

## Original Article

# Prognostic value and therapeutic implications of ZHX family member expression in human gastric cancer

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**Abstract:** Despite significant advances in the early diagnosis and effective treatment of gastric cancer, it remains the fourth most common cancer and the second leading cause of cancer-related deaths worldwide. The zinc-fingers and homeoboxes (ZHX) family of transcriptional repressors has been shown to play a role in multiple types of cancer. However, the prognostic significance of ZHX expression in patients with gastric cancer remains unclear. This work studied the association between differential expression of ZHX mRNA and outcomes in patients with gastric cancer using data from the Oncomine, CCLE, Kaplan-Meier-plotter, and cBioPortal databases. Expression of ZHX3 protein was also measured by immunohistochemistry (IHC) in gastric cancer tissues. We found that increased expression of *ZHX1* mRNA and decreased expression of *ZHX2* and *ZHX3* were correlated with better overall survival (OS) in patients with gastric cancer. Further subgroup analyses identified significant associations between *ZHX1* expression and survival in select gastric cancer patients. IHC staining confirmed that the over-expression of ZHX3 was associated with worse OS, and multivariate analyses identified ZHX3 expression as an independent prognostic factor. These results suggest that the ZHX family members may serve as distinct biomarkers and prognostic factors for patients with gastric cancer.

**Keywords:** Zinc-fingers and homeoboxes, gastric cancer, prognosis, in silico analysis

## Introduction

Gastric cancer is the fourth most common cancer and the second leading global cause of cancer-related deaths [1, 2]. To date, surgery is still the only curative therapy for patients with gastric cancer, but its 5-year survival rate is less than 25% [2]. Despite significant prior research, the molecular mechanisms underlying the initiation and progression of gastric cancer remain largely unclear. Thus, there is an urgent need for the identification of new biomarkers that could serve as therapeutic targets or prognostic indicators for patients with gastric cancer.

Our group routinely conducts integrative analyses of existing online public datasets with the goal of identifying new targets and prognostic markers for cancers. Recently, we found that the family of zinc-fingers and homeoboxes

(ZHX) transcription factors appeared to be differentially expressed in some cancers. The ZHX family contains two zinc-finger motifs and five homeobox DNA-binding domains that are localized in the cell nucleus, including ZHX1, ZHX2, and ZHX3 [3-8]. The ZHX family members are transcriptional repressors that form homodimers and heterodimers with each other and interact with the A subunit of the nuclear factor Y (NF-YA) [4-8]. Increasing evidence has suggested that the ZHX factors are major transcriptional mediators involved in events such as the development and differentiation of hematopoietic cells, maintenance of neural progenitors, and osteogenic differentiation of mesenchymal stem cells [3, 9, 10]. Differential expression of ZHX factors appears to be correlated with the development of a diverse set of neurological, hematological, and glomerular diseases [11, 12]. Prior *in vitro* and *in vivo* studies have also suggested that the ZHX family members may

participate in the initiation and progression of several cancers [3]. As such, the ZHX proteins may serve as biomarkers that could be used for cancer diagnoses, prognoses, and for therapeutic surveillance. However, to the best of our knowledge, the expression of ZHX transcription factors has not yet been studied in patients with gastric cancer. In the present study, we employed multiple *in silico* approaches to examine the prognostic value of ZHX family member expression in gastric cancer. These bioinformatics analyses made use of a set of publicly accessible databases including the Oncomine, CCLE, Kaplan-Meier-plotter, and cBioPortal databases. Further, we conducted immunohistochemistry analysis on gastric cancer tissue samples to confirm ZHX3 protein expression.

## Materials and methods

### *Oncomine database analysis*

To analyze the relative expression of specific ZHXs in a variety of malignancies, we analyzed genome-wide expression data from the online cancer microarray database Oncomine ([www.oncomine.org](http://www.oncomine.org)), which includes 715 datasets and 86,733 samples [13, 14]. Paired Student's t-test was used to compare group means. A fold-change of at least 2 with a *P*-value less than 0.01 was defined as clinically significant, as previously described [14].

### *CCLE database analysis*

The mRNA levels of distinct ZHX factors were determined in multiple cancer cell lines using the CCLE database (<http://portals.broadinstitute.org/ccle>), an online encyclopedia of gene expression, copy numbers, and massively parallel sequences gathered from 1,457 human cancer cell lines [15].

### *Kaplan-Meier-plotter survival analysis*

The prognostic value of ZHX mRNA expression was determined using the Kaplan-Meier-plotter online database (<http://kmplot.com/analysis/>), a tool for the analysis of the relationships between 54,675 genes and survival that was generated from 10,461 clinical cancer samples, including 1,065 from patients with gastric cancer [16, 17]. To investigate the overall survival (OS) of gastric cancer patients, clinical

samples were divided into high and low expression groups based on the median value of mRNA expression. Kaplan-Meier plots were created for each individual gene of interest. A *P*-value < 0.01 was considered to indicate a statistically significant result. The hazard ratio (HR) with 95% confidence intervals and the log-rank *P*-value were also calculated.

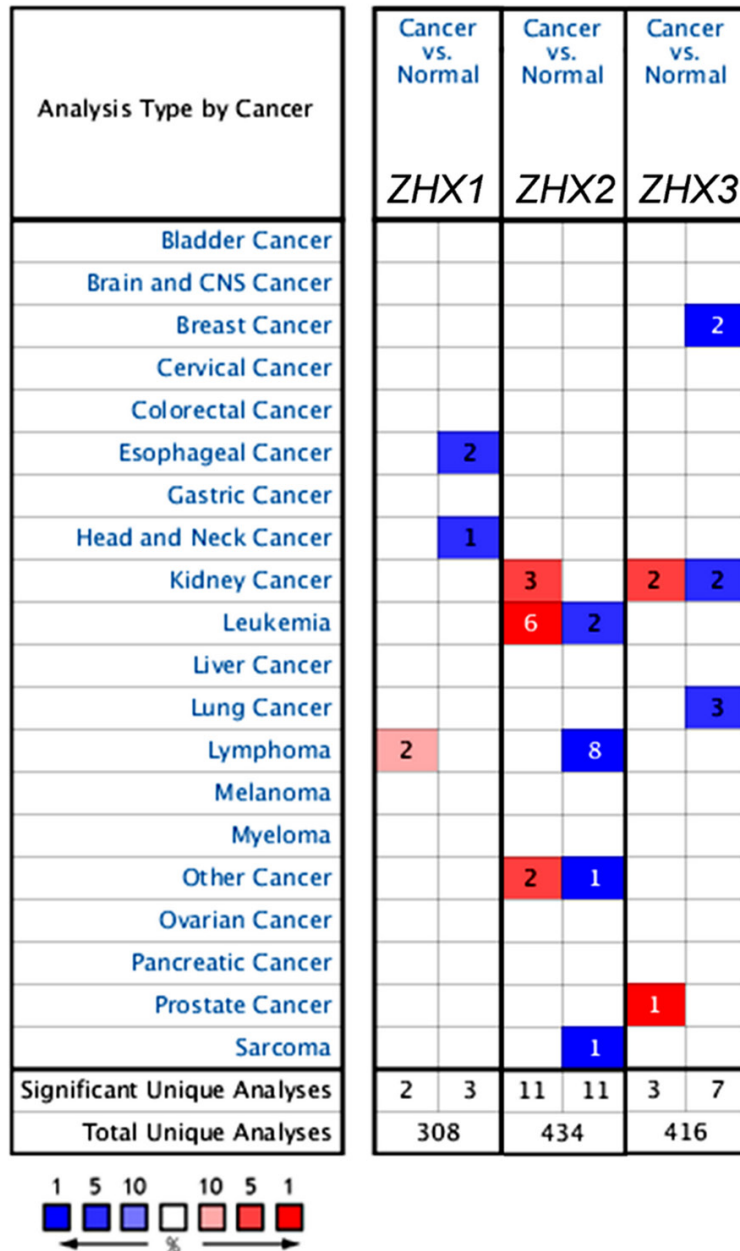
### *cBioPortal database analysis*

The impact of mutations and copy numbers of ZHX genes on OS and disease-free survival (DFS) in patients with gastric cancer was calculated using the cBioPortal online database ([www.cbioportal.org](http://www.cbioportal.org)) [18, 19]. The cBioPortal for Cancer Genomics facilitates visualization, analysis, and download of large-scale cancer genomics datasets. Additionally, we analyzed a stomach adenocarcinoma dataset (TCGA, Provisional) containing pathological and prognostic data from 478 patients.

### *Immunohistochemistry and microscopy analysis*

Immunohistochemical analysis of ZHX3 protein expression was performed using the EnVision complex method as previously described [20-23]. A set of 106 formalin-fixed, paraffin-embedded (FFPE) specimens were collected from patients with gastric cancer (median age, 53 years; range, 29-88 years) from the Affiliated Cancer Hospital of Shantou University Medical College. Following deparaffinization, rehydration, and antigen retrieval, 4- $\mu$ m sections were prepared on slides and incubated with a rabbit polyclonal anti-ZHX3 antibody (catalog no. ab84677; dilution, 1:500 in 1% milk in PBS; Abcam, Cambridge, MA, USA). Immunohistochemical staining was conducted using an EnVision antibody complex (anti-mouse/rabbit) method in conjunction with an EnVision detection kit (ZSGB-BIO, Beijing, China) with 3,3'-diaminobenzidine as the chromogenic substrate. Nuclei were counterstained with hematoxylin. Sections that were immunostained with a rabbit IgG as the primary antibody were used as negative controls.

Ten random microscopic fields per slide were evaluated at 400 $\times$  by two independent observers who were blinded to samples' clinical information. ZHX3 staining was assessed using a semi-quantitative approach, which combines



**Figure 1.** The transcription levels of ZHX family members in different cancer types. A graphic obtained from Oncomine indicates the numbers of datasets with significant over-expression (Red) or under-expression (Blue) of ZHX factors at the transcriptional level in cancer tissues relative to normal tissues. Cell color was determined by the best gene rank percentile for the analyses within the cell, and the gene rank was analyzed by percentile of target genes in the top of all genes measured in each analysis. The cut-offs for significant *P*-values and fold changes were defined as 0.01 and 2, respectively.

the staining intensity and percentage of positive cells. The mean percentage of positively stained cells was scored as follows: (0) 0-5%; (1) 5-25%; (2) 26-50%; (3) 51-75%; or (4) 76-100%. Staining intensity was categorized as follows: (0) absent; (1) weak; (2) moderate; or

(3) strong. The multiplication of staining intensity by percentage of positive cells was used as the final staining score. For statistical evaluation, tumor samples with a final staining score less than 3 were classed as low ZHX3 expression and those with scores greater than or equal to 3 were considered high ZHX3 expression.

#### Statistical analysis

Statistical analysis was performed using the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA). The correlations between the expression of ZHXs and different clinical variables were analyzed using Fisher's exact test or Pearson  $\chi^2$  test, as appropriate. Survival curves were generated using the Kaplan-Meier method with a log-rank test. The prognostic impacts of clinicopathological variables were analyzed by univariate and multivariate regression analyses with a Cox hazards model. *P* < 0.05 (two-tailed) was considered a statistically significant difference.

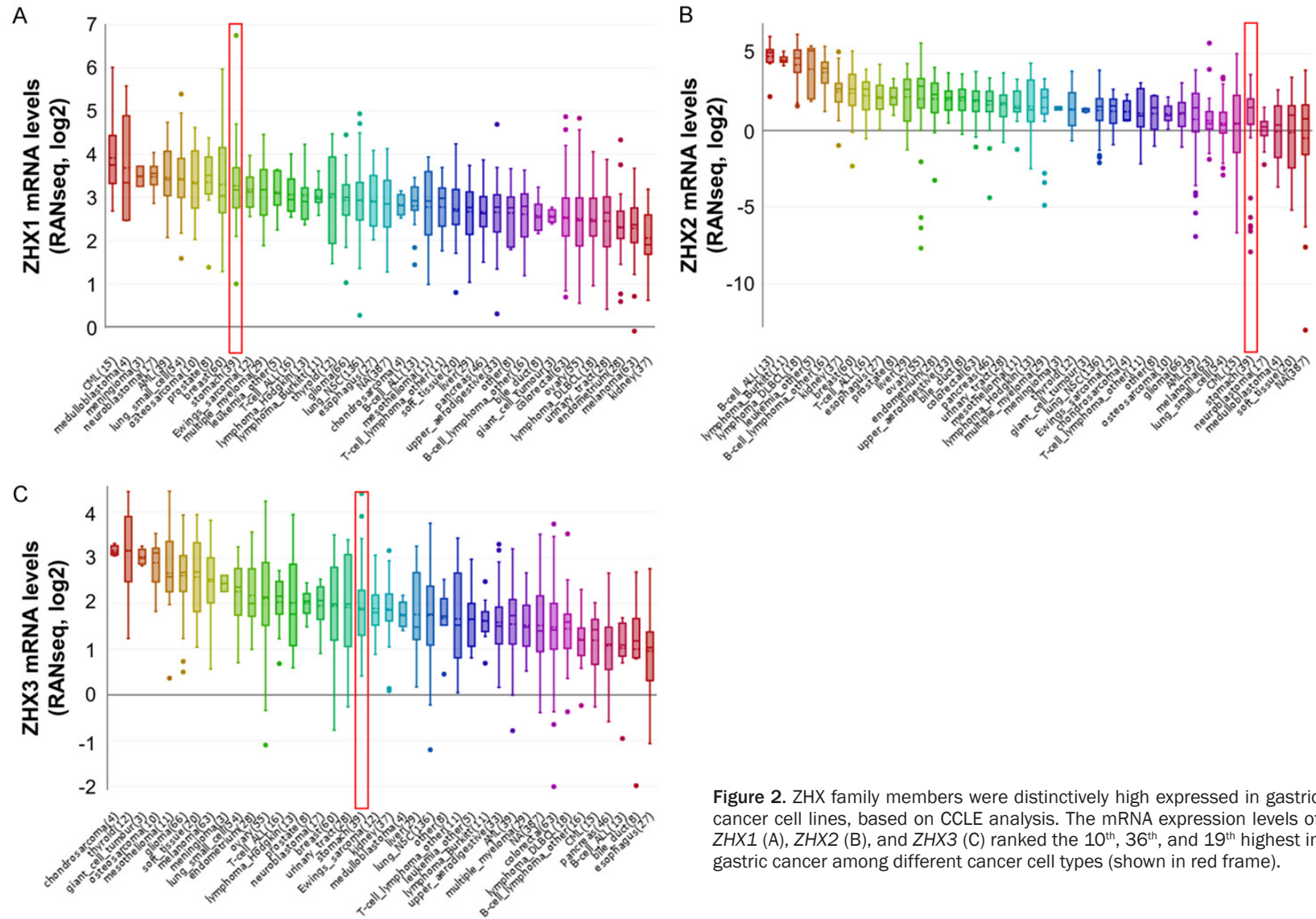
#### Results

##### *mRNA expression patterns of ZHX family members in human cancers*

Differences between the transcription of 3 ZHX factors in tumor and normal tissues were analyzed in multiple cancer types using the Oncomine database. As shown in **Figure**

**1**, the Oncomine database contained 308, 434, and 416 unique analyses for ZHX1, ZHX2, and ZHX3, respectively. We found that three unique studies revealed lower ZHX1 mRNA levels in cancer tissues than normal tissues, but two other studies found an opposite effect.

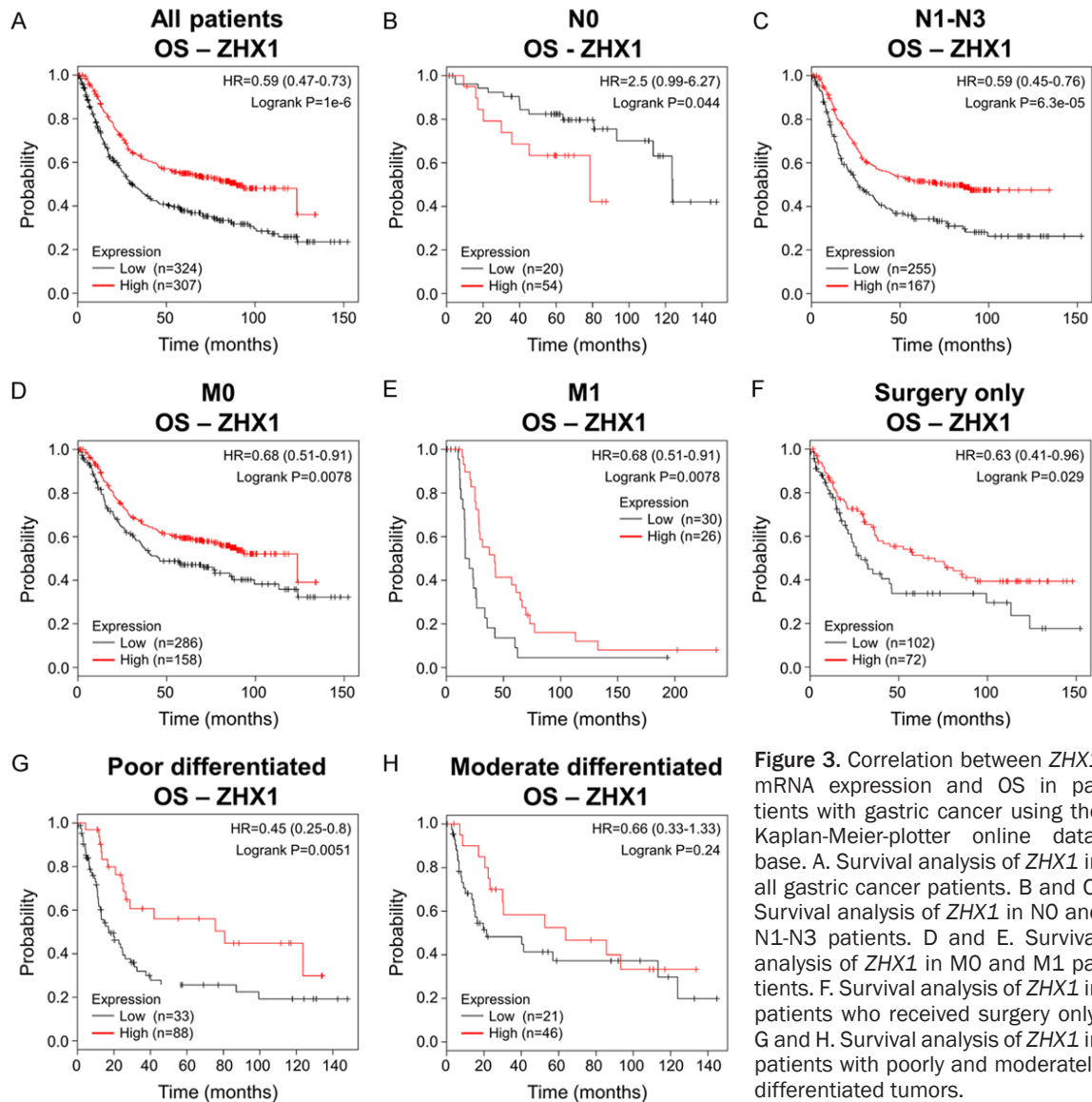
# Prognostic significance of ZHXs in gastric cancer



**Figure 2.** ZHX family members were distinctively high expressed in gastric cancer cell lines, based on CCLE analysis. The mRNA expression levels of ZHX1 (A), ZHX2 (B), and ZHX3 (C) ranked the 10<sup>th</sup>, 36<sup>th</sup>, and 19<sup>th</sup> highest in gastric cancer among different cancer cell types (shown in red frame).



## Prognostic significance of ZHXs in gastric cancer



**Figure 3.** Correlation between *ZHX1* mRNA expression and OS in patients with gastric cancer using the Kaplan-Meier-plotter online database. A. Survival analysis of *ZHX1* in all gastric cancer patients. B and C. Survival analysis of *ZHX1* in N0 and N1-N3 patients. D and E. Survival analysis of *ZHX1* in M0 and M1 patients. F. Survival analysis of *ZHX1* in patients who received surgery only. G and H. Survival analysis of *ZHX1* in patients with poorly and moderately differentiated tumors.

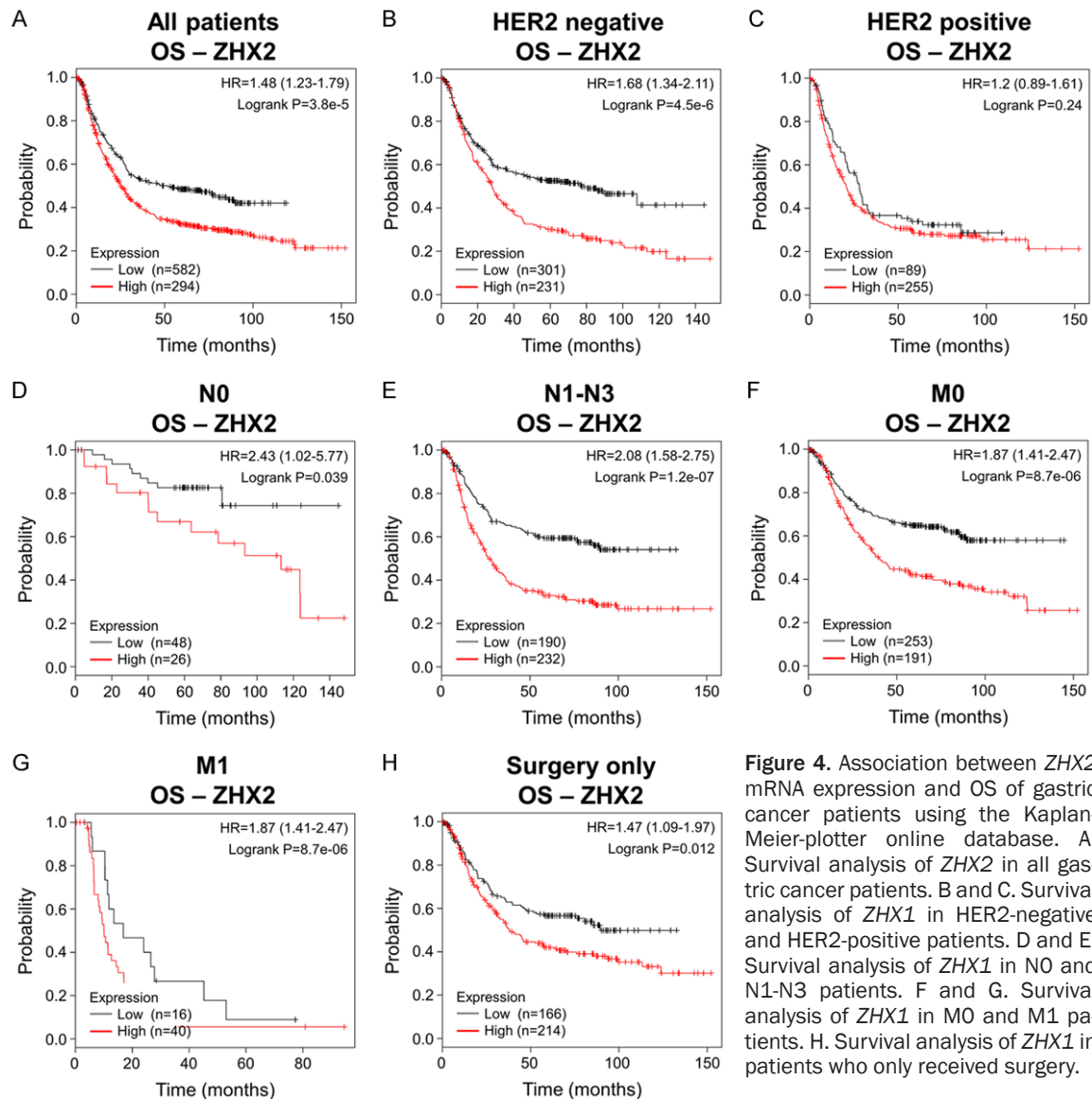
Similarly, *ZHX2* expression was decreased in 11 cancers but overexpressed in another 11 cancers. *ZHX3*, was down-regulated in seven cancers and up-regulated in three cancers. However, *ZHX* mRNA expression was not reported in the gastric cancer datasets. Analysis of the CCLE database found that *ZHX1*, *ZHX2*, and *ZHX3* mRNA expression in gastric cancer was the 10<sup>th</sup>, 36<sup>th</sup>, and 19<sup>th</sup> highest among all cancer types, respectively (Figure 2).

### Association between mRNA expression of *ZHX* factors and outcomes in patients with gastric cancer

Next, we characterized the prognostic impacts of *ZHX* family members in patients with gastric cancer via Kaplan-Meier-plotter survival analy-

sis. Increased *ZHX1* expression was associated with better OS in patients with gastric cancer (Figure 3A). Subgroup analyses revealed that high expression of *ZHX1* predicted improved OS in the subgroups of patients with or without lymph node metastases (N0 and N1-N3; Figure 3B and 3C) or distant metastasis (M0 and M1; Figure 3D and 3E). Increased *ZHX1* expression indicated a favorable rate of OS in patients who only received surgery (Figure 3F). In addition, elevated *ZHX1* was significantly associated with improved OS in patients with poorly differentiated tumors (Figure 3G), but not in those patients with moderately differentiated tumors (Figure 3H).

Similarly, we observed that low *ZHX2* mRNA expression was associated with a better rate of

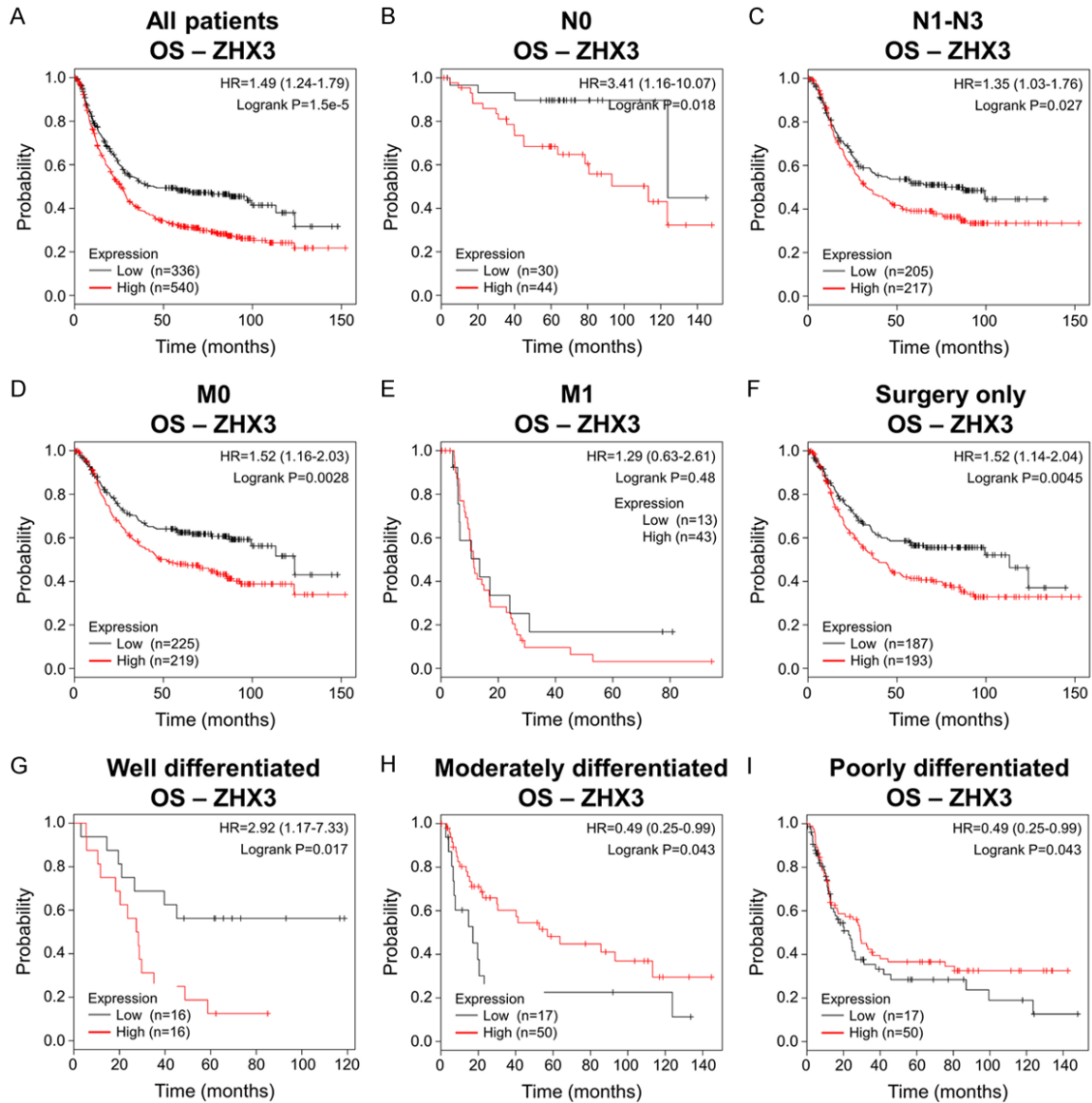


**Figure 4.** Association between ZHX2 mRNA expression and OS of gastric cancer patients using the Kaplan-Meier-plotter online database. A. Survival analysis of ZHX2 in all gastric cancer patients. B and C. Survival analysis of ZHX2 in HER2-negative and HER2-positive patients. D and E. Survival analysis of ZHX2 in N0 and N1-N3 patients. F and G. Survival analysis of ZHX2 in M0 and M1 patients. H. Survival analysis of ZHX2 in patients who only received surgery.

OS in patients with gastric cancer (**Figure 4A**). Subgroup analyses suggested that down-regulation of ZHX2 was predictive of improved OS in HER2-positive patients (**Figure 4B**), but not in HER2-negative patients (**Figure 4C**). Reduced ZHX2 expression was associated with longer OS in the subgroups of patients with or without lymph node metastases (N0 and N1-N3; **Figure 4D** and **4E**) and distant metastasis (M0 and M1; **Figure 4F** and **4G**). Attenuated ZHX2 levels indicated a favorable OS rate in patients who only received surgery, but not in patients who received additional therapy (**Figure 4H**).

Generally, down-regulation of ZHX3 was correlated with longer OS in patients with gastric

cancer (**Figure 5A**). Subgroup analyses revealed that decreased ZHX3 mRNA were associated with longer OS in subgroups of patients with or without lymph node metastases (N0 and N1-N3; **Figure 5B** and **5C**). Low ZHX3 expression also indicated favorable OS in patients without distant metastasis (**Figure 5D**), but not in those with distant metastasis (**Figure 5E**). Reduced ZHX3 expression was associated with longer OS in patients who only received surgery, but not in patients who received other treatments (**Figure 5F**). Of note, decreased expression of ZHX3 was associated with better OS rates in patients with well-differentiated tumors (**Figure 5G**), and increased expression of ZHX3 was associated with worse



**Figure 5.** Relationship between *ZHX3* mRNA expression and OS in patients with gastric cancer as determined by the Kaplan-Meier-plotter online database. A. Survival analysis of *ZHX3* in all gastric cancer patients. B and C. Survival analysis of *ZHX3* in N0 and N1-N3 patients. D and E. Survival analysis of *ZHX3* in M0 and M1 patients. F. Survival analysis of *ZHX3* in patients who only received surgery. G-I. Survival analysis of *ZHX3* in patients with well, moderately, and poorly differentiated tumors.

OS rates in patients with moderately and poorly differentiated tumors (**Figure 5H** and **5I**).

#### *Correlation between mutations in ZHX factors and patient survival*

Next, we characterized the prognostic correlation between mutations in ZHX factors and outcomes in patients with gastric cancer using the CbioPORTAL online database. The genetic alteration rates for *ZHX1*, *ZHX2*, and *ZHX3* were 11, 11, and 7%, respectively (**Figure 6A**). However,

no significant association was found between mutations in ZHX family members and patient survival, as indicated by OS and DFS (**Figure 6B-G**).

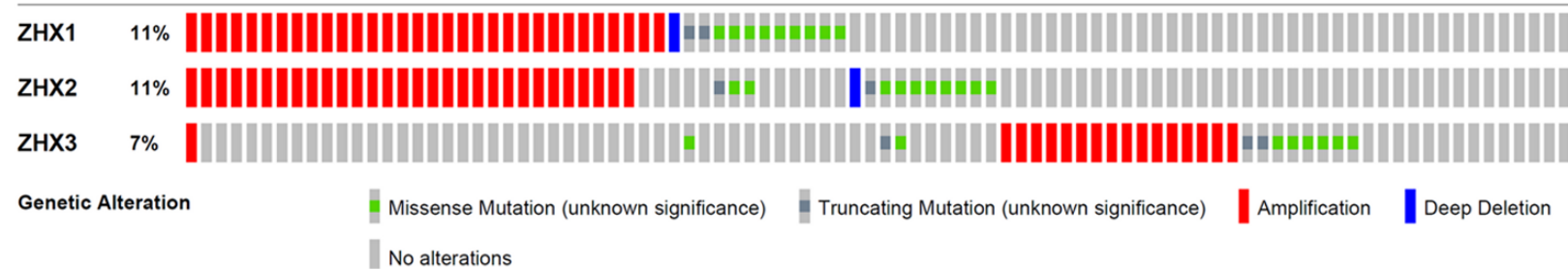
#### *ZHX3 expression is an independent prognostic factor in gastric cancer*

Last, we further investigated the expression of *ZHX3* in 106 FFPE specimens through immunohistochemistry. We observed a high level of *ZHX3* immunostaining in the nucleus of tumor

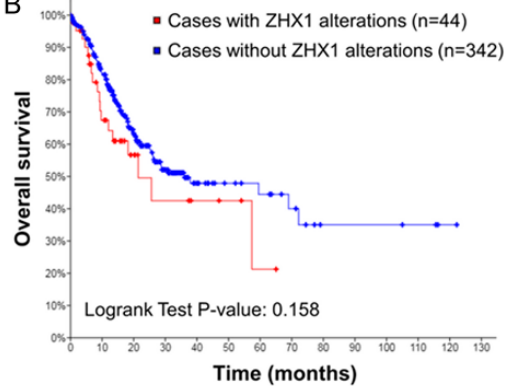
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## Oncoprint in cBioPortal

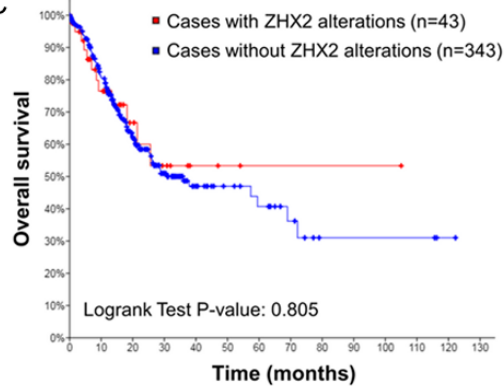
Case Set: Tumor Samples with sequencing and CNA data (393 patients / 393 samples)



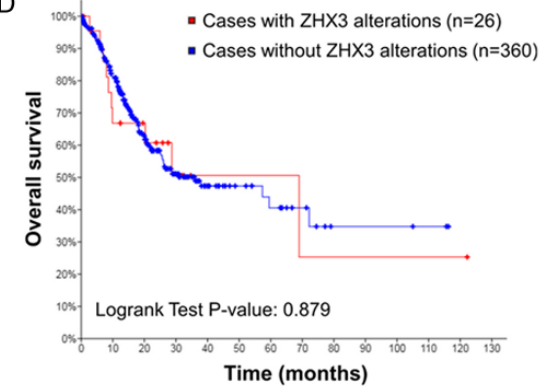
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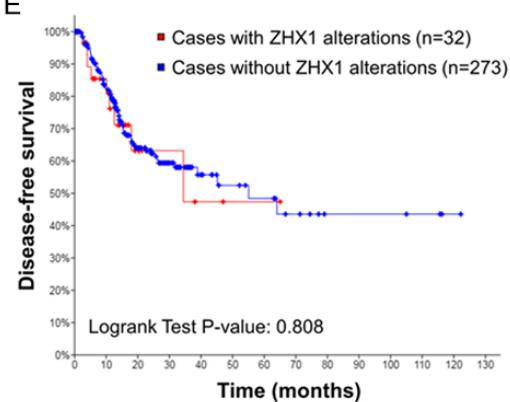
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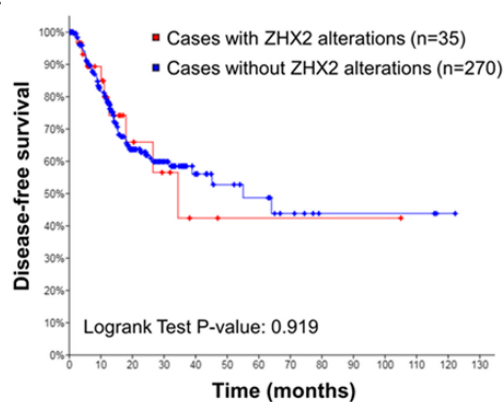
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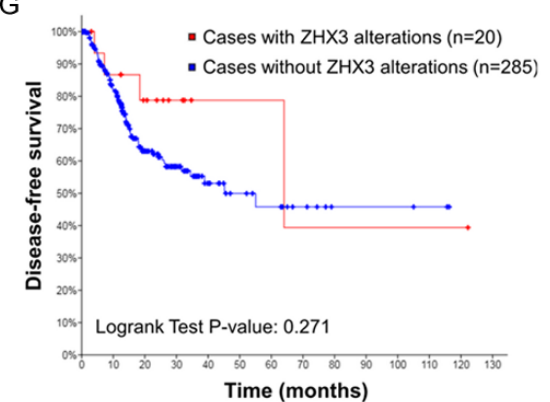
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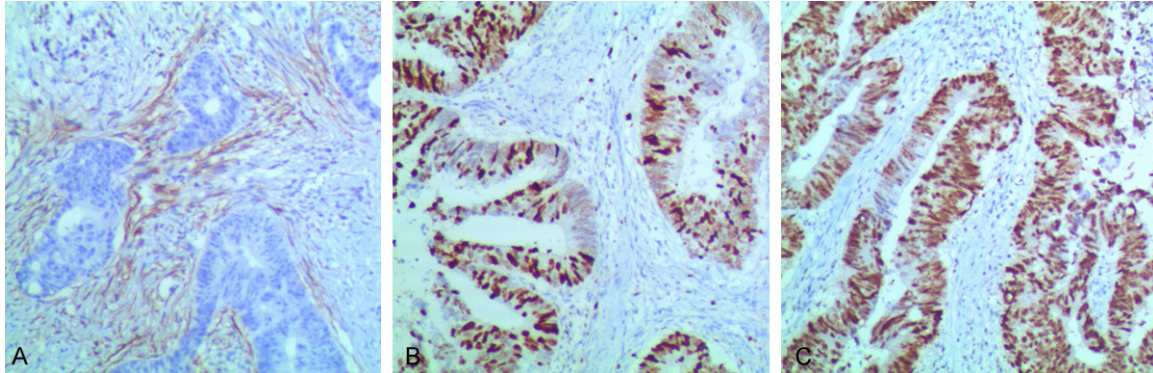
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## Prognostic significance of ZHXs in gastric cancer

**Figure 6.** Genetic alterations of ZHX genes and their association with survival of patients with gastric cancer, via analysis of the cBioPortal database. A. OncoPrint in cBioPortal represents the proportion and distribution of samples with mutations in ZHX factor genes. The figure was cropped on the right to exclude samples without mutations. B-D. The impact of genetic mutations in *ZHX1*, *ZHX2*, and *ZHX3* on rates of OS in patients with gastric cancer. E-G. The impact of mutations in *ZHX1*, *ZHX2*, and *ZHX3* on DFS in patients with gastric cancer.



**Figure 7.** Representative serial sections of gastric cancer immunohistochemical staining for ZHX3: (A) Negative staining; (B) Low ZHX3 staining; and (C) High staining. Original magnification 200 $\times$ .

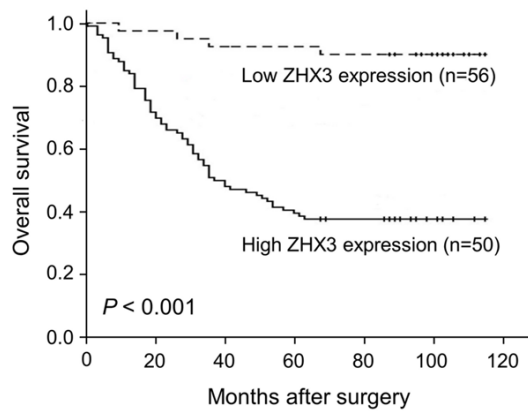
**Table 1.** Correlation between ZHX3 expression and clinicopathological variables in patients with gastric cancer

Parameters	No. of patients	ZHX3 expression		P-value
		Low, n (%)	High, n (%)	
Age				
≤60 years	56	31 (55.4)	25 (44.6)	0.697
> 60 years	50	25 (50.0)	25 (50.0)	
Sex				
Male	80	41 (51.3)	39 (48.8)	0.654
Female	26	15 (57.7)	11 (42.3)	
Tumor size				
≤5 cm	57	31 (54.4)	26 (45.6)	0.846
> 5 cm	49	25 (51.0)	24 (49.0)	
Differentiation				
Well/Moderate	43	23 (53.5)	20 (46.5)	0.932
Poor	63	33 (52.4)	30 (47.6)	
Stage of tumors				
I/II	23	16 (69.6)	5 (21.7)	0.027
III/IV	85	40 (47.1)	45 (52.9)	
Invasive depth				
T1/T2	28	16 (53.3)	12 (46.7)	0.662
T3/T4	78	40 (51.3)	38 (48.7)	
Lymph node metastasis				
N0/N1	17	15 (88.2)	2 (11.8)	0.001
N2/N3	89	41 (46.1)	48 (53.9)	
Distant metastasis				
M0	75	42 (56.0)	33 (44.0)	0.393
M1	31	14 (45.1)	17 (54.8)	

cells in 47.2% (50/106) of gastric cancer samples (**Figure 7**). Further, we found that ZHX3 over-expression was associated with lymph node metastasis and advanced tumor stage (**Table 1**). Kaplan-Meier survival analyses showed that patients with ZHX3 over-expression had worse OS compared with those with low ZHX3 expression (**Figure 8**). Univariate analysis found that larger tumor size, lymph node metastasis, advanced tumor stages, and ZHX3 expression were associated with worse OS (**Table 2**). After adjusting for confounding effects, multivariate analysis found that only tumor size, lymph node metastasis, and ZHX3 over-expression were independent significant predictors of worse OS (**Table 2**).

### Discussion

The current study is part of our ongoing endeavors to



**Figure 8.** Kaplan-Meier curves comparing the overall survival in gastric cancer patients with high and low protein expression of ZHX3. ZHX3 over-expression was significantly correlated with shorter OS for patients with gastric cancer ( $P < 0.001$ ).

discover the relationship between the expression profile of ZHX family members and outcomes in patients with cancer. The ultimate aim of these efforts is to identify novel diagnostic and prognostic biomarkers, some of which may even make sense as therapeutic targets. Identification of such factors may help guide the future clinical management of patients with gastric cancer. The *in silico* analyses presented in this work provide an in-depth view into the potential prognostic significance of ZHX factors in patients with gastric cancer.

We utilized publicly accessible online databases to systemically and comprehensively analyze the expression of ZHX family members in gastric cancers. ZHX1, the first-identified factor in this family, has previously been proposed to act as a tumor suppressor in many cancer types [24-28]. These observations are consistent with our current findings; high ZHX1 expression predicted better OS rates in patients with gastric cancer. Intriguingly, whereas we found that decreased ZHX1 expression was associated with better OS in patients without lymph node metastasis, increased ZHX1 expression was associated with better OS in patients without distant metastasis, suggesting that ZHX1 may exert different functions across patient subgroups. In addition, increased ZHX1 expression was associated with better OS rates in patients who only received surgery and in patients who had poorly differentiated tumors. However, two recent studies suggested that ZHX1 may act as an oncogene

in glioblastoma and cholangiocarcinoma [29, 30]. It has been reported that increased ZHX1 levels in tumor specimens are correlated with worse outcomes for patients with cancer [29, 30]. Differences in tissue types, histological types, detection methods, and intrinsic differences across tumor types may be accountable for these contradictory observations.

Our present findings suggest an oncogene role of ZHX2. Over-expression of ZHX2 was generally associated with worse OS in patients with gastric cancer. It has been reported that ZHX2 forms heteromeric complexes with ZHX3 [8, 31]. We observed a similar prognostic impact of ZHX2 and ZHX3 on outcomes in patients with gastric cancer; down-regulation of ZHX2 and ZHX3 mRNA expression was associated with better rates of OS. Indeed, decreased ZHX2 and ZHX3 were significantly associated with better OS in patients without lymph node metastasis or distant metastasis, suggesting that ZHX2 and ZHX3 help predict prognosis for patients with early-stage gastric cancer. In addition, their decreased expression was associated with better OS rates in patients who only received surgery. Together, these data suggest that ZHX2 and ZHX3 may act as oncogenes. This conclusion is contrary to the tumor suppressing function of ZHX2 that has been described in studies of human hepatocellular carcinoma and multiple myeloma [32-38]. This difference may be due to the complexity of the tumor microenvironment and the intrinsic differences between types of tumors. Another possible explanation is that ZHX2 and ZHX3 form heteromeric complexes with ZHX1 that exhibit diverse roles in different cancer types [24-30]. Of note, decreased ZHX3 expression was associated with a better rate of OS in patients with well-differentiated tumors, but worse OS in patients with moderately or poorly differentiated tumors. This implies that up-regulated expression of ZHX3 may be involved in the differentiation of gastric tumors. Thus, we hypothesize that ZHX2 and ZHX3 may play an essential role in the progression of gastric cancer. Our immunohistochemistry analysis of ZHX3 expression in tumor tissues validated our findings from *in silico* analyses, supporting an association between ZHX3 and worse OS. We conclude that ZHX3 protein expression may serve as a prognostic predictor for patients with gastric cancer.

**Table 2.** Univariate and multivariate Cox proportional hazards models including those variables that affected overall survival in patients with gastric cancer

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Tumor size (cm)				
> 5 vs. ≤5	2.126 (1.325-3.413)	0.002	1.751 (1.082-2.833)	0.022
Stage of tumors				
III/IV vs. I/II	2.353 (1.417-3.907)	0.001	1.246 (0.677-2.291)	0.480
Lymph node metastasis				
Positive vs. Negative	2.038 (1.188-3.496)	0.010	2.063 (1.192-3.572)	0.010
ZHX3 expression				
High vs. Low	1.737 (1.053-2.867)	0.031	1.604 (1.011-2.543)	0.031

HR, hazard ratio; CI, confidence interval.

In summary, the distinct ZHX family members have diverse impacts on the prognosis of gastric cancer and may be useful biomarkers for predicting patient outcomes or optimizing individualized therapy. Future research should elucidate the exact mechanisms by which the ZHX factors influence the initiation and progression of gastric cancer. Such studies could confirm the potential value of using ZHX factors as prognostic indicators or therapeutic targets, enabling a precision medicine approach for patients with gastric cancer.

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### Disclosure of conflict of interest

None.

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