Attenuated ZHX3 expression is predictive of poor outcome for liver cancer: Indication for personalized therapy

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Abstract. The zinc-fingers and homeoboxes (ZHX) family members have been characterized as master regulators in cancer initiation and development. The present study performed *in silico* data-mining with publicly available datasets and immunohistochemistry to assess the expression status of ZHX factors and the corresponding prognostic implications in liver cancer. Increased ZHX3 mRNA expression was associated with favorable overall survival in patients with liver cancer. Subgroups analyses revealed a significant association between the expression of ZHX factors and outcomes in select patient cohorts. Immunohistochemical analysis supported that ZHX3 expression was an independent prognostic indicator for patient survival. These results suggested that dysregulation of ZHX factors is involved in disease progression and ZHX3 expression may serve as a prognostic biomarker for liver cancer.

Introduction

Primary liver cancer remains one of the commonest types of cancers and hepatocellular carcinoma (HCC) is its major histologic subtype (1). Incidence and mortality of liver cancer are associated with the infection of viral hepatitis, which is a disease with significant geographic distributions worldwide (2). Although multiple novel techniques are now available for treatment of this heterogeneous disease, identification and validation of molecular factors that hold prognostic and therapeutic promise are urgently needed.

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Through past efforts in finding novel molecular markers associated with survival outcomes of patients with breast and gastric cancers by *in silico* data-mining analysis, it was found that the zinc-fingers and homeoboxes (ZHX) family members may be among the targets (3,4). ZHX factors, including ZHX1, ZHX2 and ZHX3, have been reported as a group of transcription factors with two zinc-finger motifs and five homeobox DNA-binding domains existing in the cell nucleus (5-10). Evidence has indicated that ZHX factors are important transcriptional regulators in downstream signaling that is involved in the osteogenic differentiation of mesenchymal stem cells, development and differentiation of hematopoietic cells and maintenance of neural progenitors (5,11,12). Misexpression of ZHX factors has been associated with development of various diseases, such as neurological, hematological and kidney diseases (5,13,14). Moreover, results from relevant studies suggest that ZHX family members are involved in initiation and development of a variety of types of cancer (3-5). The crucial roles of ZHX factors provide reason enough for them as candidate biomarkers for cancer surveillance, diagnosis and survival prediction. Nevertheless, to the best of the authors' knowledge, the prognostic values of individual ZHX factors in liver cancer remain to be elucidated. The present study examined the expression patterns of ZHX factors and the corresponding prognostic implications in liver cancer, using integrative bioinformatics analyses with a set of online available databases, including the Oncomine (http://www.oncomine.org/) (15), Tumor IMmune Estimation Resource (TIMER) 2.0 (16), Cancer Cell Line Encyclopedia (CCLE) database (http://sites.broadinstitute.org/ccle//) (3,4,17), Kaplan-Meier Plotter (http://kmplot.com/analysis/) (18,19) and cBioPortal (http://www.cbioportal.org/) (20,21). Further, immunohistochemistry was performed to confirm ZHX3 protein expression in liver cancer, as well as its association with clinicopathologic variables and survival outcomes.

Materials and methods

Oncomine database analysis. The present study analyzed the expression of distinct ZHX factors in cancers through the Oncomine database (15). When the transcriptional expression of ZHX factors in tumor tissues were compared to those in noncancerous tissues, $P<0.01$ with a fold-change = 2 was considered as statistically significant. Paired Student's t-test was used.

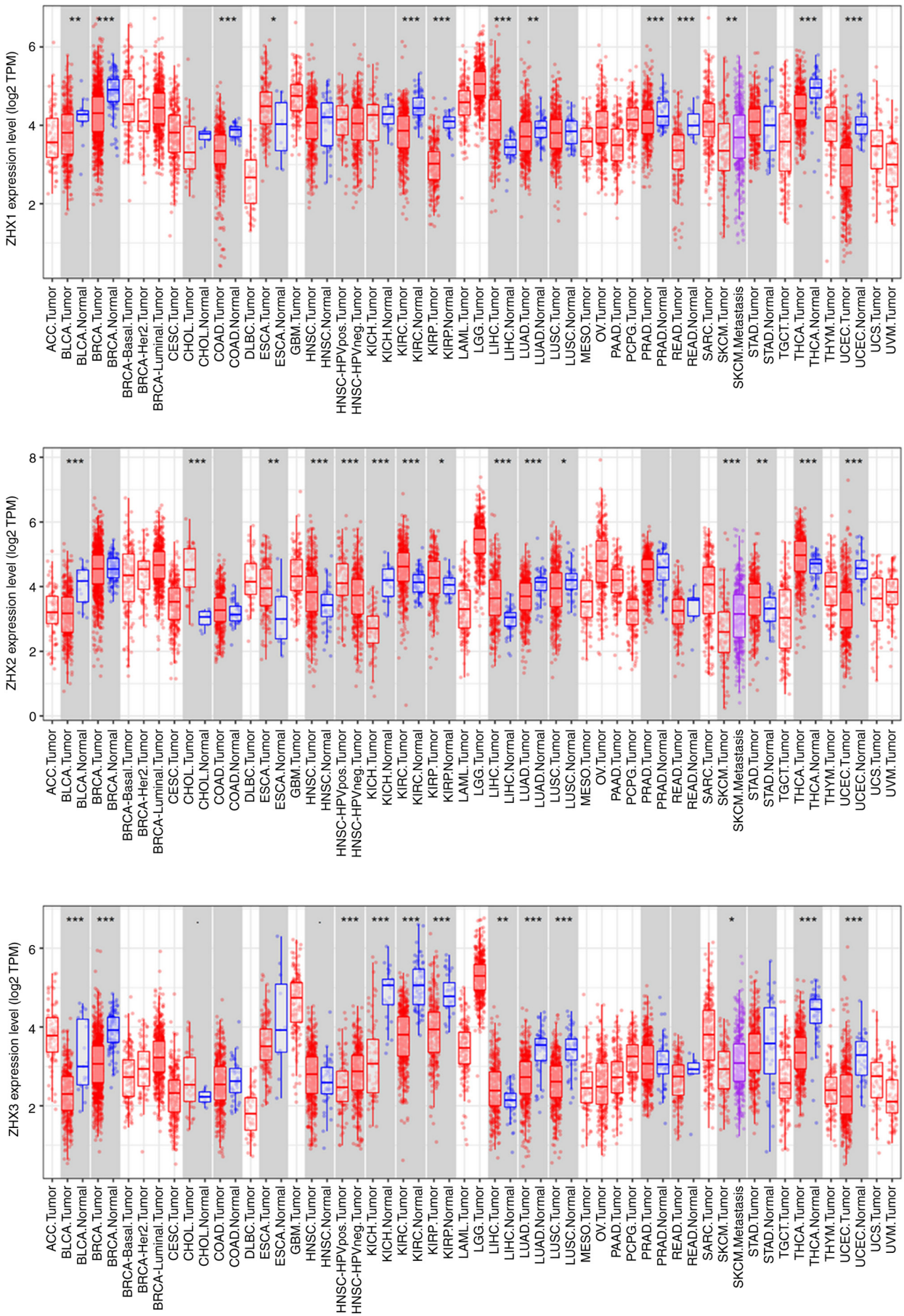

Figure 1. Transcription levels of ZHX family members in different types of cancer. Graphics obtained from the TIMER database indicates the expression status of ZHX factors at the transcriptional level in cancer tissues compared to corresponding normal tissues. *P<0.05; **P<0.01, ***P<0.001. ZHX, zinc-fingers and homeoboxes; TIMER, Tumor IMmune Estimation Resource.

Tumor IMmune Estimation Resource (TIMER) database analysis. TIMER web server is an integrated online database for comprehensive analysis of immune infiltrates through multiple types of cancer (16). In the current study, the gene expression profile of ZHX factors in multiple types of cancer were evaluated via TIMER database analysis (https://cistrome.shinyapps.io/timer/).

CCLE database analysis. The mRNA expression levels of specific ZHX factors in diverse types of cancer cell lines were determined using the CCLE database (http://portals.broadinstitute.org/ccle/), as described previously (3,4,17).

Kaplan-Meier Plotter survival analysis. The prognostic impacts of ZHX mRNA levels were analyzed using the Kaplan-Meier Plotter online database, which includes the information of 54,675 genes on survival using 10,461 clinical cancer samples, including 364 from patients with liver cancer for outcome prediction analysis (18,19). Data sources contain those from the Gene Expression Omnibus (GEO), the European Genome-phenome Archive (EGA) and the Cancer Genome Atlas (TCGA). To investigate the overall survival (OS) and relapse-free survival (RFS) rates, patients were separated into high and low-expression groups according to the median mRNA expression levels so that survival analyses were conducted to produce Kaplan-Meier plots. Hazard ratio with 95% confidence interval and log-rank P-values were calculated.

cBioPortal cancer genomics database analysis. The effects of genomic alterations of ZHX genes containing mutations and copy-number variance on OS and disease-free survival (DFS) rates in patients with liver cancer were analyzed using the cBioPortal online database (20,21). The raw data used prior to bioinformatic analysis are derived from GEO and TCGA. In the present study, OncoPrint in cBioPortal were employed to demonstrate the proportion and distribution of samples with genetic alterations in ZHX genes.

Immunohistochemistry and evaluation. The immunohistochemical staining for ZHX3 protein expression was performed using a standard EnVision complex method previously described (3,4,22,23). One tissue microarray chip containing 94 primary HCC tissues and 86 adjacent noncancerous tissues was purchased from Outdo Biotech Co., Ltd. Following deparaffinization, rehydration and antigen retrieval, 4-μm sections of tissue samples were incubated with a rabbit polyclonal anti-ZHX3 antibody (catalog no. ab84677; dilution, 1:500; Abcam) overnight at 4˚C. ZHX3 protein staining was visualized using an EnVision antibody complex (anti-Mouse/Rabbit) method with an Envision Detection kit (OriGene Technologies, Inc.) and 3,3'-diaminobenzidine as the chromogen substrate. Nuclei were counterstained with 0.5% hematoxylin for 2 min at room temperature.

A total of 10 random microscopic fields per slide (magnification, x400) were evaluated by two independent observers who were unaware of the clinical information. Immunostaining was graded semi-quantitatively by multiplication of staining intensity and percentage of positive cells. The mean percentage of positively stained cells was scored as follows: 0-5% (0); 5-25% (1); 26-50% (2); 51-75% (3); and 76-100% (4). The staining intensity was categorized as follows: Absent (0); weak (1); moderate (2); and strong (3). Tumor samples exhibiting a final staining score of <2 were defined as low ZHX3 expression and those with scores ≥2 as high ZHX3 expression.

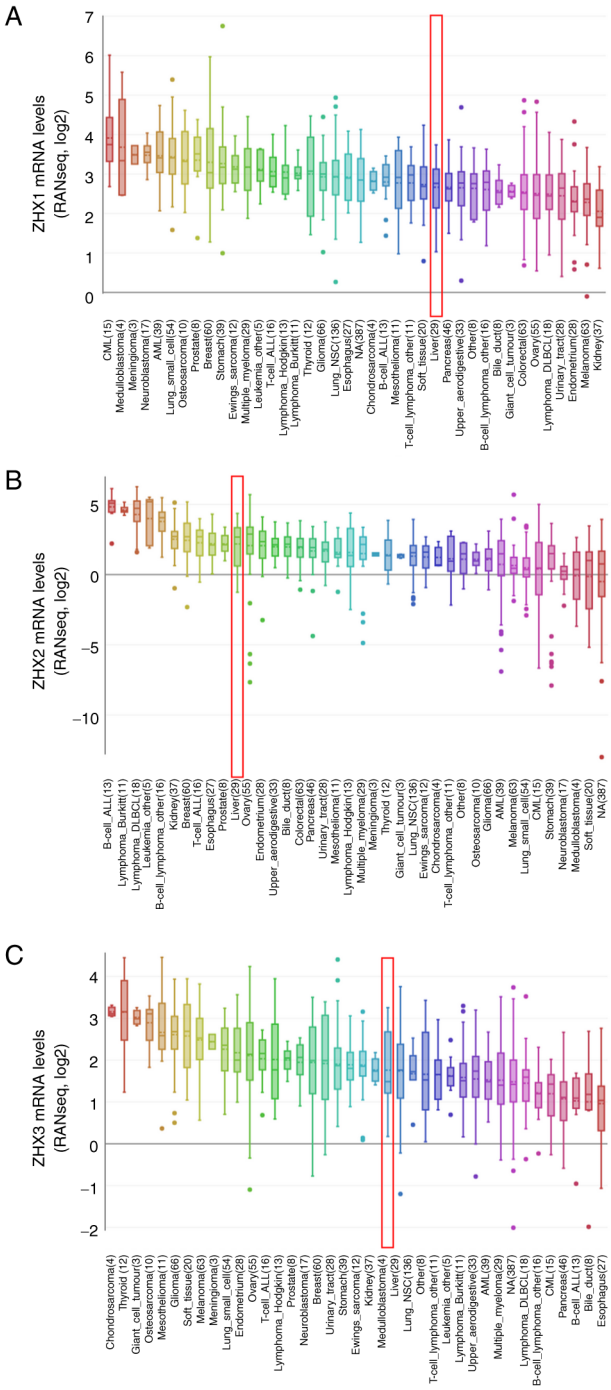

Figure 2. ZHX factors are distinctively expressed in liver cancer cell lines according to CCLE analysis. The mRNA expression levels of (A) ZHX1, (B) ZHX2 and (C) ZHX3 in liver cancer cells ranked the 27th, 11th and 23rd highest among different types of cancer (shown in red frame). ZHX, zinc-fingers and homeoboxes; CCLE, Cancer Cell Line Encyclopedia.

Statistical analysis. Statistical analyses were conducted using the SPSS 17.0 statistical software package (SPSS Inc.). Associations between the expression levels of ZHX factors

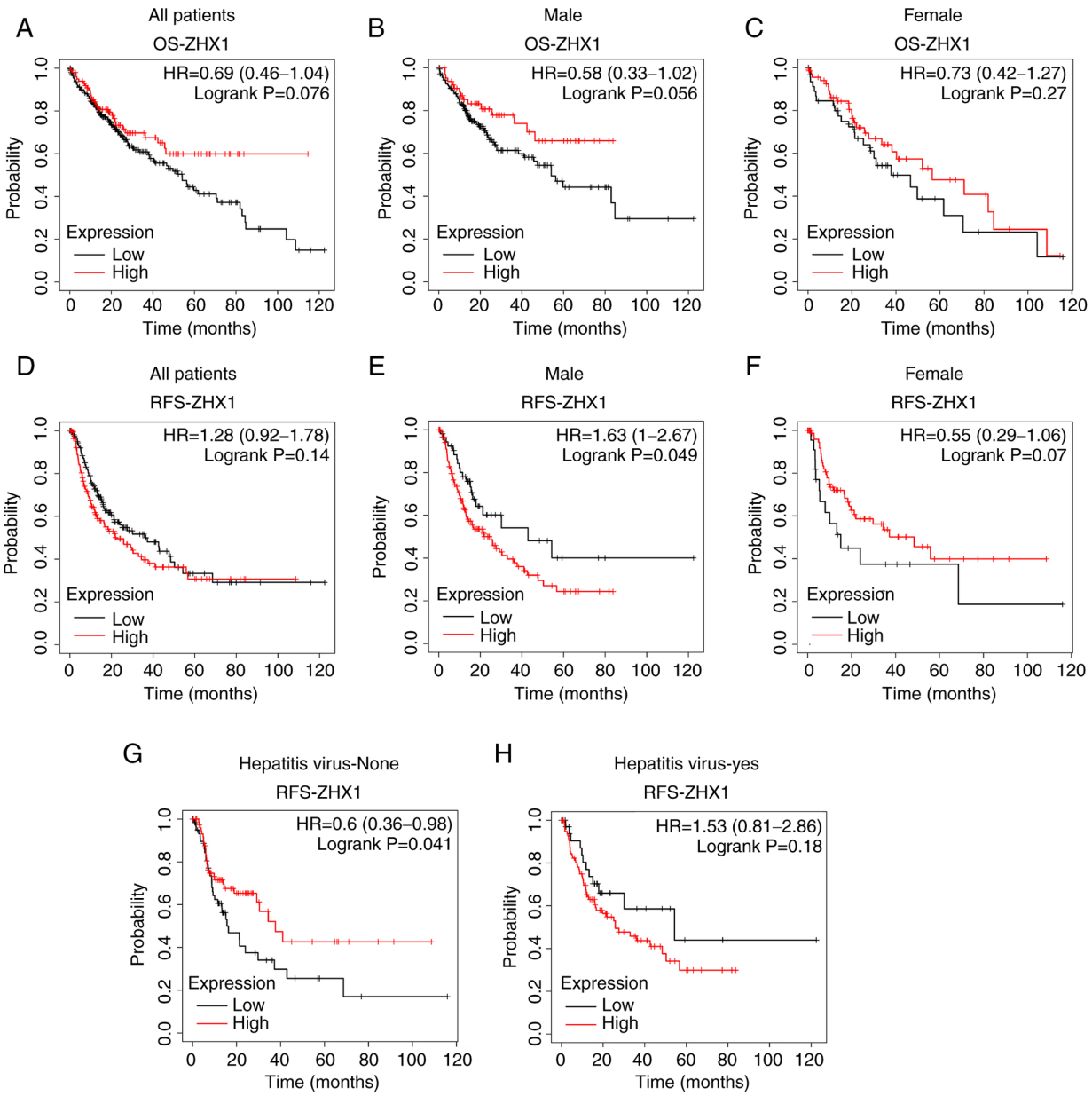

Figure 3. Correlation between ZHX1 mRNA expression and survival outcomes in patients with liver cancer using the Kaplan-Meier plotter online database. (A) OS analysis of ZHX1 in all patients. OS analysis of ZHX1 in (B) male and (C) female patients. (D) RFS analysis of ZHX1 in all patients. RFS analysis of ZHX1 in (E) male and (F) female patients. (G and H) RFS analysis of ZHX1 in patients (G) without and (H) with hepatitis virus infection. ZHX, zinc-fingers and homeoboxes; OS, overall survival; RFS, relapse-free survival.

and clinicopathological variables were assessed using the Pearson's χ^2 test or Fisher's exact test. Survival curves were produced using the Kaplan-Meier method and compared with the log-rank test. The prognostic significance of the clinicopathological variables was determined using a univariate Cox regression analysis. A Cox proportional hazards regression model for multivariate analysis was employed for factors that achieved significance in the univariate analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

mRNA expression profile of ZHX factors in human cancers. Hitherto, three ZHX factors were characterized in a variety of types of human cancer. Our previous study revealed that the Oncomine database provided a total of 308, 434 and 416 unique analyses for ZHX1, ZHX2 and ZHX3, respectively (3). However, the mRNA levels of ZHX factors were not found in liver cancer datasets. The present study thus examined the mRNA expression of ZHX factors in multiple types of cancer using the TIMER online database. The expression of all three ZHX factors was significantly higher in liver hepatocellular carcinoma (LIHC) tissues than in normal tissues (Fig. 1). Additionally, analyses from the CCLE database revealed that the mRNA levels of ZHX1, ZHX2 and ZHX3 in liver cancer cells ranked the 27th, 11th and 23rd highest across all types of cancer, respectively (Fig. 2).

Association between the expression of ZHX factors and survival outcomes. The present study next identified the

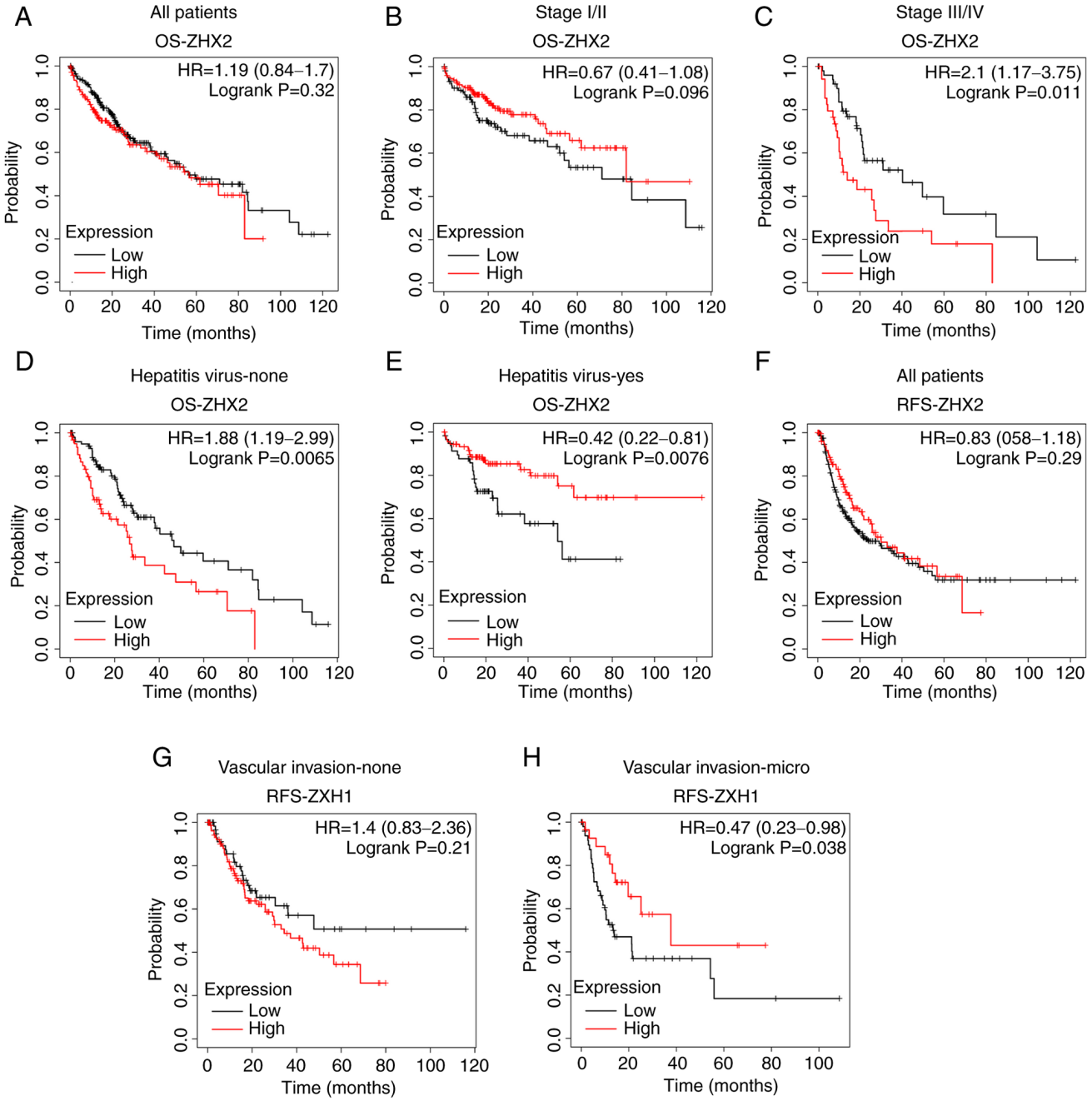

Figure 4. Association between ZHX2 mRNA expression and survival outcomes in patients with liver cancer using the Kaplan-Meier plotter database. (A) OS analysis of ZHX2 in all patients. OS analysis of ZHX2 in patient with (B) Stage I/II and (C) Stage III/IV tumors. OS analysis of ZHX1 in in patients (D) without and (E) with hepatitis virus infection. (F) RFS analysis of ZHX2 in all patients. RFS analysis of ZHX2 in patients (G) without and (H) with vascular invasion. ZHX, zinc-fingers and homeoboxes; OS, overall survival; RFS, relapse-free survival.

prognostic impacts of ZHX family members on patient outcome via Kaplan-Meier plotter survival analysis. ZHX1 mRNA level was not significantly associated with OS in patients with liver cancer (Fig. 3A). Subgroup analyses showed no significant association between ZHX1 mRNA expression and male patients or female patients (Fig. 3B and C). Similarly, ZHX1 mRNA expression was not correlated with RFS in patients with liver cancer. Low expression of ZHX1 predicted a longer RFS rate in male patients, but not in female patients (Fig. 3E and F). Increased ZHX1 expression also displayed a longer RFS rate in patients without hepatitis virus infection (Fig. 3G and H).

No significant association was observed between ZHX2 mRNA levels and OS in patients with liver cancer (Fig. 4A). Subgroup analyses suggested that decreased ZHX2 expression indicated a longer OS rate in patients with stage III/IV tumors but not in patients with stage I/II tumors (Fig. 4B and C). Decreased ZHX2 mRNA level was associated with an improved OS in patients without hepatitis virus infection (Fig. 4D), whereas increased ZHX2 expression was associated a favorable OS in patients with hepatitis virus infection (Fig. 4E). Similarly, ZHX2 expression was not significantly associated with RFS in patients with liver cancer (Fig. 4F). High expression of ZHX2 implied longer RFS times in patients

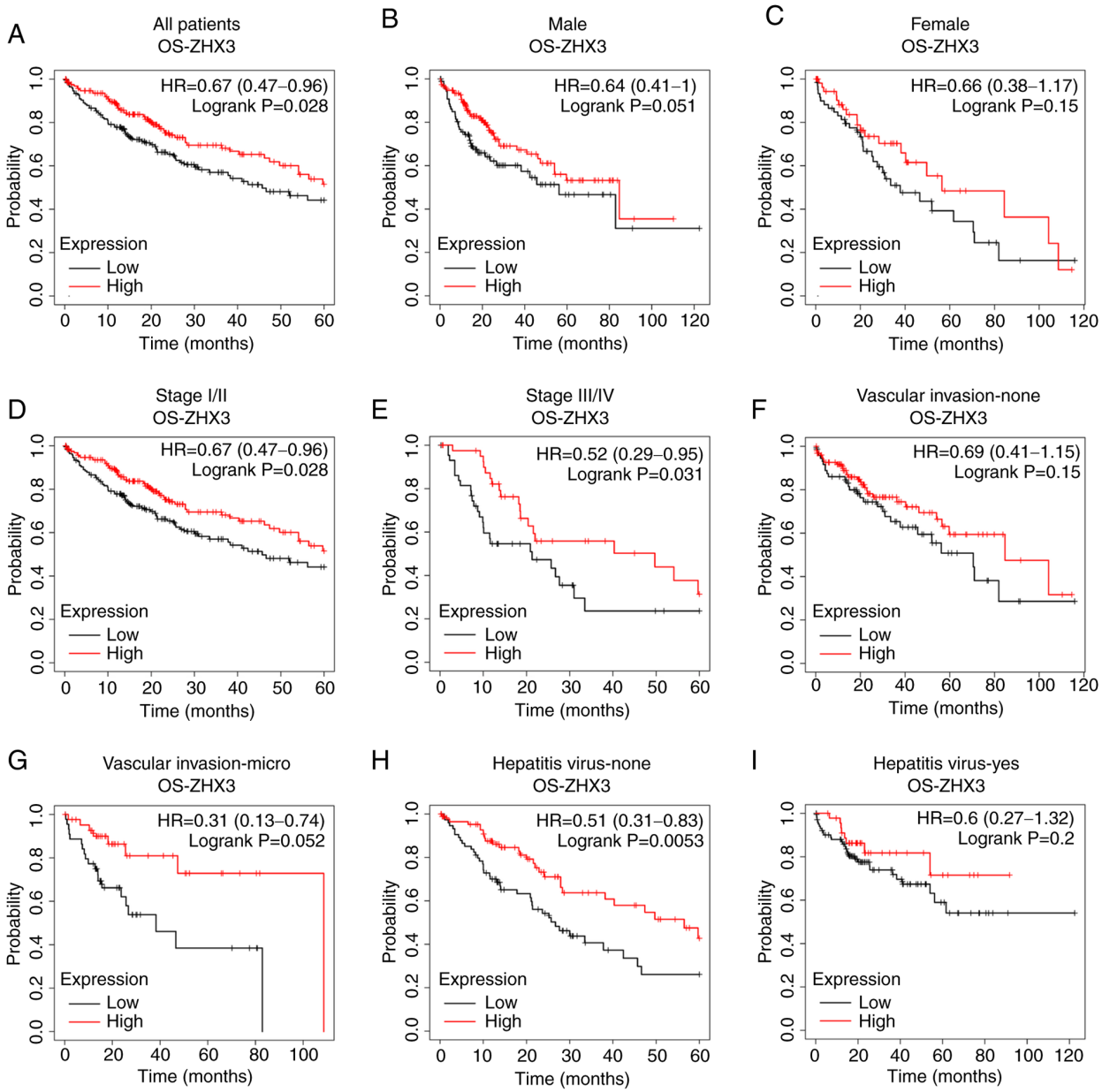

Figure 5. Relationship between ZHX3 mRNA expression and survival outcomes in patients with liver cancer using the Kaplan-Meier plotter database. (A) OS analysis of ZHX3 in all patients. OS analysis of ZHX3 in (B) male and (C) female patients. OS analysis of ZHX3 in patients with (D) Stage I/II and (E) Stage III/IV tumors. OS analysis of ZHX3 in patients (F) without and (G) with vascular invasion. OS analysis of ZHX3 in patients (H) without and (I) with hepatitis virus infection. ZHX, zinc-fingers and homeoboxes; OS, overall survival.

with micro vascular invasion, but not in those without vascular invasion (Fig. 3G and H).

Regarding ZHX3, its upregulation was found to be associated with a prolonged OS rate in patients with liver cancer (Fig. 5A). Subgroup analyses showed that no significant correlation between ZHX2 expression and OS either in male patients or in female patients (Fig. 5B and C). Increased ZHX3 expression exhibited longer OS times in patients with Stage I/II tumors and Stage III/IV tumors (Fig. 5D and E). High ZHX3 mRNA level represented an improved OS rate in patients with micro vascular invasion, but not in those without micro vascular invasion (Fig. 5F and G). In addition, elevated ZHX3 expression illustrated a longer OS in patients without hepatitis virus infection (Fig. 5H), but not in those with hepatitis virus infection (Fig. 5I).

Correlation between genetic alterations of ZHX factors and survival outcomes. The prognostic association between genetic alterations of ZHX factors and outcomes in patients with liver cancer was further characterized using the CbioPORTAL online database. The genetic alteration rates for ZHX1, ZHX 2 and ZHX3 were 10, 10 and 0.6%, respectively (Fig. 3). The genetic alteration of ZHX2 was found to be associated with OS in patient with liver cancer (Fig. 6C). Nevertheless, no other significant relationship was observed between genetic alterations of ZHX factors and patient survival, as regarding either OS or DFS (Fig. 6B and D-G).

ZHX3 expression is an independent prognostic factor in liver cancer. To support the above results, the expression status of ZHX3 protein was thus examined using one tissue microarray

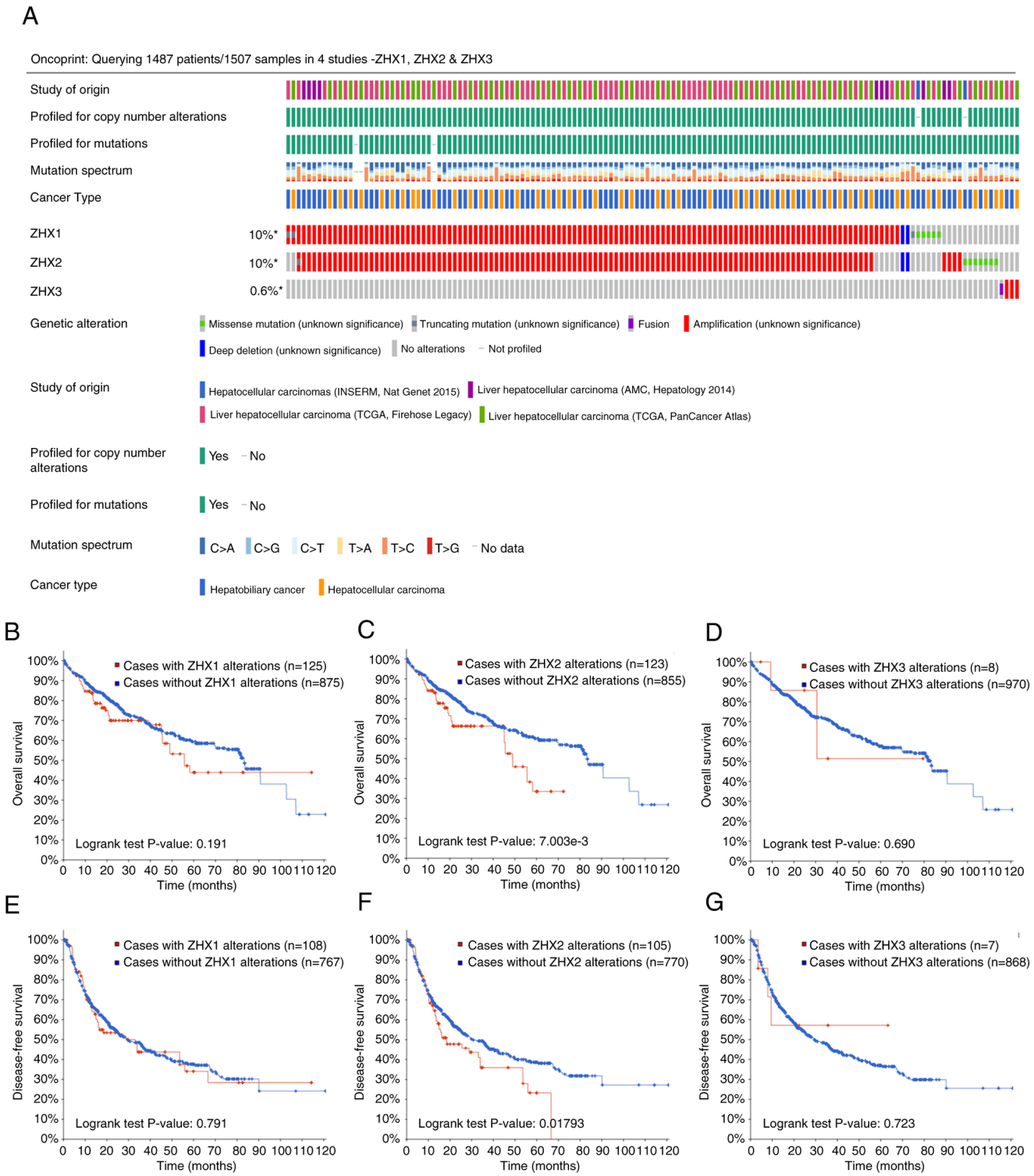

Figure 6. Genetic alterations of ZHX genes and their association with patient survival in liver cancer. (A) OncoPrint in cBioPortal demonstrated the proportion and distribution of samples with genetic alterations in ZHX genes. The figure was cropped on the right side to exclude samples without alterations. The impact of genetic alterations of (B) ZHX1, (C) ZHX2 and (D) ZHX3 on OS in patients with liver cancer. The impact of genetic alterations of (E) ZHX1, (F) ZHX2 and (G) ZHX3 on DFS in patients with cancer liver cancer. ZHX, zinc-fingers and homeoboxes; OS, overall survival; DFS, disease-free survival.

chip containing total 94 primary HCC specimens. A high level of ZHX3 protein expression primarily in the cytoplasm of cancer cells in 48.9% (46/94) of the HCC specimens tested was observed (Fig. 7). Low ZHX3 expression was found to be associated with larger tumor size, advanced TNM staging and T stage, positive thrombus status and TP53 expression (Table I). Kaplan-Meier survival analyses demonstrated that patients with high ZHX3 expression had an improved OS compared with those with low ZHX3 expression (Fig. 8A). Subgroup analyses showed that high ZHX3 expression indicated an improved OS in patients both with T1/T2 tumors and T3/T4 tumors (Fig. 8B and C). ZHX3 overexpression also exhibited

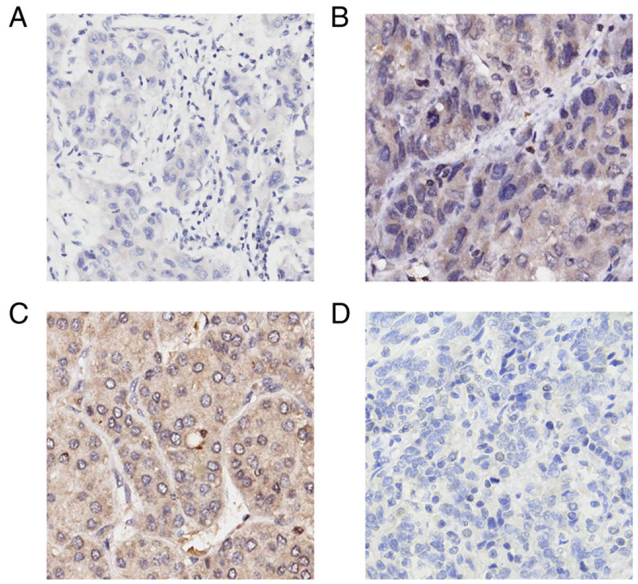

Figure 7. Representative immunohistochemical staining for ZHX3 protein in primary liver cancer tissues. (A) Weak staining; (B) Moderate staining; (C) Strong staining; and (D) no staining. Original magnification, x200. ZHX, zinc-fingers and homeoboxes.

a longer OS in patients both with Stage I/II and Stage III/IV tumors (Fig. 8D and E). In addition, elevated ZHX3 suggested an improved OS in patients with histological grade I/II tumors (Fig. 8F). In the univariate analysis, larger tumor size, advanced TNM stage, higher histological grade, positive thrombus status and ZHX3 expression were determined to be associated with an unfavorable OS (Table II). After correcting the prognostic variables obtained in the univariate analysis, only histological grade and ZHX3 expression kept the independent implication in the multivariate analysis (Table II).

Discussion

The present study is part of a continuing effort to explore molecular targets of liver cancer behaviors with reliability to predict outcome and promise as targets for directed therapy. Identification of this issue may be important to improve clinical management of liver cancer in the future. Consequently, the results of the present study using data-mining analyses as well as immunohistochemistry provided an in-depth investigation into the prognostic values of ZHX family members in patients with liver cancer.

ZHX1 has been identified as a tumor suppressor in several types of cancer (24-28). On the contrary, two reports show that ZHX1 might act as an oncogene in cholangiocarcinoma and glioblastoma (29,30). To the best of the authors' knowledge, except for our two reports (3,4), no other study has unraveled the association between ZHX1 expression and outcomes of patients with cancer. Of note, its prognostic impact on different cancers appears to be contradictory. The present authors previously reported that high ZHX1 expression predicts worse OS for breast cancer but present better OS for gastric cancer, suggesting its diverse roles in development of different types of cancer (3,4). It was inferred that different sample sources, histological types and intrinsic differences in each type of cancers may be possible to explain this disparity. Although there no relevance was found between ZHX1 expression and OS in patients with liver cancer in the present study, a prognostic value for ZHX1 was identified in subgroup analyses, i.e., a significant association between low ZHX1 mRNA levels and longer RFS in male patients as well as in patient without hepatitis virus infection.

Several studies have reported tumor-suppressor roles of ZHX2 in multiple types of cancer, including liver cancer (31-38). However, no significant association was observed between ZHX2 expression and OS or RFS in patients with liver cancer. Decreased ZHX2 expression was only observed to be correlated with an improved OS in patients with Stage III/IV tumors or an improved RFS in patients with micro vascular invasion. Dysregulation of ZHX2 has been described to function in the transcriptional inhibition of cancer markers in normal hepatocytes (31). It has been noted that gene promoter methylation-medicated silencing of ZHX2 frequent occurs in HCC and overexpression of ZHX2 suppresses proliferation and augments the chemo-sensitivity of HCC cells (32-35). It has been also reported that HBV inhibits ZHX2 expression and accelerates the proliferation of HCC cells through the activation of miR-155 and, conversely, ZHX2 represses HBV replication through epigenetic and non-epigenetic manners (35,36). These observations seem consistent with the findings of the present study, i.e., ZHX2 expression predicted better OS in patients with hepatitis infection, suggesting that ZHX2 may exert different functions according the different microenvironment during development of liver cancer.

Consistent with our previous study in breast cancer (3), attenuated ZHX3 expression was observed to be correlated with unfavorable OS in patients with liver cancer. The data also demonstrated that elevated ZHX3 was associated with an improved OS in patients with both Stage I/II and Stage III/IV tumors, suggesting that ZHX3 might be valuable in predicting the outcomes of patients with early-stage malignancy. This conclusion is contrary to the oncogene function of ZHX3 in gastric cancer in another study (4). To support the observation by *in silico* analyses, protein expression of ZHX3 was also examined by immunohistochemistry in cancer tissues. The data of the present study characterized that decreased ZHX3 levels were significantly associated with malignant properties and suggested that ZHX3 expression is an independent prognostic factor in liver cancer. Notably, the genetic alteration rate of ZHX3 was lower than that of ZHX1 and ZHX2 in liver cancer, which is similar to our previous studies in breast and gastric cancers (3,4). This lower frequency of ZHX3 gene alteration in the types of cancer that we observed suggest that ZHX3 may exert more important biological functions as an tumor suppressor gene.

In summary, the present study systematically examined the expression pattern of ZHX factors and the corresponding prognostic significance in liver cancer, based on *in silico* analysis and immunohistochemistry analyses. The results suggested that ZHX family members are distinct prognostic biomarkers for this disease. Future research should be performed to discover the exact functions of ZHX family members in liver cancer, which may support that ZHX factors could serve as prognostic predicators and promising therapeutic targets for precision medicine.

Table I. Correlation between ZHX3 expression and clinicopathological variables in liver cancer.

Parameters	No. of patients	ZHX3 expression		P-value
		Low, *n* (%)	High, *n* (%)	
Age				
≤60 years	53	29 (54.7)	24 (45.3)	0.491
>60 years	40	19 (47.5)	21 (52.5)	
NA	1			
Sex				
Male	10	3 (30.0)	7 (70.0)	0.194
Female	84	45 (53.6)	39 (46.4)	
Tumor size				
≤5 cm	39	9 (23.1)	30 (76.9)	<0.001
>5 cm	54	38 (70.4)	16 (29.6)	
NA	1			
Histological grade				
I/II	66	27 (40.9)	39 (59.1)	0.932
III	28	21 (75.0)	7 (25.0)	
TNM Stage				
I/II	43	8 (18.6)	35 (81.4)	<0.001
III/IV	43	37 (86.0)	6 (14.0)	
NA	8			
T Stage				
T1/T2	43	8 (18.6)	35 (81.4)	<0.001
T3/T4	43	37 (86.0)	6 (14.0)	
NA	8			
Thrombus				
Negative	75	36 (48.0)	39 (52.0)	0.013
Positive	7	7 (100.0)	0 (0.0)	
NA	12			
Cirrhosis				
Negative	58	31 (53.4)	27 (46.6)	0.557
Positive	36	17 (47.2)	19 (52.8)	
AFP				
Negative	39	23 (59.0)	26(41.0)	0.167
Positive	54	24 (44.4)	30(55.6)	
NA	1			
CD34				
Negative	37	17 (45.9)	20 (54.1)	0.524
Positive	55	29 (52.7)	26 (47.3)	
NA	1			
Ki67				
Negative	44	19 (43.2)	25 (56.8)	0.179
Positive	49	28 (57.1)	21 (42.9)	
NA	1			
TP53				
Negative	44	17 (38.6)	27 (61.4)	0.030
Positive	49	30 (61.2)	19 (28.8)	
PDL-1				
Negative	43	22 (51.2)	21 (48.8)	0.911
Positive	42	22 (52.4)	20 (47.6)	
NA	9			

Table I. Continued.

Parameters	No. of patients	ZHX3 expression		P-value
		Low, *n* (%)	High, *n* (%)	
CD8				
Negative	42	20 (47.6)	22 (52.4)	0.528
Positive	46	25 (54.3)	21 (45.6)	
NA	6			

NA, not available; ZHX, zinc-fingers and homeoboxes; AFP, α-fetoprotein; PDL-1, programmed death-ligand 1.

Table II. Univariate and multivariate analyses of the factors correlated with overall survival of liver carcinoma patients.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Tumor size				
>5 cm vs. ≤5 cm	2.397 (1.365-4.210)	0.002	1.002 (0.445-2.256)	0.996
TNM Stage				
III/IV vs. I/II	2.860 (1.611-5.077)	<0.001	0.824 (0.321-2.115)	0.687
Histological grade				
III/IV vs. I/II	3.401 (2.002-5.779)	<0.001	2.067 (1.07-3.995)	0.031
Thrombus				
Positive *vs*. Negative	2.644 (1.117-6.259)	0.027	1.732 (0.580-5.170)	0.325
ZHX3 expression				
Low vs. high	0.179 (0.098-0.329)	<0.001	0.173 (0.066-0.453)	<0.001

HR, hazard ratio; CI, confidence interval; ZHX, zinc-fingers and homeoboxes.

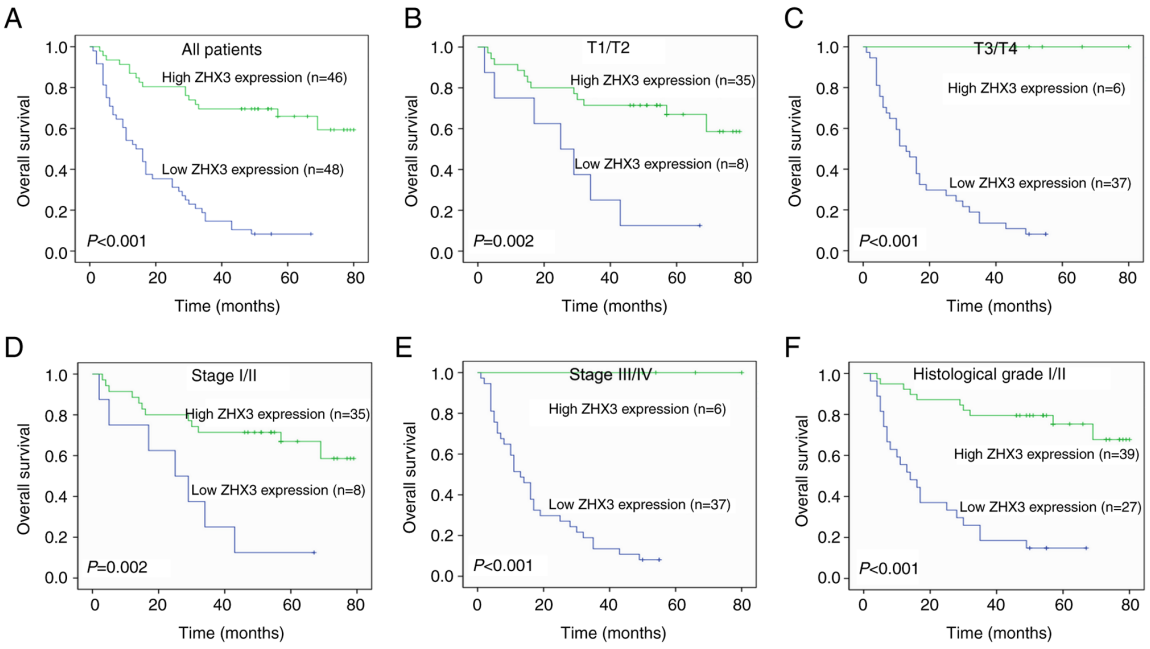

Figure 8. Kaplan-Meier curves compared the overall survival in HCC patients with high and low expression of ZHX3 protein. (A) High ZHX3 expression was significantly correlated with better OS in HCC patients (P<0.001). Survival curves of ZHX3 expression in patients with (B) T1/T2 and (C) T3/T4 tumors. Survival curves of ZHX3 expression in patients with (D) Stage I/II and (E) Stage III/IV tumors. (F) Survival curves of ZHX3 expression in patients with histological grade I/II tumors. ZHX, zinc-fingers and homeoboxes; HCC, hepatocellular carcinoma; OS, overall survival.

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Availability of data and materials

The dataset used and/or analyzed in the current study is available from the corresponding authors on reasonable request.

Authors' contributions

YY, FH and SH conceived the study, designed and performed the experiments, analyzed and interpret the data and drafted the manuscript. YY and SH confirm the authenticity of all raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol conformed to the ethical guidelines outlined in the Declaration of Helsinki and was approved by the Institutional Review Board (approval no. 07-170) of Ningxia Hui Autonomous Region People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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