen with age, gender and tumor size (P>0.05). Therefore, YEATS4 may be a candidate prognostic marker in HCC biopsy specimens.

YEATS4 promotes HCC cell proliferation and colony formation

The association between high expression levels of YEATS4 and tumor size indicates that YEATS4 may be related to the proliferation and apoptosis of HCC cells. To validate our hypothesis, we depleted endogenous YEATS4 in PLC/PRF/5 cells via the shRNA approach. CCK-8 assay showed that downregulation of YEATS4 remarkably suppressed HCC cell proliferation (**Figure 2A**). In contrast, exogenous overexpression of YEATS4 in the HCC Huh7 and MHCC-97H cell lines had opposite effects on proliferation (**Figure 2B**). An EdU assay on the same cell lines also suggested that higher YEATS4 endowed these cells with a proliferative advantage (P < 0.01, **Figure 2C**). Finally, colony formation assays indicated that YEATS4 overexpression in the Huh7 and MHCC-97H cells was positively correlated with the number and size of colonies (P < 0.01, **Figure 2D**). Therefore, the above results demonstrated that YEATS4 significantly promoted HCC cell proliferation and colony formation.

YEATS4 regulates HCC cell proliferation via TCEA1

To further elucidate the underlying mechanism of YEATS4 mediated HCC progression, we conducted transcriptomic analyses of HCC cell lines with YEATS4 overexpression or knockdown by RNA-SEQ. TCEA1 as a proliferation related protein reported in numerous malignant tumors, TCEA1 was our main focus [10, 14]. As shown in **Figure 3A**, shRNA-mediated knockdown of YEATS4 in PLC/PRF/5 cells significantly downregulated TCEA1 mRNA and protein expression, while ectopic expression of YEATS4 in the MHCC-97H cells upregulated TCEA1 (**Figure 3B**). Furthermore, the expression of the proliferation marker PCNA was correlated to that of YEATS4 and TCEA1. Based on our results and other studies, we hypothesized that YEATS4 mediated HCC cell proliferation may be dependent on TCEA1. To validate this hypothesis, we overexpressed TCEA1 in YEATS4 knockdown PLC/PRF/5 cells, and as shown in **Figure 3C**, **3D**, the proliferative impairment due to lack of YEATS4 was effectively restored by over-expression of TCEA1. Concurrently, CCK-8 assay indicated that knockdown TCEA1 abolished the cell growth promoting effect of ectopic expression of YEATS4 (**Figure 3E**, **3F**). In addition, TCEA1 mRNA expression was significantly upregulated in HCC tissue samples compared to the para-carcinoma tissues, and showed a strong positive correlation with YEATS4 mRNA levels (**Figure 3G**, **3H**, r = 0.048, P < 0.0001). Taken together, these results suggested that YEATS4 overexpression in HCC cells upregulated TCEA1, resulting in excessive proliferation.

YEATS4 blinds to TCEA1 promoter and increases its transcriptional activity

YEATS4 shares a high sequence homology with human MLLT1 and MLLT3 proteins which indicates that its encoded protein may act as a transcription factor [4]. Based on our and others' findings, we hypothesized that YEATS4 may bind to TCEA1 promoter to regulate its transcriptional activity [10, 23, 24], and tested this hypothesis *in vitro* TCEA1 by chromatin immunoprecipitation (ChIP). The ChIP assay was performed in PLC/PRF/5 cells using rabbit anti-YEATS4 monoclonal antibody and rabbit IgG antibody as controls. As shown in **Figure 3I**, **3J**, significant amplification was seen in the TCEA1 promoter (p3) region in the anti-YEATS4 precipitated samples, while none of the sequences were amplified in the control group. These results indicated the direct binding of YEATS4 to the TCEA1 promoter. The effect of YEATS4 on TCEA1 transcriptional activity was analyzed using a TCEA1-luciferase reporter assay. As shown in **Figure 3K**, **3L**, YEATS4 remarkably induced the TCEA1 pro-

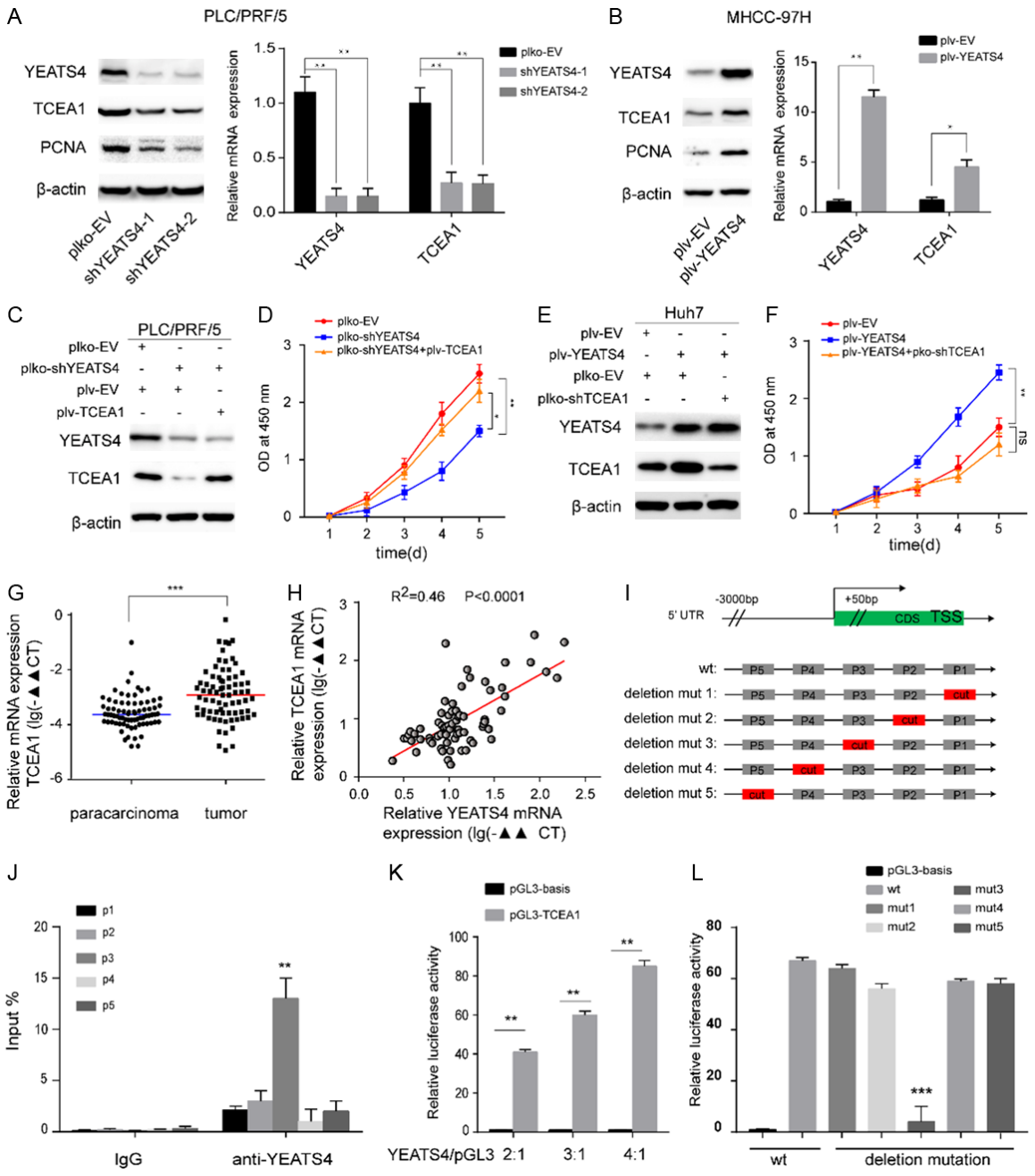

Figure 3. YEATS4 regulates HCC cell proliferation via binding to TCEA1 promoter and increasing its transcription activity. A. The protein and mRNA level of TCEA1 was up-regulated in PLC/PRL/5-shYEATS4 cells compared with PLC/PRL/5-shCtrl. Cell proliferation maker PCNA had corresponding change. B. TCEA1 mRNA and protein level were increased significantly when YEATS4 was over-expression in MHCC-97H cells and PCNA protein expression also upregulated. C, D. CCK-8 assay indicated that YEATS4 down-regulation induced weak proliferation of PLC/PRL/5 cells was strongly restored by ectopic expression of TCEA1. E, F. CCK-8 assay indicated that knockdown TCEA1 abolished the cell growth promoting effect of ectopic expression of YEATS4. G. RT-PCR test showed that TCEA1 was upregulated in HCC tissues compared with paracarcinoma tissues. H. YEATS4 mRNA level in HCC tissues was positive associated with TCEA1. I. The schematic diagram of TCEA1 promoter region for ChIP and luciferase reporter assay. We designed 5 paired random primers for TCEA1 promoter. J. The primers site3 resulted in a strong DNA amplification of the TCEA1 promoter region compare with control, which were detected by Real-time PCR method. K, L. Over-expression YEATS4 could enhance TCEA1 promoter transcription activity, but the effects could be weakened when deletion mutation of promoter sites except site3 in 293T cells. **P≤0.01, ***P≤0.001.

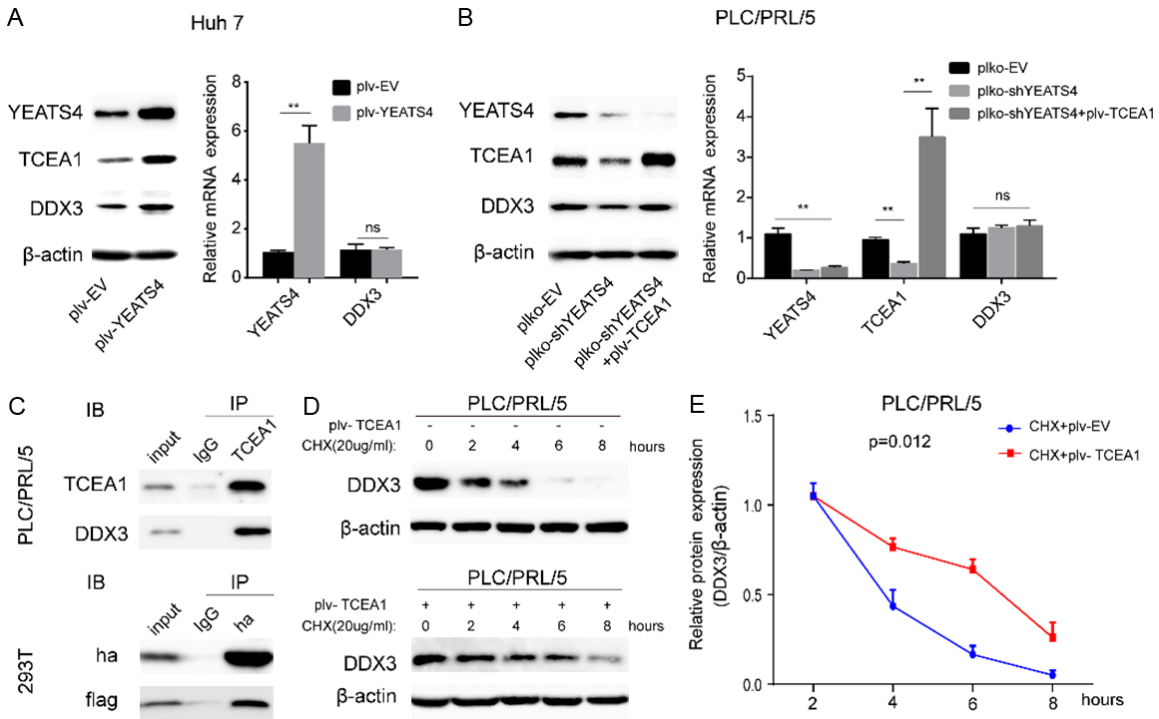

Figure 4. TCEA1 increases the sability of DDX3 protein. A. Ectopic expression of YEATS4 in Huh7 cells increased the DDX3 protein level along with increase of the expression of TCEA1, whereas it had no effect on DDX3 mRNA levels. B. Decreased expression of TCEA1 in PLC/PRL/5-shYEATS4 cells downregulated the DDX3 protein level rather than mRNA level. C. Co-immunoprecipitation experiments indicated that TCEA1 interacts with DDX3 in PLC/PRL/5 and 293T cells. D, E.TCEA1 significantly prolonged the half-life of DDX3 protein. **P≤0.01.

moter driven luciferase reporter gene activity TCEA1. However, the TCEA1 reporter gene was inactivated when the p3 sequence of the TCEA1 promoter was deleted. Taken together, YEATS4 increases the transcriptional activity of TCEA1 by directly binding to TCEA1 promoter at the specific p3 (-1200 to -1500 bp) site.

TCEA1 increases the stability of DDX3 protein

To determine the mechanism of action of TCEA1 and its possible target(s) TCEA1 vis-a-vis HCC cell proliferation, we conducted a protein mass spectrometry analysis following YEATS4 antibody co-immunoprecipitation. DDX3 has been reported in various solid tumors and promotes cancer cell proliferation and metastasis [18, 25-27]. Given that TCEA1 interacts with DDX3, we hypothesized that DDX3 may be modulated by TCEA1 and affect HCC cell proliferation. After confirming a direct physical interaction between TCEA1 and DDX3 proteins via co-immunoprecipitation and Western blotting (**Figure 4C**), we analyzed the latter's expression levels in HCC cells. DDX3 protein expression in Huh7 cells was increased following YEATS4 overexpression, although no changes were seen at the mRNA level (**Figure 4A**). Furthermore, short hairpin RNA (shRNA) mediated depletion of YEATS4 significantly reduced the DDX3 protein levels in PLC/PRF/5 cells, and this inhibition was reversed by the simultaneous ectopic expression of TCEA1 (**Figure 4B**). Based on these findings, we surmised that TCEA1 influences the stability of DDX3 protein rather than its transcription.

To evaluate the possible effect of TCEA1 on the stability of DDX3 protein, the PLC/PRF/5 cells were treated with the protein synthesis inhibitor cycloheximide (CHX). Western blotting showed that TCEA1 significantly prolonged the half-life of DDX3 (**Figure 4D**, **4E**). Taken together, TCEA1 interacted with DDX3 and increased its stability DDX3 thereby augmenting the pro-proliferative effect of YEATS4 in HCC cells.

YEATS4 accelerates the growth of xenograft tumor in vivo

To determine the *in vivo* significance of YEATS4, we constructed a xenograft model of HCC by subcutaneously transplanting the animals with

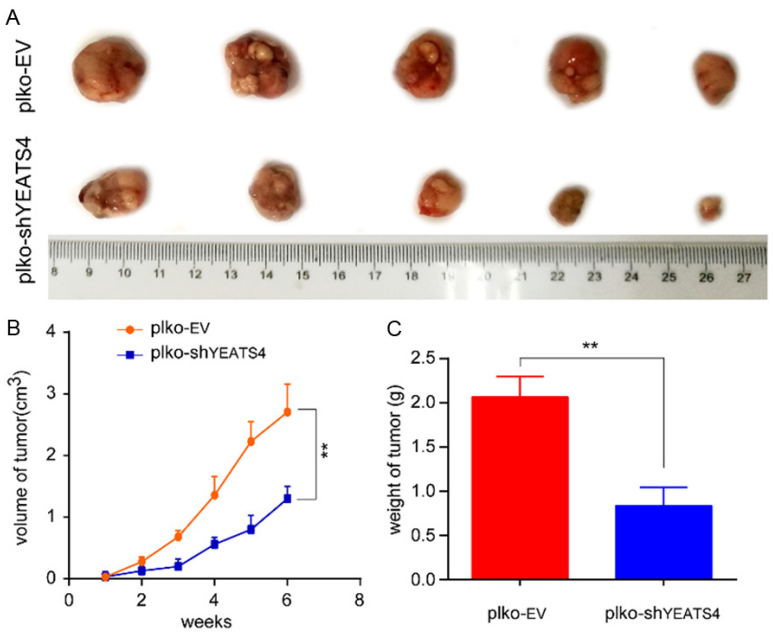

Figure 5. YEATS4 accelerates xenograft tumor growth in vivo. A. The volume of tumors of PLC/PRL/5-shYEATS4 group was smaller than control group. B. Growth curves of Ctrl and PLC/PRL/5-shYEATS4 models. C. Tumor weight in the PLC/PRL/5-shYEATS4 group was smaller than that in the control group.

equal numbers of PLC/PRL/5-plko-shYEATS4 and PLC/PRL/5-plko-EV cells. Tumors were successfully formed in both groups after 1 week, and tumor growth was monitored for two months. The tumors of the PLC/PRL/5-plko-shYEATS4 group had a slower growth rate than those of the PLC/PRL/5-plko-EV group (**Figure 5B**). At the end of the observation period, the animals were sacrificed, and the tumors were removed and weighed. As shown in **Figure 5A** and **5C**, the tumor size and weight of the PLC/PRL/5-plko-shYEATS4 group was significantly less compared to that of the control group ($P < 0.01$). These findings indicated that YEATS4 accelerated xenograft tumor growth *in vivo*, consistent with the *in vitro* data.

Discussion

Several aberrantly expressed genes have been identified in HCC, including YEATS4, which is usually amplified in astrocytomas and glioblastomas and was originally identified in the glioblastoma multiform cell line TX3868 [3, 28]. An increasing number of studies have found a correlation between YEATS4 mRNA or protein expression and cancers like colorectal cancer, NSCLC, ovarian cancer, gastric cancer, pancreatic cancer and glioblastoma [5-9, 29, 30]. However, its functions in HCC, a malignant tumor with high morbidity and mortality [31], is relatively poorly understood.

We have reported for the first time that YEATS4 mRNA and protein are up-regulated in HCC tissues compared to non-tumor tissues (**Figure 1A-C**), and higher YEATS4 levels are significantly associated with large tumor size, poor differentiation and distant metastasis (**Table 1**), which is consistent with previous reports on other solid tumors [3, 5, 7, 9]. Overexpression of YEATS4 might also be an independent risk factor for HCC prognosis, and YEATS4 is a potential pretreatment prognostic marker in liver biopsy specimens. The pro-proliferative and pro-invasive effects of YEATS4 is dependent on the deregulation of p53 and Wnt pathways [6, 7], and previous studies have also shown that YEATS4 acts as a transcription factor [8]. Consistent with this, we were able to demonstrate that the oncogenic role of YEATS4 in HCC was associated with its binding to, and the transcriptional activation of the TCEA1 promoter. Furthermore, YEATS4 mediated HCC cell proliferation was also dependent on TCEA1 expression, thereby showing for the first time that TCEA1 is a target gene of YEATS4TCEA1.

Although previous studies have shown a TCEA1 significant upregulation of TCEA1 in HCC cells [14], the underlying mechanism, especially TCEA1 with respect to proliferation, remains unclear. We found that TCEA1 increased HCC cell proliferation by interacting with DDX3, and that TCEA1 upregulated DDX3 protein levels by enhancing its stability rather than its transcription. TCEA1 the exact mechanism of TCEA1 mediated regulation of DDX3 stability, whether direct or indirect, remains to be elucidated.

The primary role of DDX3, an RNA helicase, is post-transcriptional mRNA processing [32]. Previous studies have shown that DDX3 plays an oncogenic role in HCC [20, 27], and various other tumors [17, 20, 26, 33, 34]. Mechanistically, DDX3 inhibits apoptosis [35], and

promotes cell cycle progression [26, 36], migration and invasion [33, 37]. In addition, DDX3 is dysregulated in hepatitis virus-associated HCC and is involved in cell proliferation, although the regulatory mechanism of DDX3 in HCC remains to be validated. Our results showed that DDX3 was the active target of TCEA1 and accelerated HCC cell proliferation *in vitro* and *in vivo*. Aberrantly high levels of YEATS4 in HCC cells upregulated TCEA1, which induced an increase in DDX3 protein levels TCEA1 thereby resulting in higher proliferation rates.

In conclusion, we have shown the oncogenic role of the YEATS4/TCEA1/DDX3 axis in HCC for the first time, and presented YEATS4 as a potential therapeutic target and prognosis maker for HCC.

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Disclosure of conflict of interest

None.

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References

[1] Ullrich A and Miller A. Global response to the burden of cancer: the WHO approach. Am Soc Clin Oncol Educ Book 2014; e311-315.

[2] Hasegawa K, Kokudo T and Kokudo N. Current evidence and topics of diagnosis and treatment for hepatocellular carcinoma. Nihon Shokakibyo Gakkai Zasshi 2017; 114: 1585-1592.

[3] Fischer U, Heckel D, Michel A, Janka M, Hulsebos T and Meese E. Cloning of a novel transcription factor-like gene amplified in human glioma including astrocytoma grade I. Hum Mol Genet 1997; 6: 1817-1822.

[4] Debernardi S, Bassini A, Jones LK, Chaplin T, Linder B, de Bruijn DR, Meese E and Young BD. The MLL fusion partner AF10 binds GAS41, a protein that interacts with the human SWI/SNF complex. Blood 2002; 99: 275-281.

[5] Ji S, Zhang Y and Yang B. YEATS domain containing 4 promotes gastric cancer cell proliferation and mediates tumor progression via activating the wnt/beta-catenin signaling pathway. Oncol Res 2017; 25: 1633-1641.

[6] Jixiang C, Shengchun D, Jianguo Q, Zhengfa M, Xin F, Xuqing W, Jianxin Z and Lei C. YEATS4 promotes the tumorigenesis of pancreatic cancer by activating beta-catenin/TCF signaling. Oncotarget 2017; 8: 25200-25210.

[7] Pikor LA, Lockwood WW, Thu KL, Vucic EA, Chari R, Gazdar AF, Lam S and Lam WL. YEATS4 is a novel oncogene amplified in non-small cell lung cancer that regulates the p53 pathway. Cancer Res 2013; 73: 7301-7312.

[8] Kim YR, Park MS, Eum KH, Kim J, Lee JW, Bae T, Lee DH and Choi JW. Transcriptome analysis indicates TFEB1 and YEATS4 as regulatory transcription factors for drug resistance of ovarian cancer. Oncotarget 2015; 6: 31030-31038.

[9] Tao K, Yang J, Hu Y and Deng A. Knockdown of YEATS4 inhibits colorectal cancer cell proliferation and induces apoptosis. Am J Transl Res 2015; 7: 616-623.

[10] Ito T, Seldin MF, Taketo MM, Kubo T and Natori S. Gene structure and chromosome mapping of mouse transcription elongation factor S-II (Tcea1). Gene 2000; 244: 55-63.

[11] Kugawa F and Aoki M. Genomic cloning of Xenopus TFIIS (TCEA1) and identification of its transcription start site. DNA Seq 2002; 13: 55-60.

[12] Zali H and Rezaei Tavirani M. Meningioma protein-protein interaction network. Arch Iran Med 2014; 17: 262-272.

[13] Park KS, Cha Y, Kim CH, Ahn HJ, Kim D, Ko S, Kim KH, Chang MY, Ko JH, Noh YS, Han YM, Kim J, Song J, Kim JY, Tesar PJ, Lanza R, Lee KA and Kim KS. Transcription elongation factor Tcea3 regulates the pluripotent differentiation potential of mouse embryonic stem cells via the Lefty1-Nodal-Smad2 pathway. Stem Cells 2013; 31: 282-292.

[14] Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y and Nakamura Y. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001; 61: 2129-2137.

[15] Leitao AL, Costa MC and Enguita FJ. Unzippers, resolvers and sensors: a structural and functional biochemistry tale of RNA helicases. Int J Mol Sci 2015; 16: 2269-2293.

[16] Hathaichoti S, Visitnonthachai D, Ngamsiri P, Niyomchan A, Tsogtbayar O, Wisessaowapak C, Watcharasit P and Satayavivad J. Paraquat induces extrinsic pathway of apoptosis in A549 cells by induction of DR5 and repression of anti-apoptotic proteins, DDX3 and GSK3 expression. Toxicol In Vitro 2017; 42: 123-129.

[17] Heerma van Voss MR, Brilliant JD, Vesuna F, Bol GM, van der Wall E, van Diest PJ and Raman V. Combination treatment using DDX3 and PARP inhibitors induces synthetic lethality in BRCA1-proficient breast cancer. Med Oncol 2017; 34: 33.

[18] Wu DW, Lin PL, Wang L, Huang CC and Lee H. The YAP1/SIX2 axis is required for DDX3-mediated tumor aggressiveness and cetuximab resistance in KRAS-wild-type colorectal cancer. Theranostics 2017; 7: 1114-1132.

[19] Botlagunta M, Vesuna F, Mironchik Y, Raman A, Lisok A, Winnard P Jr, Mukadam S, Van Diest P, Chen JH, Farabaugh P, Patel AH and Raman V. Oncogenic role of DDX3 in breast cancer biogenesis. Oncogene 2008; 27: 3912-3922.

[20] Chang PC, Chi CW, Chau GY, Li FY, Tsai YH, Wu JC and Wu Lee YH. DDX3, a DEAD box RNA helicase, is deregulated in hepatitis virus-associated hepatocellular carcinoma and is involved in cell growth control. Oncogene 2006; 25: 1991-2003.

[21] Huang JS, Chao CC, Su TL, Yeh SH, Chen DS, Chen CT, Chen PJ and Jou YS. Diverse cellular transformation capability of overexpressed genes in human hepatocellular carcinoma. Biochem Biophys Res Commun 2004; 315: 950-958.

[22] Li J, Zhang S, Hu Q, Zhang K, Jin J, Zheng X, Yin Z and Wang X. The NKD1/Rac1 feedback loop regulates the invasion and migration ability of hepatocarcinoma cells. Sci Rep 2016; 6: 26971.

[23] Carriere L, Graziani S, Alibert O, Ghavi-Helm Y, Boussouar F, Humbertclaude H, Jounier S, Aude JC, Keime C, Murvai J, Foglio M, Gut M, Gut I, Lathrop M, Soutourina J, Gerard M and Werner M. Genomic binding of Pol III transcription machinery and relationship with TFIIS transcription factor distribution in mouse embryonic stem cells. Nucleic Acids Res 2012; 40: 270-283.

[24] Flynn RA, Almada AE, Zamudio JR and Sharp PA. Antisense RNA polymerase II divergent transcripts are P-TEFb dependent and substrates for the RNA exosome. Proc Natl Acad Sci U S A 2011; 108: 10460-10465.

[25] Bol GM, Xie M and Raman V. DDX3, a potential target for cancer treatment. Mol Cancer 2015; 14: 188.

[26] Heerma van Voss MR, Vesuna F, Trumpi K, Brilliant J, Berlinicke C, de Leng W, Kranenburg O, Offerhaus GJ, Burger H, van der Wall E, van Diest PJ and Raman V. Identification of the DEAD box RNA helicase DDX3 as a therapeutic target in colorectal cancer. Oncotarget 2015; 6: 28312-28326.

[27] Li HK, Mai RT, Huang HD, Chou CH, Chang YA, Chang YW, You LR, Chen CM and Lee YH. DDX3 represses stemness by epigenetically modulating tumor-suppressive miRNAs in hepatocellular carcinoma. Sci Rep 2016; 6: 28637.

[28] Fischer U, Meltzer P and Meese E. Twelve amplified and expressed genes localized in a single domain in glioma. Hum Genet 1996; 98: 625-628.

[29] Fu Q, Cheng J, Zhang J, Zhang Y, Chen X, Xie J and Luo S. Downregulation of YEATS4 by miR-218 sensitizes colorectal cancer cells to L-OHP-induced cell apoptosis by inhibiting cytoprotective autophagy. Mod Pathol 2016; 36: 3682-3690.

[30] Lee JC, Lu TP, Changou CA, Liang CW, Huang HN, Lauria A, Huang HY, Lin CY, Chiang YC, Davidson B, Lin MC and Kuo KT. Genomewide copy number analysis of Mullerian adenosarcoma identified chromosomal instability in the aggressive subgroup. Mod Pathol 2016; 29: 1070-1082.

[31] Greten TF and Sangro B. Targets for immunotherapy of liver cancer. J Hepatol 2017; [Epub ahead of print].

[32] Tanner NK and Linder P. DExD/H box RNA helicases: from generic motors to specific dissociation functions. Mol Cell 2001; 8: 251-262.

[33] Chen HH, Yu HI, Cho WC and Tarn WY. DDX3 modulates cell adhesion and motility and cancer cell metastasis via Rac1-mediated signaling pathway. Oncogene 2015; 34: 2790-2800.

[34] Wu DW, Lee MC, Wang J, Chen CY, Cheng YW and Lee H. DDX3 loss by p53 inactivation promotes tumor malignancy via the MDM2/Slug/E-cadherin pathway and poor patient outcome in non-small-cell lung cancer. Oncogene 2014; 33: 1515-1526.

[35] Li Y, Wang H, Wang Z, Makhija S, Buchsbaum D, LoBuglio A, Kimberly R and Zhou T. Inducible resistance of tumor cells to tumor necrosis factor-related apoptosis-inducing ligand receptor 2-mediated apoptosis by generation of a blockade at the death domain function. Cancer Res 2006; 66: 8520-8528.

[36] Bol GM, Vesuna F, Xie M, Zeng J, Aziz K, Gandhi N, Levine A, Irving A, Korz D, Tantravedi S, Heerma van Voss MR, Gabrielson K, Bordt EA, Polster BM, Cope L, van der Groep P, Kondaskar A, Rudek MA, Hosmane RS, van der Wall E, van Diest PJ, Tran PT and Raman V. Targeting DDX3 with a small molecule inhibitor for lung cancer therapy. EMBO Mol Med 2015; 7: 648-669.

[37] Sun M, Song L, Zhou T, Gillespie GY and Jope RS. The role of DDX3 in regulating Snail. Biochim Biophys Acta 2011; 1813: 438-447.

Table S1. Primers sequences

	Primer name	F: 5'-3'	R: 5'-3'
RT-PCR	YEATS4	TGAAAGACCTGTAACCCTGTATC	CATCATTGCTGTTGGGTCTTG
	TCEA1	TGGACAAGATGGTGCAGAAG	GCAGTAATTCCAGGGTCATAGG
	DDX3	GGTATTAGCACCAACGAGAGAG	TATCGGCACCACCATAAACC
ChIP	P1	CACCTAGAAGAGTGACTGGAAC	TTCAGTTTGGAGGGAACATACT
	P2	AAGGTAGCGGAGAGAGAGTT	AGCCTGTGTTGTTGGGTTAT
	P3	CTCCAAAGCCCATCCTTCTAA	GAGCAAGACTCCGTCTCAAA
	P4	CCGGAGCTCTTTCAGTATCATC	GAGGGAATAAGCCAAGAAGGAG
	P5	TGTGGAAACTCATCTCCTTAACA	TGCAGTCACATCACAGGAC
PGL3.0	Promoter	CCAAGTGCTCTCCACTCAGGCTC	TTTACTCTCCCGCCGGAGTCA
PLV-puro	YEATS4	GCTCTAGAATGTTCAAGAGAATGGCCGAA	CCGCTCGAGTTATATGTCTTTTGCTTGGT
	ha-YEATS4	GCTCTAGAATGTACCCATACGACGTCCCAGACTACGCTTTCAAGAGAATGGCCGAA	CCGCTCGAGTTATATGTCTTTTGCTTGGT
	flag-TCEA1	GCTCTAGAATGGATTACAAGGATGACGACGATAAGGAGGACGAAGTGGTCCGCTT	TCAACAGAACTTCCATCGATTTCC
PLKO.1	shYEATS4-1	CCGGTAACCCTGTATCATTTGCTAAAGCTTCAAGAGAGCTTTAGCAAATGATACAGGGTTTTTTTTG	AATTCAAAAAAAACCCTGTATCATTTGCTAAAGCTCTCTTGAAGCTTTAGCAAATGATACAGGGTTA
	shYEATS4-2	CCGGTAAGGGTGTTACTATCGTTAAACCTTCAAGAGAGGTTTAACGATAGTAACACCCTTTTTTTTG	AATTCAAAAAAAAGGGTGTTACTATCGTTAAACCTCTCTTGAAGGTTTAACGATAGTAACACCCTTA
	shTCEA-1	CCGGTGTGGAGCATTGGATTTGCTAAAGGTTCAAGAGACCTTTAGCAAATCCAATGCTCCATTTTTTG	AATTCAAAAAATGGAGCATTGGATTTGCTAAAGGTCTCTTGAACCTTTAGCAAATCCAATGCTCCACA
	shTCEA-2	CCGGTATGGCTAGTGATGAGCTGAAAGATTCAAGAGATCTTTCAGCTCATCACTAGCCATTTTTTTG	AATTCAAAAAAATGGCTAGTGATGAGCTGAAAGATCTCTTGAATCTTTCAGCTCATCACTAGCCATA